Published in final edited form as:

Chem Biol Drug Des. 2011 August ; 78(2): 211–223. doi:10.1111/j.1747-0285.2011.01136.x.

Targeting Metallo-proteins by Fragment-Based Lead Discovery

Sherida Johnson¹, Elisa Barile¹, Biancamaria Farina¹, Angela Purves¹, Jun Wei¹, Li-Hsing Chen¹, Sergey Shiryaev¹, Ziming Zhang¹, Irina Rodionova¹, Arpita Agrawal², Seth M. Cohen², Andrei Osterman¹, Alex Strongin¹, and Maurizio Pellecchia^{1,*}

¹ Sanford-Burnham Medical Research Institute, 10901 N. Torrey Pines Rd., La Jolla, California 92037, USA

² Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093, USA

Abstract

It has been estimated that nearly one third of functional proteins contain a metal ion. These constitute a wide variety of possible drug targets including metalloproteinases, dehydrogenases, oxidoreductases, hydrolases, deacetylases or many others in which the metal ion is either of catalytic or structural nature. Despite the predominant role of a metal ion in so many classes of drug targets, current high-throughput screening techniques do not usually produce viable hits against these proteins, likely due to the lack of proper metal binding pharmacophores in the current screening libraries. Herein we describe a novel fragment based drug discovery approach using a metal targeting fragment library that is based on a variety of distinct classes of metal-binding groups designed to reliably anchor the fragments at the target's metal ions. We show that the approach can effectively identify novel, potent and selective agents that can be readily developed into metalloprotein-targeted therapeutics.

Introduction

Fragment-based lead design (FBLD), or also called fragment-based drug discovery (FBDD), is becoming a well established approach for the discovery of biologically active compounds. FBLD involves the rapid screening of libraries of relatively few (hundreds to thousands) fragment compounds against a target of interest (1-5). Once fragments are identified that bind to a target, generally one of two approaches are pursued: a) a single fragment can be elaborated in order to obtain a tight binder, or b) multiple fragments binding at distinct sites can be connected by an appropriate linker to obtain a potent inhibitor (Fig. 1a and 1b) (2, 4, 5). In most cases, fragments are structurally characterized in the bound form either by X-ray crystallography (6), or by high-field NMR techniques (7). Compared to high-throughput screening (HTS), FBLD is purported to have several advantages, which include a more efficient exploration of chemical diversity space and higher ligand efficiencies (LE) (8-14). LE is the binding free energy for a ligand per number of heavy atoms, which represent the goodness of the fit between the ligand and the receptor (8, 13). Monitoring the ligand efficiency during the fragment or hit maturation process helps in assessing the additional contribution that each given functionality brings to the overall activity of a compound series (3, 8).

One of the earliest applications of FBLD was against the zinc(II)-dependent matrix metalloproteinase-3 (MMP-3, also known as stromelysin) (5). In this early study, an

* To whom correspondence should be addressed. mpellecchia@sanfordburnham.org. Telephone: (858)646-3159. Fax: (858)795-5225.

acetohydroxamic acid zinc-binding group (ZBG) was used to bind to the catalytic zinc(II) ion (partially occupying one binding site), while a series of hydrophobic, aromatic fragments were examined for their ability to occupy the S1' selectivity pocket (schematically illustrated in Fig. 1c) (5). The weakly interacting second site binding hydrophobic fragments (K_d s in the high μ M range) were then linked to the acetohydroxamic acid moiety (K_d in the mM range) to generate ligands with nanomolar affinity for MMP-3 (Fig. 1c) (5, 15). Despite this early, important application of FBLD to metalloproteinases, only a limited number of subsequent studies have actively applied FBLD to such targets. Furthermore, there are only a few studies that describe the development of libraries that are specifically designed to target metalloproteins (16-19).

In order to demonstrate the utility and advantages of applying FBLD to metallo-targets, a focused library of about 500 fragments was synthesized. The compounds were selected based on their ability to bind metal ions, by using various different ZBG anchors (20, 21). By incorporating a ZBG into each member of the library, it is anticipated that initial binding fragments are found that more efficiently occupy a first site around the metal ion (Fig. 1d). For each metal anchoring motif, a diverse set of small substituents was appended in order to generate a diverse fragment library. In this way, a set of molecular metal-coordinating fragments is obtained that are highly amenable to elaboration into potent and selective inhibitors (Fig. 1d). Hence, a library was assembled using a variety of fragments, each derivatized with a potential metal chelating group (Fig. 2), including not only the well known hydroxamic acid, but other less investigated ZBGs (20-22). Specifically, the library contained about 200 hydroxamates, (one of them is a known LF inhibitor synthesized in house (23)), 48 were commercially available in which 9 are known HDAC inhibitors, 23 reverse hydroxamates (limited due to availability of starting materials), 96 sulfonylhydroxamates, 64 maltol derivatives and 23 thiomaltol derivatives (22). Although it has been reported that hydroxamic acids have potentially limiting pharmacokinetic properties possibly due to the hydrolysis of the hydroxamic acid to the corresponding shorter and less active carboxylic acid, simple strategies can be employed to eliminate the metabolic liability of hydroxamate-containing inhibitors by modifying the substituents around the hydroxamate moiety (15, 24-27). As a consequence, there has been a successful histone deacetylase (HDAC) inhibitor, known as Vorinostat (Zolinza[®], formerly known as SAHA) that has been recently approved by the FDA for treatment of the cutaneous T-cell lymphoma (28), while other hydroxamate inhibitors for other metalloproteins are at various stages in clinical development (14, 17, 29). Nonetheless, we believe it is advantageous to explore additional ZBGs as illustrated in Fig. 2.

