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HTS by NMR for the Identification of Potent and Selective Inhibitors of Metalloenzymes

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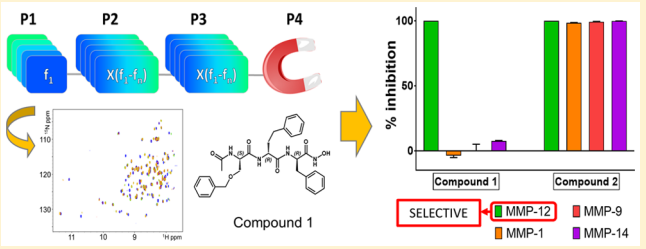
Supporting Information

ABSTRACT: We have recently proposed a novel drug discovery approach based on biophysical screening of focused positional scanning libraries in which each element of the library contained a common binding moiety for the given target or class of targets. In this Letter, we report on the implementation of this approach to target metal containing proteins. In our implementation, we first derived a focused positional scanning combinatorial library of peptide mimetics (of approximately 100,000 compounds) in which each element of the library contained the metal-chelating moiety hydroxamic acid at the C-terminal. Screening of this library by nuclear magnetic resonance spectroscopy in solution allowed the identification of a novel and selective compound series targeting MMP-12. The data supported that our general approach, perhaps applied using other metal chelating agents or other initial binding fragments, may result very effective in deriving novel and selective agents against metalloenzyme.



KEYWORDS: *HTS by NMR, FBDD, FBLD, MMP, focused positional scanning libraries*

While initially introduced as a way to weed out false positives, biophysical methods such as protein nuclear magnetic resonance (NMR) spectroscopy have increasingly played a major role in *de novo* drug discovery campaigns in the past decade. These approaches have the invaluable advantage to enable the unambiguous identification and characterization of the binding properties of test molecules to a given protein target, without relying on convoluted indirect biochemical assays.[1−4] Hence, these methods found fertile ground in guiding the design of lead compounds and drug candidates in recent years. In the well-known and clever application termed "SAR by NMR" (Structure Activity Relationships by NMR), pairs of low molecular weight and low affinity binders are identified using NMR chemical shift mapping techniques. Subsequently, guided by structural studies, these "fragments" are chemically linked to obtain potent bidentate compounds.[5,6] Recent successful implementations of the approach led to the design of antagonists of Bcl-2 and Bcl-xL (ABT-737)[7] that culminated in the current clinical agent ABT-199[8,9] (also known as venetoclax, one of the first antagonist of protein−protein interactions to reach the clinic and FDA approval). Of note is that conventional high throughput screening (HTS) campaigns using biochemical screens against the same targets failed to produce viable hits.[7] In its original implementation, the SAR by NMR strategy was applied to the metalloproteinase MMP-3, or stromelysin.[5] In the approach, the first binding fragment was simply acetohydroxamic acid, a common metal chelator that binds with millimolar affinities to most zinc metalloproteinases. Hence, in the presence of this small molecule, an NMR-based second site screen was performed to identify additional small molecules that bound in subpockets adjacent to the acetohydroxamic acid. This screen led to a biphenyl compound binding to MMP-3 with double-digit micromolar affinity.

After structural characterization of the ternary complex using NMR spectroscopy with ^{15}N-labeled protein, the two fragments were chemically linked to derive a low nanomolar inhibitor of this enzyme. Arguably, this approach opened the way to what is currently known as fragment-based drug discovery (FBDD) that has since evolved with the inclusion of other biophysical approaches, notably X-ray crystallography, surface plasmon resonance (SPR), and isothermal titration calorimetry (ITC), and computational docking. Fragment-hit optimization strategies have also evolved including fragment-evolution and fragment-merging strategies, which seem nowadays the preferred optimization approaches compared to the above-described fragment-linking.[10−18] As an alternative to these strategies, we have recently proposed a novel approach, termed HTS by NMR,[19] in which the principles of positional scanning combinatorial chemistry[20−23] and fragment-based drug design are combined with protein-NMR spectroscopy[1] to iteratively

