Published in final edited form as:

Anal Biochem. 2019 February 15; 567: 30–37. doi:10.1016/j.ab.2018.12.004.

Development of high throughput screening methods for inhibitors of ClpC1P1P2 from *Mycobacteria tuberculosis*

Hugo Fraga^{a,b,c,d,*}, Beatriz Rodriguez^a, Ana Bardera^a, Concha Cid^a, Tatos Akopian^e, Olga Kandror^e, Annie Park^e, Gonzalo Colmenarejo^a, Joel Lelievre^a, and Alfred Goldberg^a

^aDiseases of the Developing World, Global Health R&D, GlaxoSmithKline, Severo Ochoa 2, 28760 Tres Cantos, Spain

^bDepartment Cell Biology, Harvard Medical School, USA

^cInstitut de Biologie Structurale, Grenoble, France

^dDepartamento de Bioquímica, Faculdade de Medicina da Universidade do Porto, Portugal

^eSchool of Public Health, Harvard Medical School, USA

Abstract

Tuberculosis affects about 100 million people worldwide and causes nearly 2 million deaths annually. It has been estimated that one third of all humans is infected with latent *Mycobacterium tuberculosis* (*Mtb*). Moreover, *Mtb* has become increasingly resistant to available antibiotics. Consequently, it is important to identify and characterize new therapeutic targets in *Mtb* and to synthesize selective inhibitors. ClpP1, ClpP2 and their associated regulatory ATPases, ClpX and ClpC1 are required for the growth of *Mtb* and for its virulence during murine infection and are highly attractive drug targets, especially since they are not present in the cytosol of mammalian cells, and they differ markedly from the mitochondrial ClpP complex. The importance of these proteins in *Mtb* is emphasized by the existence of several natural antibiotics targeting this system. In order to find new inhibitors of ClpC1P1P2 system, we developed an assay based on the ATP-dependent degradation of a fluorescent protein substrate. The hits obtained were further characterized with a set of secondary assays to identify precise targets within a complex. A large library of compounds was screened and led to the identification of a ClpC1 ATPase inhibitor demonstrating that this approach can be used in future searches for anti-TB agents.

Keywords

Mycobacterium tuberculosis; ClpP; ClpC1; ClpX; Screening

1. Introduction

Despite extensive efforts to combat Tuberculosis (TB) each year, ten million people are infected, and two million die from TB. Furthermore, TB has become increasingly resistant to available antibiotics, and current therapeutics remain largely ineffective containing the

* to whom correspondence should be addressed: hfraga@med.up.pt.

disease. Therefore, it is important to identify new targets and to find selective inhibitors. Ideal targets should be enzymes that are essential for bacterial viability and differ markedly from human enzymes. Among the most interesting anti TB drug targets to emerge in the past decade is the proteolytic complex formed by ClpP1P2 and its specific activators ClpX and ClpC1 [1,2].

First described by the Maurizi and Goldberg labs 40 years ago [3,4], ClpP is a tetradecameric structure composed of two heptameric rings forming a hollow cylinder with 14 proteolytic sites compartmentalized within its central chamber. While ClpP alone is able to rapidly hydrolyse peptides, the degradation of large proteins requires the presence of an hexameric AAA+ ATPase complex, such as ClpA, ClpX or ClpC. These ATPases activate the Clp Proteases, but also bind proteins substrates, unfold them, and translocate them into the proteolytic compartment. Unlike most bacteria and mitochondria, *Mtb* contains two *clp* genes, *clpP1* and *clpP2*, both of which are essential for viability and infectivity in *Mtb* [1]. Recently, we showed that the active enzyme is a 2-ring tetradecameric complex [2]. While neither ClpP1 nor ClpP2 by itself has proteolytic activity, when mixed together in the presence of a dipeptide activator, they form the active complex containing one ClpP1 and one ClpP2 ring. Whereas the mechanism of this activation is still unclear, Xray structures of the complex in the presence of the activator Benzoyl-Leu-Leu (Bz-LL), an N-terminally blocked dipeptide, show that it binds in opposite orientations in ClpP1 and ClpP2 [5]. While in ClpP1, Bz-LL binds with the C-terminal leucine side chain in the S1 pocket. One C-terminal oxygen is close to the catalytic serine, whereas the other contacts backbone amides in the oxyanion hole, in ClpP2, Bz-LL binds with the benzoyl group in the S1 pocket, and the peptide hydrogen bonded between parallel strands [5].