In order to evaluate the effectiveness of our approach we tested our library against a variety of Zn(II)-metalloproteins, including anthrax lethal factor (LF), botulinum neurotoxin A (BoNT/A), gelatinase-2 (MMP-9), stromelysin (MMP-3), histone deacetylase 1 and 8 (HDAC1, 8), and matrix metalloproteinases MT1-MMP and MT6-MMP. Enzymatic assays identified 'hits' for all targets, including unique fragment hits for the majority of them. In addition, the metalloproteins showed different distributions of hits for each ZBG, indicating that the choice of ZBG contributes substantially to the binding specificity. Some fragments proved to be scaffold precursors for known clinical candidates as metalloproteinase inhibitors, while others could be easily optimized into potent and selective compounds. We further proved that the approach could also be applicable to other metal-ions such as the Mn^{2+} present in the active site of the bacterial essential enzyme 1-deoxy-D-xylulose-5-phosphate reductoisomerase, chosen as an example (DXR; IspC) (30). Hence, the findings reported here show that our approach and the derived metalloprotein-focused library for FBLD will be a significant source of lead compounds for the development of new inhibitors for a large variety of drug targets.

Results and discussion

In order to evaluate the potential of the designed library for the identification of viable fragment hits, we profiled it against a variety of Zn-metalloproteins including two bacterial toxins (LF and BoNT/A), human metalloproteinases (MMP -3, -9, MT1-MMP, and MT6-MMP) and two hydrolases (HDAC1 and 8) as well as a Mn-metalloprotein, DXR. Each compound was tested in an enzyme inhibition assay and hits were defined as those that exhibited >50% inhibition at 100 μ M and IC_{50} values were subsequently obtained for the most potent compounds (Table 1) with the exception of DXR hits, which were initially found via NMR screening using T1 ρ NMR experiments and further validated using a colorimetric assay. Overall, the screening of our fragment library gave 14 hits against LF (4% hit rate), 19 hits against BoNT/A (4.8% hit rate), 9 hits against MMP2 (2% hit rate), 16 hits against MMP3 (4% hit rate), 81 hits against HDAC1 (17% hit rate), 189 hits against HDAC8 (34% hit rate) and 47 hits (11%) against DXR (see below for our criteria for definition of hits against this target).

Several striking observations can be made: first, compounds show rather marked inhibition constants (in the low micromolar range) and very good ligand efficiencies; second, both cross-reactive and rather selective fragments could be identified; third, fragment selectivity is a function of both the ZBG and the scaffold that carries it. Hence, unlike in typical FBLD approaches, the library produces hits that are nearly comparable in activity and selectivity to what one could obtain in the successful HTS campaigns against these targets (31-33). Moreover, the ligand efficiencies are significantly higher than both typical FBLD and HTS hits (8). Furthermore, since different metal chelating groups have varying affinities for different targets, the fragments are expected to have an intrinsically higher selectivity than typical FBLD hits (34-36). At the same time, we expect that our approach would lead to fragments that could be rapidly optimized into potential lead candidates, either by fragment maturation (Fig. 1d) or by fragment merging (Fig. 1e). While these methods work best when supported by the experimentally derived structure of the binary complexes (either by X-ray(6) or NMR spectroscopy(7)), recently Chen and Shoichet (37) reported that virtual docking could also effectively supplement these endeavors. Likewise, in our approach, these tasks could be enormously supported by structure-based computational modeling, given that positioning the hit fragments in the binding site would be facilitated by the geometry of the metal coordination.

For example, in devising inhibitors against Anthrax lethal factor (LF), fragments **4** and **5** were found as hits in our kinetic assay screen (Fig 3). These two fragments, **4** and **5** could have been used together with the X-ray structure of the enzyme for further optimizations (Fig. 3a) (23). The evolution of these fragments would then follow simple medicinal chemistry guided by the structure of the docked hits as schematically illustrated in Fig. 3. Proper evolution of these fragments would indeed lead to compounds **18** and **19** with IC_{50} values in the low nanomolar range and with high selectivity for LF (23, 38). In particular, compound **19** (Fig. 3a) has been reported to be a viable clinical candidate against Anthrax (23).

In addition to the fragment maturation strategy, we mentioned that optimized compounds could be designed by merging two weak inhibitory fragments (Fig. 1e). In the current example, compounds **20** (IC_{50} = 100 μ M) and **3** (IC_{50} = 47 μ M), found as hits in the kinetic screen against LF (Fig. 3b) were merged leading to the low micromolar compound **22** (Fig. 3b and 3c). Docking studies performed using the X-ray coordinates of LF (PDB-ID 1YQY) suggest that compound **22** fits nicely into the binding pocket of the enzyme, placing the hydroxamate moiety coordinating the metal ion, extending into the adjacent S1' pocket (Fig. 3e). Other fragments with the same scaffold were in the library, but were not chosen due to

lack of activity in the kinetic assay. To demonstrate this, we merged compound **3** with an inactive compound (compound **21**, $IC_{50} > 100 \mu M$), which did not result in a molecule with significantly improved affinity (compound **23**, Fig. 3b).

To further analyze the efficacy of compound **22** in cell, we confirmed its ability of protecting macrophages from LF/PA induced cell death (31, 39), and in preventing MEK1 cleavage in Hela cells (Fig. 3d) (40). These data further corroborate the underlying principle of FBLD that the combination of two albeit weak binder fragments can result in high affinity ligands. Overall, the SAR of this fragment indeed led to potent LF inhibitors, exhibiting selectivity, cell permeability, and efficacy *in vivo*.

Screening the fragment library against the other metalloprotease targets also resulted in significant hits (50% inhibition at $50 \mu M$), which could ultimately be optimized. In particular, screening the library against BoNT/A gave 19 hits that were predominately the thiomaltol derivatives, with the exception of three compounds (one hydroxamate, compound **9**, Table 1; two maltol derivatives, $IC_{50} > 10 \mu M$), the top hits were compounds **11-13** (Table 1). This same trend was also observed for MMP-9 in which we identified 22 total hits comprising of 17 thiomaltol derivatives, two maltol derivatives (IC_{50} 30-40 μM) and three hydroxamate derivatives (compound **5**, Table 1, and two other hydroxamates with IC_{50} 's $> 30 \mu M$). Hits identified against MMP-3 were mainly the hydroxamate analogues in which compounds **7**, **10** and **11** represent the most potent compounds with IC_{50} values of 1.0, 3.0 and 4.0 μM respectively. In the case of HDAC1 and HDAC8, the hits were mainly the hydroxamate derivatives such as compound **8** (Table 1) and analogues thereof (not shown).