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identify and optimize antagonists from collections of >100,000 peptide mimetics.[19,24−26]

The approach seems also particularly effective in the fragment-hit to lead optimization stages, when a positional scanning library is generated from an initial weak binder, perhaps common to a class of protein targets and/or previously identified from a FBDD campaign,[19,24,25] and tested by biophysical methods including not only NMR but also ITC.[27] Indeed, we recently demonstrated that testing a positional scanned library using the HTS by NMR approach revealed qualitatively a ranking that not only confirmed the known binding consensus motif for the BIR3 domain of the X-linked Inhibitor of Apoptosis Protein (XIAP), but also identified compounds that closely resembled a clinical agent (GDC-0152)[25] that targets it. Hence, to assess if this approach could be implemented to target metalloproteinases, we first derived a focused positional scanning (POS) combinatorial library of peptide mimetics (of approximately 100,000 compounds) where each element of the library contained the metal-chelating moiety hydroxamic acid at the C-terminal (Figure 1A). The

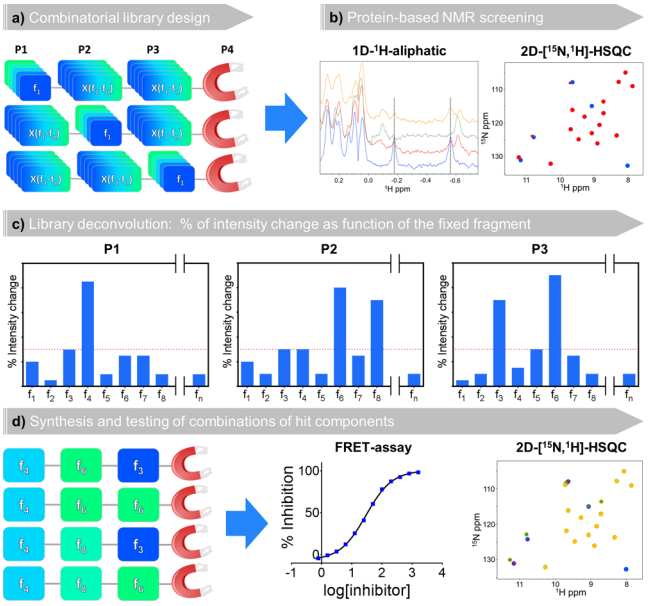

Figure 1. Schematic representation of the HTS by NMR approach as applied to metalloenzymes. (a) Focused positional scanning (fPOS) library of tripeptoids is designed and synthesized. Our implementation consisted of a three-position fPOS library, each with an hydroxamate as metal chelating group. (b) Library is next screened by collecting either 1D-^{1}H-aliph, or 2D-(^{15}N, ^{1}H) correlation spectra of the target macromolecule. (c) Significant perturbations in the NMR spectra of the target are subsequently ranked as a function of the fixed *f*n element at each position. (d) Finally, individual compounds are synthesized with a proper combination of P1, P2, and P3 *f*n elements causing the largest perturbations, and tested using NMR binding assays and/or biochemical assays.

synthesis of these agents was easily attained using traditional solid phase synthesis protocols and using an fmoc-hydroxylamine-2-chlorotrityl resin that after cleavage with 94% of trifluoroacetic acid (TFA) delivered the desired agents with the hydroxamate at the C-terminus. In our implementation, we used a combination of 46 natural and non-natural f_n amino acids (n = 1−46, Supplementary Figure S1) to generate 46 mixtures of f_nXX-CONHOH, 46 mixtures of Xf_nX-CONHOH, and 46 mixtures of XXf_n-CONHOH (Figure 1A), where X represents all the 46 amino acids. Hence, each mixture contained approximately 46 × 46−2100 compounds. Subsequently, each mixture was tested against the metalloproteinase MMP-12 using sensitive protein−NMR screening methods,[19] as these approaches present a number of unique advantages over any other assays. The approach consists in testing each mixture using 1D ^{1}H aliphatic and 2D (^{15}N,^{1}H) so-fast HMQC correlation spectra (Figure 1B) and report the observed chemical shift perturbations and/or changes in intensity as a function of given f_n element at each of the three positions (Figure 1C). Top ranking elements are therefore selected and individual compounds are synthesized and tested using NMR and subsequently also biochemical assays to assess potency and selectivity (Figure 1D). In practice, each mixture was dissolved in deuterated DMSO and diluted to a final concentration of 500 μM (~240 nM for each individual peptide-mimetics) into a buffer containing 10 μM hMMP-12 catalytic domain.