In addition to genetic evidence that these four Clp proteins are essential for viability, the relevance of ClpC1 and ClpP1P2 as *Mtb* targets has been reinforced by the recent discovery of several natural antibiotics that kill *Mtb* targeting this system. Antibiotics like ecumicin [6], cyclomarin [7] and lassomycin [6] are among the most powerful anti TB molecules to emerge recently. However, due to their complex multiring structures, these natural compounds are challenging for medical chemistry. In another approach, peptide boronates designed based on the preferred substrate sequences of ClpP1P2 and able to inhibit ClpP protein degradation have also displayed interesting anti *Mtb* properties with MIC₅₀ in the low micromolar range [8]. Considering the chemical diversity and the variety of mechanisms of action displayed by ClpC1 and ClpP1P2 inhibitors, it is likely that small molecules with more amenable chemical properties will also be able to block ClpP1P2 activity and represent valid drug candidates. Furthermore, as ClpP is important for the virulence of other pathogenic bacteria as *Listeria monocytogene* [9] and *Staphylococcus aureus* [10] and mitochondrial ClpP was recently associated to human acute myeloid Leukemia and obesity [11,12], new ClpP inhibitors could also be useful for the treatment of other human pathologies.

With the objective of enlarging the collection of ClpP inhibitors as chemical biology tools and to identify novel pharmacological leads, we developed a sensitive assay for the ATP-dependent degradation of fluorescent proteins by ClpP1P2 together with the ClpC1

hexameric ATPase complexes. This assay was complemented with a set of secondary assays for further selection.

2. Materials and Methods

2.1 Protein expression and purification

ClpP⁻ *E. coli* B121 carrying plasmids encoding ClpP1 and ClpP2 [13] and *E. coli* B121 carrying a ClpC1 plasmid [6] were used for recombinant expression. Proteins were grown and expressed using autoinduction media in 30 liters fermenters at a constant temperature of 30 °C for ClpP1 and ClpP2 and 16 °C for ClpC1. ClpC1 yield was particularly sensitive to the expression temperature used. Proteins ClpP1, ClpP2 and ClpC1 were purified as described previously [2]. GFPssra was expressed and purified as described [14].

2.2 GFPssra degradation by ClpC1P1P2 and ClpXP1P2

ClpC1P1P2 GFPssra degradation was measured taking advantage of the intrinsic fluorescence of GFPssra. The standard protocol for 384 well plates (Corning 3820) consisted of 2 steps. Unless otherwise noted all concentrations refer to the final volume of 15 μ l. A first step where ClpC1 (0.4 μ M hexamer), ClpP1P2 (0.7 μ M tetradecamer), the activator Bz-LL (2.5 mM) [5], GFPssra (5.5 μ M) in HEPES pH 7.6 (20 mM, Sigma), KCl (100 mM, Sigma), Triton X-100 (0.22 mM, Sigma) and MgCl₂ (40 mM, Sigma) in total volume of 10 μ l were incubated for at least 15 minutes with the test compound (max DMSO concentration of 4%). In a second step 5 μ l of ATP (10 mM, buffered to pH 7.6) or water (column 18) were added. The mixture was rapidly centrifuged and fluorescence decay as a result of GFPssra degradation was measured in a ViewLux Microplate Imager (Perkin Elmer) using 480 $\pm$ 20 nm for excitation and 540 $\pm$ 25 nm for emission. Protein degradation was measured after incubation for 120 minutes at 23 °C (ViewLux does not allow temperature control). For the calculation of the reaction rate, a lag period of 10 minutes was excluded, and only the linear range of the curve was considered. Following HTS, GFPssra degradation by ClpC1P1P2 was also measured using an excitation wave length of 440 nm and emission of 509 nm (cutoff 495 nm, PTM medium), at 37 °C for 1 hour in Spectramax M5 (Molecular Devices). In the case of ClpXP1P2, instead of ClpC1, ClpX was used to activate proteolysis. The protein concentrations used were: ClpX 0.3 μ M hexamer, ClpP1P2 0.15 μ M tetradecamer and GFPssra 0.15 μ M.