The results from screening the library via $T_{1\rho}$ NMR against DXR led to 47 hits by monitoring the broadening or decrease in intensity of the peaks of the compounds ($200 \mu M$) in the presence of a substoichiometric ($5 \mu M$) amount of protein, compared to the spectra of the ligands without protein. Of the 47 hits, 35 were hydroxamates, one sulfonyl hydroxamate, four reverse hydroxamates, five maltol derivatives and two thiomaltol derivatives. As an example, compound **24** shown in Fig. 4a represents the outcome of the $T_{1\rho}$ screen against DXR as described in the material and methods section. These 47 compounds were then tested using a colorimetric enzymatic assay in which six of the 47 compounds were confirmed to inhibit DXR in the low μM range (Table 2). Docking studies were performed on all hits in which compound **24** gives a representation of how well it fits inside the pocket showing the hydroxamate moiety chelating the Mn^{2+} (Fig. 4b).

Hence, with several examples we demonstrated that this fragment library can rapidly and efficiently lead to potent inhibitors of metalloproteins. In fact, similar fragment maturation strategies could be applied to any other hits reported in Table 1 leading to a rapid generation of lead candidates for these targets. For example, we recently reported on the discovery and maturation of hydroxamic acid based inhibitors of MT6-MMP using this strategy (41). Moreover, further potent, non-hydroxamic acid based compounds **15-17** were obtained that effectively inhibit MT6-MMP and MT1-MMP cleavage of α -1-antitrypsin (Fig. 5). The maturation of these compounds into nanomolar inhibition was achieved with very limited SAR guided by the docked structure of initial hits. Of note is that the docking was performed with a homology-modeled structure, given that the X-ray coordinates of MT6-MMP are not yet available (Fig. 5). Given their role in tumor progression and neurodegeneration (41), inhibitors of MT1-MMP and MT6-MMP hold great potential for the development of novel therapeutic agents.

In summary, screening this library composed of hydroxamates, reverse hydroxamates, sulfonyl hydroxamates, hydroxypyranones, hydroxypyridinethiones and sulfonamide acids resulted in an array of hits with the exception of the reverse hydroxamates. In conclusion,

we have devised a novel metal-targeted fragment library and demonstrated that its application in FBLD projects lead to novel, selective and highly efficient ligands that could be matured into lead candidates. Finally, because it has been estimated that nearly one third of drug targets contain a metal ion (42), we envision that our approach will find wide utility in targeting a variety of additional drug targets for which HTS has notoriously failed in producing viable hits such as dehydrogenases, carboxylases, oxidoreductases, transcription factors, and potentially many others as recently demonstrated by a similar approach (61).

Methods

Preparation of Fragment Library

Starting materials and solvents were purchased and used without further purification from commercial suppliers (Sigma-Aldrich, Alfa Aesar, Fisher Scientific, Maybridge, Acros, TCI, Cambridge Isotope Laboratories, Inc., and others). Compound **5** was purchased from AKOS Consulting and Solutions GmbH (Germany).

The library was constrained to be in line with the general 'rule of three' (43) for fragments: a) the MW of all fragments were generally less than 300 Da; b) excluding the ZBG, the number of H-bond donors and H-bond acceptors was less than 3; and c) high aqueous solubility was sought for ease of screening. As such, the library consisted of small molecule derivatives containing one of several basic ZBGs: hydroxamic acids, hydroxysulfonic acids, reverse hydroxamates, 3,4-hydroxypyridinones, and 3,4-hydroxypyridinethiones (Fig. 2). These groups were selected because of their established ability to bind metal ions and prior identification as effective ZBGs for use in metalloproteinase inhibitors (20-22).

The syntheses of both hydroxypyridinones and hydroxypyridinethiones have been well established by several reported procedures (22).

Similarly, the hydroxamates, reverse hydroxamates and sulfonylhydroxamates were all synthesized according to previous literature reports (44-50). In the synthesis of the hydroxamic acids, different protocols were utilized, including conversion of the acid to the acyl chloride using oxalyl chloride or thionyl chloride (44, 45), or coupling the acid with the hydroxamic acid via EDC coupling reagent along with cyanuric chloride (46), or using derivatized esters that were converted to the hydroxamic acid using 50% hydroxylamine in dimethylacetamide (DMA) / methanol (51). Two types of reverse hydroxamates were incorporated into this library, N-arylacetoxyhydroxamates and N-hydroxyformanilides. The N-acetoxyhydroxamates were synthesized in two steps starting from the nitro compound to the hydroxylamine using raney nickel followed by acetylation with acetyl chloride (48). N-hydroxyformanilides were obtained using commercially available nitroso compounds and converting them to the reverse hydroxamate using glyoxylic acid as described previously (52). Similar to the procedure of the hydroxamic acids, the sulfonylhydroxamates were obtained starting from commercially available sulfonyl chloride in the presence of hydroxylamine hydrochloride and sodium bicarbonate (50). However, several of the sulfonylhydroxamates are easily hydrolyzed and are not stable in DMSO stock solutions, but stable in powder form. Full experimental details on the synthesis of compounds **16-21** were carried out as described in Kim, K. et al. (53). Compounds **10-12** were synthesized as described in Levin, J. et al. (54). Characterization of the compounds is reported in the supplementary material. Molecular docking studies were performed as described in Johnson et al. (31).

Metalloenzyme Assays

MAPKKide® assay for LF—The fluorescence peptide cleavage assay was performed in a 96 well plates (100 μ L/well) in which each reaction mixture contained MAPKKide® (4 μ M) and LF (50 nM) (List Biological Laboratories) in 20 mM Hepes, pH 7.4, and the screening compounds (mixture of 20; each compound at 10 μ M). Kinetics of the peptide cleavage was examined for 30 min by using a fluorescent plate reader (Victor V, Perkin Elmer, Waltham, MA) at excitation and emission wavelengths of 485 and 535 nm, respectively and IC₅₀ values were obtained by dose response measurements. For selected compounds, Lineweaver-Burk analysis was also carried out to verify that the compounds are competitive against the substrate. The K_m and V_{max} values of the MAPKKide® cleavage by LF were determined at 25 °C by using the same experimental condition described above for the fluorescence screening assay but with increasing MAPKKide® concentrations (10, 5, 2.5 μ M). The K_i and K_{m(app)} were calculated at 5 and/or 10 μ M inhibitor concentration.