The buffer consisted of 40 mM TRIS at pH 7.2, 300 mM NaCl, 10 mM $CaCl_2$, and 0.1 mM $ZnCl_2$, and contained 200 mM of acetohydroxamic acid, a weak inhibitor of MMP-12 that prevented the self-proteolysis of the target protein. For each sample, NMR experiments were conducted including 1D ^{1}H NMR and 2D (^{15}N,^{1}H) so-fast HMQC (Figure 2). Rank ordering of the mixtures (Figure 2; see also Supplementary Figure S1 for the structure of each *f1*−*f46* element) revealed that several mixtures induced significant changes in the signal intensity in both NMR spectra, typical of a slow exchange regime suggesting tight binding of the positive mixtures. The identified preferred library elements at each position were *f*17 (*O*-benzyl-L-serine) and *f*9 (D-homophenylalanine) in P1; *f*9, *f*17, *f*28 (D-phenylalanine), and *f*38 (L-tryptophan) in P2; and *f*28 and *f*9 in P3 (Figure 2). Mapping the chemical shift perturbations on the three-dimensional structure of MMP-12 (PDB ID 5LAB) revealed that, as expected, most perturbed signals corresponded to residues that were located in the substrate binding site of the enzyme, around the metal ion (Figure 3). Interestingly, and as expected, the XX*f*28-CONHOH mixture induced larger perturbations on residues closer to the Zn^{2+}, while the *f*17XX-CONHOH induced perturbations in a region that is more distant.

Hence, based on these data, we synthesized compound **1** (*f*17-*f*9-*f*28-CONHOH; Figure 3C) and tested it first using 1D-^{1}H aliphatic and 2D-(^{15}N,^{1}H) so-fast HMQC titrations (Figure 3D,E). In addition, we subsequently tested the compound in a biochemical assay against MMP-12, side by side with the pan-MMP inhibitor **2** as a control (GM6001, Figure 4A).[28−30] Both 1D ^{1}H aliphatic and 2D-(^{15}N,^{1}H) so-fast HMQC titrations showed that the binding of compound **1** with the target protein was in slow exchange in the NMR time scale. Measuring the chemical shift differences of peaks in the free versus bound forms (Figure 3), we estimated an upper limit for the off rate for the complex, k_{off} < 50 s^{-1}. Assuming a diffusion limited on the rate of 10^9 M^{-1} s^{-1}, a dissociation constant value for the complex can be estimated to be K_d < 50 nM.

Enzyme activity inhibition assay using MMP-12 and the fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Enzo Life Science) revealed that compound **1** was a competitive inhibitor for MMP-12 with a remarkable IC_{50} value of 54.7 nM. As expected, the control compound **2** (GM6001), tested side by side, displayed an IC_{50} value of 5.2 nM, in close agreement with that reported in the literature for this nonselective MMP inhibitor (Figure 4A). These data