2.3 ClpP1P2 peptidase Assay

The ClpP1P2 Peptidase assay quantified the hydrolysis of the peptide PKM-amc by ClpP1P2 in the presence of Bz-LL. Unless otherwise noted all concentrations refer to the final volume of 15 μ l. In all plate columns (Corning 3820) except 18, we dispensed 9 μ l mixture containing ClpP1P2 complex (15 nM) and Bz-LL (2.5 mM) in Tris pH 7.6 (50 mM, Sigma), KCl (100 mM, Sigma), Triton X-100 (0.22 mM, Sigma). In column 18, the negative control, the ClpP1P2 complex was excluded. In a second step, 6 μ l of the substrate PKMamc to a final concentration of 10 μ M were added, the plate briefly centrifuged, and the increase in fluorescence, excitation 350 nm and emission 450 nm (cutoff 435 nm, PTM medium), was measured at 37 °C for 1 hour in a Spectramax M5 (Molecular Devices).

2.4 ClpC1 ATPase Assay

The ATPase assay quantified basal ATP hydrolysis by ClpC1. Unless otherwise noted all concentrations refer to the final volume of 15 μ l. In an UV/Vis transparent plate (Greiner 784101), we dispensed a 10 μ l mixture containing ClpC1 (90 nM), phosphoenolpyruvate (1mM, Sigma), DTT (1 mM, Sigma), NADH (0.3 mM, Sigma), PK/LD (20 U/ml, Sigma), $MgCl_2$ (4 mM, Sigma), Tris (50 mM pH 7.6, Sigma), KCl (100 mM, Sigma), Triton X-100 (0.216 mM, Sigma). In a second step 5 μ l of ATP (1 mM, buffered to pH 7.6) or water (column 18) were added. The mixture was rapidly centrifuged, and absorbance at 340 nm was monitored at 37 °C for 2h in a Spectramax M5 (Molecular Devices).

2.5 ClpC1P1P2 FITC-casein Assay

FITC degradation by ClpC1 and ClpP1 and ClpP2 was measured taking advantage of the increase in fluorescence as quenched fluorescein is released. The standard protocol for 384 plates (Corning 3184) consisted of 2 steps (unless otherwise notices all concentrations refer to the final volume of 15 μ l). A first step where ClpC1 monomer (2.5 μ M), ClpP1P2 monomer (10 μ M), the activator Bz-LL (2.5 mM) [5], FITC-casein (5.5 μ M) in HEPES pH 7.6 (50 mM, Sigma), KCl (100 mM, Sigma), Triton X-100 (0.22 mM, Sigma) and $MgCl_2$ (40 mM, Sigma) in total volume of 10 μ l were incubated for at least 15 minutes with the test compound (max DMSO concentration of 4%). In a second step ATP (10 mM, buffered to pH 7.6) or water (column 18) were added. The mixture was rapidly centrifuged and fluorescence increase as a result of FITC-casein degradation was measured using excitation 440 nm and emission 509 nm (cutoff 495 nm, PTM medium), at 37 °C for 1 hour in Spectramax M5, Molecular Devices.

2.6 HepG2 cytotoxicity assay

Actively growing HepG2 cells were removed from a T-175 TC flask using 5 mL Eagle's MEM (containing 10% FBS, 1% NEAA, 1% penicillin/streptomycin) and dispersed in the medium by repeated pipetting. Seeding density was checked to ensure that new monolayers were not >50% confluent at the time of harvesting. Cell suspension was added to 500 mL of the same medium at a final density of 1.2×10^5 cells.mL⁻¹. This cell suspension (25 μ L, typically 3000 cells per well) was dispensed into the wells of 384-well clear-bottom plates (Greiner, 781091). Prior to addition of the cell suspension, the screening compounds (250 nL) were dispensed into the plates with an Echo 555 Liquid Handler (LabCyte). Plates were allowed to incubate at 37°C at 80% relative humidity for 48 h under 5% CO₂. After the incubation period, the plates were allowed to equilibrate at room temperature for 30 min before proceeding to develop the luminescent signal. The signal developer, CellTiter-Glo (Promega), was equilibrated at room temperature for 30 min and added to the plates (25 μ L per well) using a Multidrop dispenser (ThermoFisher). The plates were left for 10 min at room temperature for stabilization and were subsequently read using a ViewLux Microplate Imager (Perlin Elmer).