MMP-9 assay—This assay was performed as outlined in the Anaspec MMP Assay kit (Cat. No. 71151/71155). The fluorescence peptide cleavage assay was performed in a 96 well plate (50 μ L/well) in which each reaction mixture contained 5-FAM/QXLTM520 (60 μ L; diluted 1:100 in assay buffer) and MMP-9 (10 mg/mL; pro-MMP-9 are first activated with 1mM APMA for 2 hrs respectively) in Enzolyte™ 520 MMP-9 assay buffer and the screening compounds at 20 μ M. Kinetics of the peptide cleavage was examined every five minutes for 30 min by using a fluorescent plate reader (Victor V, Perkin Elmer, Waltham, MA) at excitation and emission wavelengths of 485 and 535 nm, respectively and % inhibition values were obtained. Similarly, MMP-3 assay was performed as we recently described (22).

SNAPTide® assay for BoNT/A—The fluorescence peptide cleavage assay (50 μ L) was performed in a 96 well plates in which each reaction mixture contained SNAPTide® (8 μ M) and Botulinum Neurotoxin Type A (5 nM) (List Biological Laboratories) in 20 mM Hepes, 0.1% Tween 20, pH 8.2, and the screening compounds. Kinetics of the peptide cleavage was examined for 30 min by using a fluorescence plate reader (Victor™² V, Perkin Elmer) at excitation and emission wavelengths of 485 and 535 nm, respectively and IC₅₀ values were obtained by dose response measurements. The K_m and V_{max} values of the SNAPTide® cleavage by BoTN/A were determined at 25 °C by using the same experimental condition described above for the fluorescence screening assay, but with increasing SNAPTide® concentrations (100, 60, 30, 10, 1 μ M).

HDAC1 and 8 assays—HDAC1 and 8 inhibitors were identified by using the Enzo Life Sciences' HDAC1 and 8 Fluorimetric Drug Discovery Kit (Cat. No. BML-AK511/AK518). The assay is carried out in two steps in a 96 well plate. First, a tetrapeptide substrate containing an acetylated lysine side chain, Fluor de Lys™ SIRT-1 (5 mM) for HDAC1 and Fluor de Lys™ HDAC8 (25 mM) for HDAC8, is incubated with the enzyme for 45 min. (0.1 mg HDAC1; 37 °C and 0.5U HDAC8; 30 °C). In the second step, the deacetylated substrate is allowed to react with the Fluor de Lys™ Developer II in order to produce a fluorophore. The fluorescence is measured by using a fluorescent plate reader (Victor V, Perkin Elmer, Waltham, MA) at excitation and emission wavelengths of 355 and 460 nm, respectively. Initially, the test samples were screened at 50 μ M and IC₅₀ values were obtained by dose-response measurements.

DXR Assay—Enzymatic activity was measured at 37 °C in 200 μ L assay mixture: MnCl₂ 0.5 mM, NADPH 200 mM, TRIS pH 8.5 100mM, Deoxy-xylulose 5-phosphate 250 mM, BSA 10mg/ml. Concentration of substrate was chosen at K_m value (55). Specific activity of enzyme was ~1 mmol/mg/min. Inhibition was measured in comparison with activity of

enzyme in presence of 1% DMSO. The oxidation of NADPH was monitored at 340 nm by using a Beckman DTX-880 multimode microplate reader. Consumption of substrate for activity measurements was less than 10%. Fosmidomycin, a known DXR inhibitor, used as a positive control showed the value of IC_{50} expected from published IC_{50} 8.2 nM (56).

DXR NMR Screen—NMR spectra were acquired on a 700 MHz Bruker Avance spectrometer equipped with a TCI-cryoprobe. All 1D 1H experiments were carried out with 100 μM of testing compounds in Tris buffer (20mM Tris buffer pH 7.5, 20mM Mn^{2+}) in the presence or the absence of 10 μM DXR protein. 1D $T_{1\rho}$ experiments were measured with a variable spin-lock time of 10 ms, 200 ms or 1000 ms, a recycle delay of 1.5 sec, and water suppression based on the watergate sequence. Data was analyzed with aid of 1D proton spectrum of the individual and hits was further confirmed by testing individual compound.

Alpha-1-Antitrypsin (AAT) SDS gel assay—Inhibitors block the cleavage of $\alpha 1$ -anti-trypsin by MT1-MMP and MT6-MMP. $\alpha 1$ -anti-trypsin (2 μM) was co-incubated for 1 h 37 °C with MMPs (0.02 μM each) in 50 mM HEPES - 10 mM $CaCl_2$ - 1 μM $ZnCl_2$, pH 6.8. The digests were analyzed using SDS-PAGE. Where indicated, GM6001 (0.5 μM) and the novel compounds (at 5 and 25 mM each) were added to the reactions.

Cell-based assays

MEK-1 cleavage—The N-terminal MEK1 antibody (anti-MEK1, N-terminal) was obtained from Calbiochem and the β -actin antibody (Monoclonal-Anti- β -actin) was purchased from Sigma. Anti-rabbit and anti-mouse-horseradish peroxidases were purchased from GE Healthcare (UK). HeLa (Human cervical carcinoma) cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum (FBS, Omega Scientific) and 1% penicillin/streptomycin (Omega Scientific) at 37 °C in a humidified incubator containing 5% CO_2 . Triplicate samples of approximately 5.0×10^4 HeLa cells were plated in individual wells of a 48-well plate and incubated for 48h. The cells were exposed, for 90 minutes at 37 °C, to a pre-incubated solution of 2.5×10^{-8} M PA_{83} , 5×10^{-9} M LF and test compounds in serum-free DMEM medium. The analyzed inhibitors were dissolved in DMSO reaching a final DMSO concentration of 1%. Controls included untreated cells and LF-PA-only treated cells. Cells were later lysed and directly collected with reducing gel sample buffer and analyzed for MEK1 cleavage by immunoblotting as previously described.(40)