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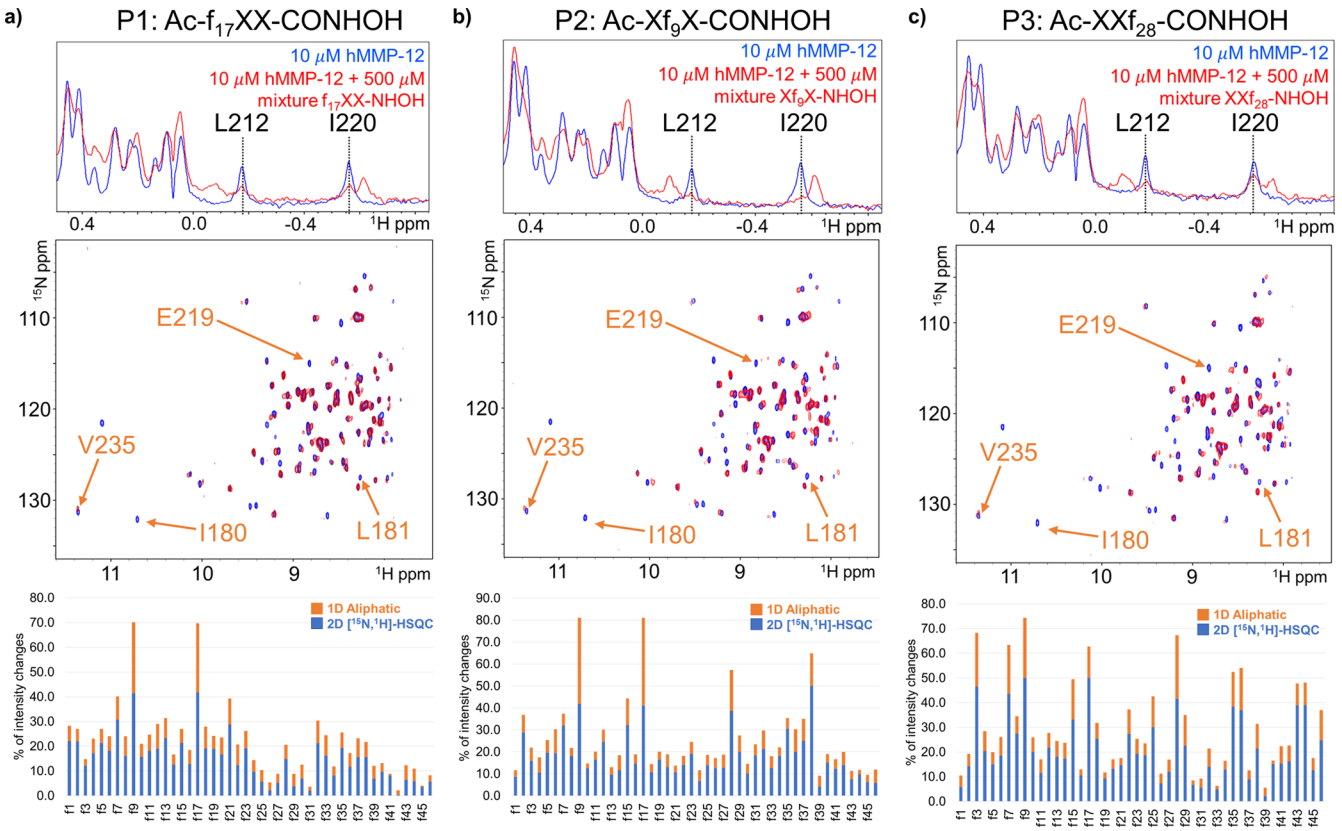

Figure 2. Application of the focused HTS by NMR strategy for targeting MMP-12. 1D-^{1}H-aliph and 2D-(^{15}N, ^{1}H) so-fast HMQC spectra of MMP-12 collected in the absence and presence of the indicated mixture with one fixed position in P1 (a), P2 (b), and P3 (c), respectively. The spectra were acquired with 10 μM hMMP-12 in the absence (blue) and presence (red) of 500 μM of each mixture. The mixture tested in panel (a) contained *O*-Bzl-L-serine in position P1 (f_{17}XX-CONHOH); the mixture in panel (b) contained a D-homophenylalanine in position P2 (Xf_9X-CONHOH); the mixture in panel (c) contained a D-phenylalanine in position P3 (XXf_{28}-CONHOH). At the bottom of panels (a–c) the percentage of NMR peak intensity changes are reported as a function of the fixed f_n element in position P1, P2, and P3, respectively, as described in the experimental procedures.

clearly suggested that the approach was successful in identifying a novel agent with nanomolar potency against the MMP-12 out of 100,000 molecules within the combinatorial library. Subsequently, in order to assess if the agent possessed also enhanced selectivity compared to GM6001, we tested compounds **1** and **2** side by side against a panel of closely related MMPs. When tested at 1 μM, compound **2** (GM6001) inhibited all the MMPs tested, again in agreement with the low selectivity of this control agent (Figure 4B). Likewise, compound **1** was very effective in inhibiting MMP-12 at 1 μM and at 55 nM. However, unlike compound **2** (GM6001), our agent only minimally inhibited MMP-1, MMP-9, MMP-13, and MMP-14 even at 1 μM, while it inhibited appreciably only the most closely related metalloproteinase (MMP-3, with ~78% inhibition at 1 μM and ~19% at 55 nM, respectively; Figure 4B).