3. Results and Discussion

3.1 Assay Development

ClpC1, ClpP1 and ClpP2 are highly promising drug targets. Our goal was the elaboration of a specific assay suitable for high-throughput screening (HTS) for the identification of inhibitors of these enzymes. In the presence of Bz-LL, independently purified Clp1 and Clp2 assemble *in vitro* into a ClpP1P2 complex able to degrade small peptides [2]. The degradation of these small peptides, when coupled to a fluorescent group as 7-amino-4-methylcoumarin (amc), allows the measurement of ClpP1P2 peptidase activity [2]. Indeed, as peptidyl-amc shows negligible fluorescence, when ClpP cleaves the bond between the peptide moiety and amc, strongly fluorescent amc is released, and the enzymatic reaction is traced by measuring the alteration of fluorescence of the reaction mixture [15]. Despite its simplicity, this approach, which was used in a previous screen for *S. aureus* ClpP inhibitors [16], has limitations. First, *in vivo* ClpP is not a peptidase, and the peptides occupy a smaller region on the enzyme than the full-length substrate protein, which probably reduces the chance to identify non-active site inhibitors. Second, the peptidase assay uses only the protease part of the system, it does not allow the identification of compounds that inhibit ClpP associated ATPases, ClpX and ClpC1 or that bind to chaperone-ClpP interface. Finally, some compounds might, as the natural antibiotics acyledepsipeptides (ADEPS) bind to the ATPase-ClpP interface open the axial pore of the protease – and will not block peptide hydrolysis but activate it [17,18]. As an alternative, the incapacity of latent *E. coli* ClpP to efficiently degrade proteins was also explored with moderate success to identify potential ADEP-like activators of ClpP [19,20].

In order to screen for inhibitors of the complex of ClpC1 and ClpP1P2 from *Mtb*, we proposed to develop an assay based on the ATP-dependent degradation of a full protein by the ATPase protease complexes. This assay would allow a proper identification of compounds able to interfere with the multiple steps in protein degradation, a complex process that includes: protein binding ClpC1 or ClpX, unfolding dependent on its ATPase activity, ClpC1 and ClpX ClpP1P2 association, and protein degradation by ClpC1P1P2. Moreover, the ClpP system is characterized by a high degree of allosteric and conformational control between the ATPase and ClpP, a complexity that can only be explored with an assay that includes simultaneously both its components. We and others previously described that together with ClpX, ClpP1P2 degrades GFPssrA-like ClpXP from *E. coli* [2,21]. GFP offer advantages over other substrates due to its intrinsic fluorescence, which can be used as a direct reporter for activity. At the time this work was carried out no ClpC1 specific recognition signal was known, but reports from other groups suggested that ClpC1 could also recognize GFPssra and catalyze its unfolding and degradation in association with ClpP1P2 through less efficiently than ClpX [21]. Taking this in consideration, we first tested if GFPssra could be used as an ClpC1P1P2 substrate. Indeed, despite the low substrate affinity compared to ClpX [21], ClpC1 in association with ClpP1P2 in the presence of 2.5 mM of the BzLL activator could catalyze the degradation of GFPssra with an apparent K^M of $11.3 \pm 1.5 \mu\text{M}$ GFPssra (Fig1a). The ATP dependence for ClpC1P1P2 GFPssra degradation displayed evidence of cooperativity with half maximal

activity at 1.4 ± 0.1 mM (Fig1b, 1c), a value significantly lower than the one previously described for ClpC1 ATPase activity [5,21].