Macrophage protection assay—RAW 264.7 Murine monocyte macrophages (4.5×10^4 cells/well) were plated into 96-well tissue culture plates in Hyclone DMEM (4500 mg/L Glucose, 110g/L Sodium Pyruvate) supplemented with 5% fetal bovine serum, 2mM Glutamax (Invitrogen, Carlsbad, CA), 1% penicillin/streptomycin (Omega Scientific). Cells were cultured overnight at 37 °C in a humidified incubator containing 5% CO_2 . They were replenished with fresh serum-free medium (0.1ml/well) and exposed to a pre-incubated solution of test compounds (increasing concentrations from 0.015 to 33.3 mM), PA_{83} (500 ng/mL) and LF (37.5 ng/mL). The analyzed inhibitors were dissolved in DMSO reaching a final DMSO concentration of 1%. Controls included untreated cells, LF-PA-only treated cells and cells treated with the known LF inhibitor **15**. After incubation for 3.5 hours at 37 °C, cell viability was assessed using ATPlite assay from Perkin Elmer (Waltham, MA). Each datum point represents triplicates of each concentration in one experiment. Viability was normalized to control cells which were treated with the vehicle, DMSO.

Protein expression of DXR—*E.coli* strain JW0168 from the ASKA collection (57) was used for recombinant DXR protein overexpression. Pre-culture was grown in LB overnight

with chloramphenicol 50ug/ml. 40ul of pre-culture was used to inoculate 50 ml LB in 250 ml flask. After the growth was reaching optical density 0.8 at 600 nm induction with IPTG 0.1 mM was produced. After shaking 4 hours at 37 °C cells were collected, lysed and Ni-NTA column purification was performed.(58) Protein was mixed with EDTA 1mM and buffer changed by Bio-Spin chromatography column. Protein SDS-polyacrilamide gel is shown in Fig.1 and concentration of protein after buffer exchange was 2.8 mg/ml. Concentration of protein was measured by Bradford assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the National Institutes of Health (NIH), grants AI070494, AI059572 to MP, and CA083017 to AS and MP, for generous support. We also thank Dr. Spyroulias and his laboratory at the Department of Pharmacy, University of Patras, Greece for the kind gift of the expression plasmid for the catalytic domain of LF used for the supplementary data.

References

1. Erlanson DA, McDowell RS, O'Brien T. Fragment-based drug discovery. *J Med Chem.* 2004; 47:3463–3482. [PubMed: 15214773]
2. Shuker SB, Hajduk PJ, Meadows RP, Fesik SW. Discovering high-affinity ligands for proteins: SAR by NMR. *Science.* 1996; 274:1531–1534. [PubMed: 8929414]
3. Carr RA, Congreve M, Murray CW, Rees DC. Fragment-based lead discovery: leads by design. *Drug Discov Today.* 2005; 10:987–992. [PubMed: 16023057]
4. Siegal G, Ab E, Schultz J. Integration of fragment screening and library design. *Drug Discov Today.* 2007; 12:1032–1039. [PubMed: 18061882]
5. Hajduk PJ, Greer J. A decade of fragment-based drug design: strategic advances and lessons learned. *Nat Rev Drug Discov.* 2007; 6:211–219. [PubMed: 17290284]
6. Jhoti H, Cleasby A, Verdonk M, Williams G. Fragment-based screening using X-ray crystallography and NMR spectroscopy. *Curr Opin Chem Biol.* 2007; 11:485–493. [PubMed: 17851109]
7. Pellecchia M, Bertini I, Cowburn D, Dalvit C, Giralt E, Jahnke W, James TL, Homans SW, Kessler H, Luchinat C, Meyer B, Oschkinat H, Peng J, Schwalbe H, Siegal G. Perspectives on NMR in drug discovery: a technique comes of age. *Nat Rev Drug Discov.* 2008; 7:738–745. [PubMed: 19172689]
8. Bembek SD, Tounge BA, Reynolds CH. Ligand efficiency and fragment-based drug discovery. *Drug Discov Today.* 2009; 14:278–283. [PubMed: 19073276]
9. 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; 41:856–864. [PubMed: 11410068]
10. Schuffenhauer A, Ruedisser S, Marzinzik AL, Jahnke W, Blommers M, Selzer P, Jacoby E. Library design for fragment based screening. *Curr Top Med Chem.* 2005; 5:751–762. [PubMed: 16101415]
11. Mestres J, Veeneman GH. Identification of “latent hits” in compound screening collections. *J Med Chem.* 2003; 46:3441–3444. [PubMed: 12877579]
12. Gribbon P, Sewing A. High-throughput drug discovery: what can we expect from HTS? *Drug Discov Today.* 2005; 10:17–22. [PubMed: 15676295]
13. Kuntz ID, Chen K, Sharp KA, Kollman PA. The maximal affinity of ligands. *Proc Natl Acad Sci U S A.* 1999; 96:9997–10002. [PubMed: 10468550]
14. Fattori D, Squarcia A, Bartoli S. Fragment-based approach to drug lead discovery: overview and advances in various techniques. *Drugs R D.* 2008; 9:217–227. [PubMed: 18588353]
15. Hajduk PJ, Shuker SB, Nettesheim DG, Craig R, Augeri DJ, Betebenner D, Albert DH, Guo Y, Meadows RP, Xu L, Michaelides M, Davidsen SK, Fesik SW. NMR-based modification of matrix