To further rationalize the observed potency and selectivity of our agent, we performed molecular docking studies using Gold (Cambridge Crystallographic Data Centre; www.ccdc.cam.ac.uk) and the X-ray structures of various MMPs. In particular, compound **1** was docked into the binding pocket of hMMP-12 catalytic domain (PDB ID 5LAB), using constrain parameters for the coordination of the hydroxylamine moiety with the Zn^{2+} atom. These constraints uniquely placed the ligands in the binding site, hence avoiding the requirement of obtaining further experimental constrains. The obtained docked model of compound **1** was superimposed into the X-ray structures of the other MMPs (the PDB IDs used for MMP-1, MMP-3, MMP-9, MMP-13, and MMP-14, were 1HFC, 4G9L, 1GKC, 4JP4, and 1BQQ, respectively). While the surroundings of the metal ion seemed fairly well conserved among these proteinases, hence, because all able to accommodate the P3 residue close to the hydroxylamine moiety, surface variations were more evident in areas that would be occupied by the P1 residue. For example, docking compound **1** in the X-ray structures of MMP-12 and the closely related MMP-1 (50.8% sequence identity in the catalytic domain) (Figures 4C,D), revealed that the substructures in P3 and P2 could occupy equivalent sites in both enzymes, close to the metal ion, while the P1 moiety of the compound protruded in a more peripheral area that was quite different within the tested MMPs (Figure 4C,D and Supplementary Figure S2), hence likely conferring the observed selectivity to our compound. Indeed, in the current pose, a poor fit is found between the residues in P1 and even P2 of compound **1** with MMP-1, corroborating the experimentally determined selectivity of the agent (Figure 4C,D).

The search of novel potent and selective metalloenzyme inhibitors has been fervid in the past two decades, and most studies pointed at selectivity as one of the likely most desirable properties of MMP inhibitors.[31] Thus, novel technologies that address potency and selectivity are still needed. In these regards, active research in the field include the search for allosteric inhibitors[32] or in deriving novel metal chelating groups.[33−37] MMP-12 is involved in inflammatory processes in

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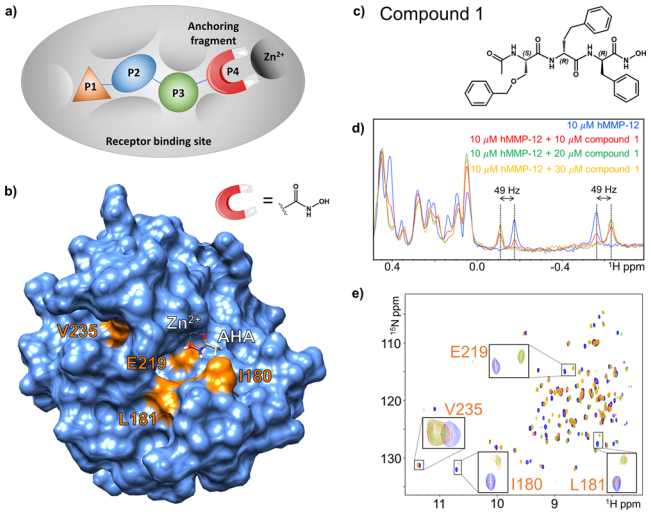