- metalloproteinase inhibitors with improved bioavailability. *J Med Chem.* 2002; 45:5628–5639. [PubMed: 12477346]
16. Wang J, Uttamchandani M, Li J, Hu M, Yao SQ. Rapid assembly of matrix metalloprotease inhibitors using click chemistry. *Org Lett.* 2006; 8:3821–3824. [PubMed: 16898826]
17. Flipo M, Beghyn T, Charton J, Leroux VA, Deprez BP, Deprez-Poulain RF. A library of novel hydroxamic acids targeting the metallo-protease family: design, parallel synthesis and screening. *Bioorg Med Chem.* 2007; 15:63–76. [PubMed: 17070058]
18. Sternson SM, Wong JC, Grozinger CM, Schreiber SL. Synthesis of 7200 small molecules based on a substructural analysis of the histone deacetylase inhibitors trichostatin and trapoxin. *Org Lett.* 2001; 3:4239–4242. [PubMed: 11784187]
19. Price S, Osbourn SE. Solid-phase synthesis of N-formylhydroxylamines (reverse/retro-hydroxamates). *Org Lett.* 2005; 7:3761–3763. [PubMed: 16092869]
20. Jacobsen FE, Lewis JA, Cohen SM. The Design of Inhibitors for Medicinally Relevant Metalloproteins. *Chem Med Chem.* 2007; 2:152–171. [PubMed: 17163561]
21. Jacobsen FE, Lewis JA, Cohen SM. A new role for old ligands: discerning chelators for zinc metalloproteinases. *J Am Chem Soc.* 2006; 128:3156–3157. [PubMed: 16522091]
22. Agrawal A, Johnson SL, Jacobsen JA, Miller MT, Chen LH, Pellecchia M, Cohen SM. Chelator fragment libraries for targeting metalloproteinases. *ChemMedChem.* 2010; 5:195–199. [PubMed: 20058293]
23. Shoop WL, Xiong Y, Wiltse J, Woods A, Guo J, Pivnichny JV, Felcetto T, Michael BF, Bansal A, Cummings RT, Cunningham BR, Friedlander AM, Douglas CM, Patel SB, Wisniewski D, Scapin G, Salowe SP, Zaller DM, Chapman KT, Scolnick EM, Schmatz DM, Bartizal K, MacCoss M, Hermes JD. Anthrax lethal factor inhibition. *Proc Natl Acad Sci U S A.* 2005; 102:7958–7963. [PubMed: 15911756]
24. Bouchain G, Delorme D. Novel hydroxamate and anilide derivatives as potent histone deacetylase inhibitors: synthesis and antiproliferative evaluation. *Curr Med Chem.* 2003; 10:2359–2372. [PubMed: 14529479]
25. Michaelides MR, Dellaria JF, Gong J, Holms JH, Bouska JJ, Stacey J, Wada CK, Heyman HR, Curtin ML, Guo Y, Goodfellow CL, Elmore IB, Albert DH, Magoc TJ, Marcotte PA, Morgan DW, Davidsen SK. Biaryl ether retrohydroxamates as potent, long-lived, orally bioavailable MMP inhibitors. *Bioorg Med Chem Lett.* 2001; 11:1553–1556. [PubMed: 11412979]
26. Curtin M, Glaser K. Histone deacetylase inhibitors: the Abbott experience. *Curr Med Chem.* 2003; 10:2373–2392. [PubMed: 14529480]
27. Pikul S, Ohler NE, Ciszewski G, Laufersweiler MC, Almstead NG, De B, Natchus MG, Hsieh LC, Janusz MJ, Peng SX, Branch TM, King SL, Taiwo YO, Mieling GE. Potent and selective carboxylic acid-based inhibitors of matrix metalloproteinases. *J Med Chem.* 2001; 44:2499–2502. [PubMed: 11472202]
28. Attenni B, Ontoria JM, Cruz JC, Rowley M, Schultz-Fademrecht C, Steinkuhler C, Jones P. Histone deacetylase inhibitors with a primary amide zinc binding group display antitumor activity in xenograft model. *Bioorg Med Chem Lett.* 2009; 19:3081–3084. [PubMed: 19410459]
29. Aranapakam V, Davis JM, Grosu GT, Baker J, Ellingboe J, Zask A, Levin JJ, Sandanayaka VP, Du M, Skotnicki JS, DiJoseph JF, Sung A, Sharr MA, Killar LM, Walter T, Jin G, Cowling R, Tillett J, Zhao W, McDevitt J, Xu ZB. Synthesis and structure-activity relationship of N-substituted 4-arylsulfonylpiperidine-4-hydroxamic acids as novel, orally active matrix metalloproteinase inhibitors for the treatment of osteoarthritis. *J Med Chem.* 2003; 46:2376–2396. [PubMed: 12773042]
30. Dhiman RK, Schaeffer ML, Bailey AM, Testa CA, Scherman H, Crick DC. 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (IspC) from *Mycobacterium tuberculosis*: towards understanding mycobacterial resistance to fosmidomycin. *J Bacteriol.* 2005; 187:8395–8402. [PubMed: 16321944]
31. Johnson SL, Chen LH, Harbach R, Sabet M, Savinov A, Cotton NJ, Strongin A, Guiney D, Pellecchia M. Rhodanine derivatives as selective protease inhibitors against bacterial toxins. *Chem Biol Drug Des.* 2008; 71:131–139. [PubMed: 18221310]

32. Johnson SL, Chen LH, Pellecchia M. A high-throughput screening approach to anthrax lethal factor inhibition. *Bioorg Chem.* 2007; 35:306–312. [PubMed: 17320146]
33. Schepetkin IA, Khlebnikov AI, Kirpotina LN, Quinn MT. Novel Small Molecule inhibitors of Anthrax Lethal Factor Identified by High-Throughput Screening. *J Med Chem.* 2006; 49:5232–5244. [PubMed: 16913712]
34. Yan YL, Miller MT, Cao Y, Cohen SM. Synthesis of hydroxypyrrone- and hydroxythiopyrrone-based matrix metalloproteinase inhibitors: developing a structure-activity relationship. *Bioorg Med Chem Lett.* 2009; 19:1970–1976. [PubMed: 19261472]
35. Agrawal A, de Oliveira CA, Cheng Y, Jacobsen JA, McCammon JA, Cohen SM. Thioamide Hydroxypyrothiones Supersede Amide Hydroxypyrothiones in Potency against Anthrax Lethal Factor. *J Med Chem.* 2009
36. Agrawal A, Romero-Perez D, Jacobsen JA, Villarreal FJ, Cohen SM. Zinc-binding groups modulate selective inhibition of MMPs. *ChemMedChem.* 2008; 3:812–820. [PubMed: 18181119]
37. Chen Y, Shoichet BK. Molecular docking and ligand specificity in fragment-based inhibitor discovery. *Nat Chem Biol.* 2009; 5:358–364. [PubMed: 19305397]
38. Johnson, AT.; Jiao, GS.; Kim, S. Hydroxamic acid derivatives of phenoxy-acetic acids an analogs useful as therapeutic agents for treating anthrax poisoning. PanThera Biopharma, LLC; United States: 2008.
39. Johnson SL, Chen LH, Barile E, Emdadi A, Sabet M, Yuan H, Wei J, Guiney D, Pellecchia M. Structure-activity relationship studies of a novel series of anthrax lethal factor inhibitors. *Bioorg Med Chem.* 2009; 17:3352–3368. [PubMed: 19359184]
40. Rainey GJ, Wigelsworth DJ, Ryan PL, Scobie HM, Collier RJ, Young JA. Receptor-specific requirements for anthrax toxin delivery into cells. *Proc Natl Acad Sci U S A.* 2005; 102:13278–13283. [PubMed: 16141341]
41. Radichev IA, Remacle AG, Shiryayev SA, Purves AN, Johnson SL, Pellecchia M, Strongin AY. Biochemical characterization of the cellular glycosylphosphatidylinositol-linked membrane type-6 matrix metalloproteinase. *J Biol Chem.* 2010; 285:16076–16086. [PubMed: 20308072]
42. Butler A. Acquisition and utilization of transition metal ions by marine organisms. *Science.* 1998; 281:207–210. [PubMed: 9660742]
43. Congreve M, Carr R, Murray C, Jhoti H. A ‘rule of three’ for fragment-based lead discovery? *Drug Discov Today.* 2003; 8:876–877. [PubMed: 14554012]
44. Booher RN, Kornfeld EC, Smalstig EB, Clemens JA. Synthesis of D-oxa tricyclic partial ergolines as dopamine agonists. *J Med Chem.* 1987; 30:580–583. [PubMed: 3102743]
45. Summers JB, Mazdiyasni H, Holms JH, Ratajczyk JD, Dyer RD, Carter GW. Hydroxamic acid inhibitors of 5-lipoxygenase. *J Med Chem.* 1987; 30:574–580. [PubMed: 3820229]
46. Giacomelli G, Porcheddu A, Salaris M. Simple one-flask method for the preparation of hydroxamic acids. *Org Lett.* 2003; 5:2715–2717. [PubMed: 12868897]
47. Miller, TA.; Witter, DJ.; Belvedere, S. Thiophene and Benzothiophene Hydroxamic Acid Derivatives. A61K. Organization, W. I. P., editor. United States: 2005. p. 1-186.
48. Boge N, Kruger S, Schroder M, Meier C. A New Short and Efficient Synthetic Route to C8-N-Acetylarylamine 2'-Deoxyguanosine Phosphoramidites. *Synthesis.* 2007:3907–3914.
49. Corbett MD, Corbett BR. Reaction of nitroso aromatics with glyoxylic acid. A new path to hydroxamic acids. *J Org Chem.* 1980; 45:2834–2839.
50. Scozzafava A, Supuran CT. Carbonic anhydrase and matrix metalloproteinase inhibitors: sulfonated amino acid hydroxamates with MMP inhibitory properties act as efficient inhibitors of CA isozymes I, II, and IV, and N-hydroxysulfonamides inhibit both these zinc enzymes. *J Med Chem.* 2000; 43:3677–3687. [PubMed: 11020282]
51. Miller, TA.; Witter, DJ.; Belvedere, S. Thiophene and Benzothiophene Hydroxamic Acid Derivatives. A61K. Aton Pharma, Inc.; United States: 2005.
52. Corbett MD, Corbett BR. Reaction of Nitroso Aromatics with Glyoxylic Acid. A New Path to Hydroxamic acids. *J Org Chem.* 1980; 45:2834–2839.
53. Kim M, Kim JY, Song K, Kim J, Lee J. Convenient total synthesis of taranabant (MK-0364), a novel cannabinoid-1 receptor inverse agonist as an anti-obesity agent. *Tetrahedron.* 2007; 63:12845–12852.

54. Levin, JI.; Rush, TSI.; Lovering, F.; Hu, Y.; Li, J.; Li, W.; Wu, JJ.; Hotchandani, R.; Xiang, JS.; Du, X.; Cole, DC.; Tam, SY. Preparation of amino acid biarylsulfonamides as metalloproteinase inhibitors. Wyeth; US: 2005. p. 119
55. Kuzuyama T, Takahashi S, Takagi M, Seto H. Characterization of 1-deoxy-D-xylulose 5-phosphate reductoisomerase, an enzyme involved in isopentenyl diphosphate biosynthesis, and identification of its catalytic amino acid residues. *J Biol Chem.* 2000; 275:19928–19932. [PubMed: 10787409]
56. Kuzuyama T, Shimizu T, Takahashi S, Seto H. Fosmidomycin, a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase in the nonmevalonate pathway for terpenoid biosynthesis. *Tetrahedron Lett.* 1998; 39:7913–7916.
57. Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, Mori H. Complete set of ORF clones of *Escherichia coli* ASOA library (A Complete Set of *E. coli* K-12 ORF Archive): Unique Resources for Biological Research. *DNA Res.* 2005; 12:291–299. [PubMed: 16769691]
58. Yang C, Rodionov DA, Rodionova IA, Li X, Osterman AL. Glycerate 2-kinase of *Thermotoga maritima* and genomic reconstruction of related metabolic pathways. *J Bacteriol.* 2008; 190:1173–1182.
59. Becattini B, Culmsee C, Leone M, Zhai D, Zhang X, Crowell KJ, Rega MF, Landshamer S, Reed JC, Plesnila N, Pellicchia M. Structure-activity relationships by interligand NOE-based design and synthesis of antiapoptotic compounds targeting Bid. *Proc Natl Acad Sci U S A.* 2006; 103:12602–12606. [PubMed: 16891420]
60. Siliphaivanh P, Harrington P, Witter DJ, Otte K, Tempest P, Kattar S, Kral AM, Fleming JC, Deshmukh SV, Harsch A, Secrist PJ, Miller TA. Design of novel histone deacetylase inhibitors. *Bioorg Med Chem Lett.* 2007; 17:4619–4624. [PubMed: 17555962]
61. Jacobsen JA, Fullagar JL, Miller MT, Cohen SM. Identifying Chelators for Metalloprotein Inhibitors Using a Fragment-Based Approach. *J Med Chem.* 2011; 54:591–602. [PubMed: 21189019]

Figure 1.