Figure 3. Identification of MMP-12 inhibitor compound **1**. (a) Schematic representation of the MMP-12 targeting focused positional scanning library. (b) Surface representation of the crystal structure of the catalytic domain of hMMP-12 in complex with acetohydroxamic acid (PDB ID 1Y93). The residues within the binding pocket that are indicated and highlighted in orange have been used to rank and select f_n elements for each position. (c) Chemical structure of compound **1** obtained by the combination of the f_n elements for each position ($f_{17}f_9f_{28}$-CONHOH), which induced the largest perturbations in the spectra of MMP-12 (Figure 2). (d) NMR 1D-^{1}H-aliph spectra of hMMP-12 (10 μM) collected in the absence (blue) and presence of 10 μM (red), 20 μM (green), and 30 μM (yellow), respectively, of compound **1**. Changes in intensity of the aliphatic signals are typical of a slow-exchange regime, and with a separation of ~50 s^{-1} between the unbound and bound form. (e) 2D-(^{15}N, ^{1}H) so-fast HMQC spectra hMMP-12 (10 μM) collected in the absence (blue) and presence of compound **1** at 10 μM (red), 20 μM (green), and 30 μM (yellow), respectively. The four residues within the binding pocket are highlighted. Slow exchange in the NMR time scale was also observed in these spectra.

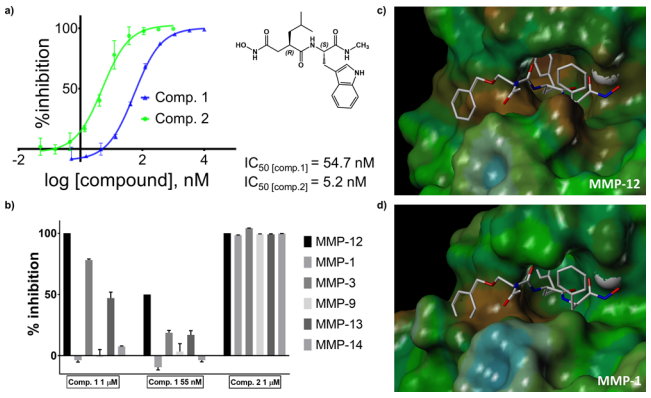

Figure 4. Enzyme inhibition and structural basis for selectivity of compound **1**. (a) FRET-based dose response curves for compound **1** (blue) and for the reference compound **2** (GM6001, green). IC_{50} values of 54.7 and 5.2 nM for compounds **1** and **2**, respectively, were obtained. The chemical structure of reference compound **2** is also shown. (b) Enzyme inhibition studies of compounds **1** and compound **2** against a panel of closely related MMPs. (c) Docked structure of compound **1** into the crystal structure of hMMP-12 catalytic domain (PDB ID 5LAB) using Gold (Cambridge Crystallographic Data Centre). (d) Docked pose of compound **1** as panel (c) but placed into the crystal structure of MMP-1 (PDB ID 1HFC).

asthma and in chronic obstructive pulmonary disease (COPD), and recent studies have reported the potential benefit of potent inhibitors of this enzyme in reducing the inflammatory response associated with exposure to cigarette smoke in mice,[38] while in humans with asthma and COPD,[39] MMP-12 gene variant activation was associated with disease severity. In a recent phase II trial, the dual MMP-12/MMP-9 inhibitor AZD1236 was tested in a randomized short trial (6 weeks) on moderate to severe COPD, and it showed an acceptable safety profile, although the therapeutic efficacy could not be demonstrated given the limited duration of the study.[40] In addition, FP-025 (Foresee Pharmaceutical) reported on an ongoing phase I trial of their MMP-12 inhibitor to assess the safety and pharmacokinetics. These studies and past experience with MMP inhibitors, underline that novel strategies to derive novel, potent, and selective MMP antagonists are still needed. Hence, we believe that our general approach as described in Figure 1, perhaps applied using other metal chelating agents[35,37] or other initial binding fragments, may support these endeavors. Finally, we are confident that the identified agent may be worthy of further iterative structure−activity relationship (SAR) studies aimed at further optimizing potency, selectivity, and ADME properties of this novel series for further pharmacological and efficacy studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00483.

Detailed experimental procedures, list of used elements of the library, and docked structures of compound **1** (PDF)

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Notes

The authors declare no competing financial interest.

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