Schematic representation of various FBLD approaches. The evolution of fragments into optimized hit compounds generally follows one of two routes involving either a) maturation of the initial fragment or b) linking two fragments occupying adjacent sites.⁽⁵⁹⁾ The latter approach was successfully utilized c) for the metalloproteinase MMP-13.⁽¹⁵⁾ The present approach provides initial ZBG-containing fragment hits that can likewise be matured using either d) the evolution or e) merging two fragments occupying non-overlapping sites.

Figure 2.
Depiction of the metal chelating groups chosen for the synthesis of the library.

Figure 3.

Application of the FBLD approach to the discovery and optimization of LF inhibitors. Inhibition constants and ligand efficiencies, expressed in kcal/mol, are reported. a) Chemical structures of fragment “hits” **4** and **5** against LF; the panel also reports the chemical structures of LF inhibitors **13**, **14** and **15** (23, 38). b) Chemical structures of the merged compounds **18** and **19** and of the fragments from which these were derived. c). Dose response curve for the inhibition of LF by compound **18**. d). Dose response protective effect of compound **18** (EC₅₀ = 7.2 μM) from LF/PA induced cell death of macrophage RAW cells. The inhibition of LF induced cleavage of MEK1 in HeLa cells is also reported. Known LF inhibitor **15** and the FBLD derived compound **18** are equally effective in inhibition LF induced MEK1 cleavage in HeLa cells. e) Molecular model of compound **18** docked into the catalytic pocket of lethal factor (PDB ID 1YQY). The protein surface was generated with MOLCAD and color coded according to cavity depth (brown, deep; blue, shallow) with the Zn²⁺ ion shown in magenta.

Figure 4. Screening results for compound **24** against DXR. a) T1ρ NMR spectra of compound **24** (100 μM) in buffer without protein (black) and with protein (10 μM) (red). Peak intensities reductions are highlighted. b) Molecular surface of DXR in complex with compound **24**. The surface is generated with MOLCAD and color coded based on cavity depth: blue (shallow), brown (deep). The metal ion is depicted as a purple sphere. (PDB ID 2JCZ)

Figure 5.

Application of the FBLD approach to the discovery and optimization of MT6-MMP and MT1-MMP inhibitors. a) Inhibition of Alpha1-Antitrypsin (AAT) cleavage by catalytic domains of MT1-MMP and MT6-MMP at 5 and 25 μ M. The non-specific peptide inhibitor GM6001 was also tested (0.5 mM). b) Molecular surface of MT6-MMP in complex with compound **12**. The surface is generated with MOLCAD and color coded based on cavity depth: blue (shallow), brown (deep). The metal ion is depicted as a purple sphere. The model was obtained by using the 3D structure of MMP-2 (26% identities) and 3D Swiss Protein Modeler (PDB ID 1YQY).

Table 1

Table of hits identified in the screening via kinetic assay against LF, MMP-3, MMP-9, BoNT/A, HDAC1, HDAC8, MT1-MMP and MT6-MMP. Each fragment is color coded according to the scheme reported in Fig. 2. For active compounds, the ligand efficiencies are also reported. Fragment **6** has been previously published to inhibit the histone deacetylase proteins.(60)

#	STRUCTURE	IC ₅₀ , μM (LE, Kcal mol ⁻¹)							
		LF	MMP-3	MMP-9	BoNT/A	HDAC1	HDAC8	MT1-MMP	MT6-MMP
1		8.0 (0.32)	>50	>50	>100	>50	>50	>100	ND
2		8.3 (0.50)	>50	>50	>100	>50	>50	>100	ND
3		47.0 (0.46)	>50	>50	>100	18.6 (0.50)	5.8 (0.55)	>100	ND
4		34.0 (0.51)	>50	>50	>100	14.8 (0.55)	9.1 (0.58)	>100	9.3 (0.58)
5		32.0 (0.34)	7.4 (0.39)	33.4 (0.34)	>100	>50	>50	>100	62.5 (0.32)
6		32.0 (0.31)	>50	>50	>100	>50	>50	>100	>100
7		30.0 (0.41)	1.08	>50	>100	>50	>50	6.0	1.0

#	STRUCTURE	IC ₅₀ , μM (LE, Kcal mol ⁻¹)							
		LF	MMP-3	MMP-9	BoNT/A	HDAC1	HDAC8	MT1-MMP	MT6-MMP
8		>100	>50	>50	>100	3.8 (0.53)	0.40 (0.63)	>100	ND
9		>100	>50	>50	25.0 (0.39)	>50	>50	>100	ND
10		>100	2.9 (0.47)	>50	>100	>50	>50	>100	ND
11		3.0 (0.34)	4.0 (0.34)	2.0 (0.36)	>100	ND	ND	>100	ND
12		>50	33	17.5	5.6	ND	ND	ND	ND
13		>50	NT	>50	6.4	>50	>50	>100	>100
14		>50	NT	>50	5.2	>50	>50	>100	>100
15		34.9 (0.20)	ND	5.85 (0.24)	ND	>50	>50	1.2 (0.27)	0.29 (0.30)

#	STRUCTURE	IC ₅₀ μM (LE, Kcal mol ⁻¹)							
		LF	MMP-3	MMP-9	BoNT/A	HDAC1	HDAC8	MT1-MMP	MT6-MMP
16		19.7 (0.21)	ND	ND	ND	>50	>50	0.26 (0.29)	0.69 (0.27)
17		49.1 (0.18)	ND	ND	ND	>50	>50	0.77 (0.26)	0.03 (0.32)

Table 2

Table of hits targeting DXR.

#	Structure	LE (kcal mol ⁻¹)	IC ₅₀ (μM)
24		0.37	50±20*
25		0.26	70 ± 11
26		0.45	2.31 ±0.22
27		0.50	0.716 ±0.048
28		0.40	5.21 ± 0.79
29		0.48	1.19 ± 0.13

* Complete inhibition could not be obtained, hence reported IC₅₀ is an approximate value.