While the drop in GFP fluorescence provided was measurable, the low affinity of ClpC1 for GFPssra was problematic for assay development. Indeed, as the concentration of GFPssra increases, deviations occur, and the plot of emission against (Fig1d) fluorophore concentration becomes non-linear. In addition, even with large concentrations of ClpC1P2 and saturating ATP the final GFPssra fluorescence after 90 minutes was still 35% of the initial value, which limited the assay window for an endpoint assay (Fig 1b). To resolve these limitations, we established a kinetic assay where the change in fluorescence readout over time was measured. For this purpose, limiting the detection of ATP-competitive inhibitors, we employed high concentrations of ATP (10 mM) coupled to high concentrations of ClpC1 and ClpP1P2. This kinetic approach offered several advantages: it significantly increased Z' compared the endpoint mode assay (0.29 end point versus 0.7-0.8 in the kinetic mode), it excluded autofluorescence compounds (false positives) and eliminated assay artifacts due to differences in initial dispensed volumes. Further, we adapted the assay for HTS format using a 2-step protocol adapted to automatic dispensers and 384 well plates (Fig 2a). The protocol included a first step where the GFPssra, ClpC1 and the ClpP1P2 were mixed in the presence of Bz-LL in the absence of ATP. Although active ClpP1P2 will be formed under these conditions, without ATP ClpC1 is unable to form an active hexamer, and no GFPssra degradation is observed. In a second step – following incubation with the test compound (or DMSO), ATP was added to the mixture, the samples were centrifuged to remove air bubbles and settle the mixture, and the drop in GFPssra fluorescence was measured at room temperature (23 °C). The degradation rate was calculated from the fluorescence measured at different time points. As a proof of concept, to evaluate the feasibility of our assay for HTS we proceeded validating the GFPssra assay using Bortezomib, an inhibitor of the proteasome that also targets ClpP1P2 [22] and 3,4-Dichloroisocoumarin (DCC), a non-specific serine protease inhibitor [23]. Both compounds could be reproducibly detected when randomly placed in a 384 well plate. Using the same protocol, we could obtain dose response curves for DCC and the ClpC1 inhibitor ecumicin [6]. Curiously the maximal response obtained for ecumicin was never 100% with 20% of the residual activity remaining. Shown in figure 2b are the dose response curves for DCC and ecumicin.

3.2 High- throughput screening

Having established a robust protocol, we tested the assay against the GSK compound collection. As the protein available was insufficient for screening the entire GSK library of compounds, which includes over 1.8 million compounds, a smaller set of compounds representative of the diversity of the GSK collection was selected. The selection criteria are described in the flow chart in Fig 3a. In addition, we also screened known inhibitors of *Mtb* (the 'TB Box' set) [24,25], a selection of compounds previously described as capable of inhibiting the growth of BCG or *Mtb*. The libraries were screened using a single dose of 40 μ M of the test compound. Positive hits were defined as the compounds whose signals were at least three standard deviations from the mean of the general sample population corresponding to 12.3% inhibition. The excellent quality of the screening setup was shown

by the Z-factor parameter that averaged 0.81 (Fig 3b). From the 2208 compounds identified in the primary screen, 476 were confirmed in duplicate. Following hit confirmation, we selected a list of 19 compounds that caused more than a 50 % inhibition at the concentration of 40 μ M for further hit characterization.

Hit follow-up characterization included 3 components: evaluation of toxicity versus mammalian cells (HepG2), inhibition of *Mtb*H37rV growth [24], and biochemical characterization using a set of secondary assays (described below). The evaluation of toxicity versus mammalian cells and the inhibition of *Mtb* H37Rv growth were used to define the selectivity window of the hits obtained – this defines how good the hit inhibits *Mtb* growth without being toxic to a mammalian cell line as previously described [25]. Biochemical characterization included assays to identify the specific targets of the hits in the ClpC1P1P2 complex. The assays used were: 1) a peptidase assay 2) ATPase assay 3) FITC-casein assay and 4) ClpX assay.

3.3 Peptidase Assay

As referred above, in the absence of a regulatory ATPase, ClpP1P2 complex can catalyse the hydrolysis of small peptides that can diffuse into the proteolytic chamber. In order to identify the inhibitors that acted specifically on ClpP1P2, we adapted a peptidase assay to HTS format [8]. In this concern, we selected the substrate, Ac-Pro-Lys-Met-amc (PKMamc), with a high specificity constant k_{cat}/K_M [8]. As PKMamc (10 μ M) is hydrolysed by ClpP1P2, the increase in fluorescence as a result of coumarin release is quantified and inhibitors identified by a reduction in the reaction rate (Fig 4a). The excellent quality of this secondary screening setup is reflected by the Z-factor parameter that averaged 0.85.

3.4 ATPase Assay

To identify compounds that reduce the hydrolysis of ATP by ClpC1, we measured the release of ADP as a result of ClpC1 basal ATPase activity [2,26]. This ADP is regenerated into ATP by the coupled pyruvate kinase (PK) and lactate dehydrogenase LDH) enzymes, which results in the consumption of NADH and the formation of NAD⁺. The difference in the absorption between the oxidized and reduced forms allows the reaction to be followed by monitoring the drop-in absorbance at 340 nm (Fig 4b). The quality of this secondary screening setup is reflected by the Z-factor parameter that averaged 0.66.

3.5 FITC-casein Assay

Previous reports have shown that ClpC1 is able to recognize intrinsically unfolded proteins with exposed hydrophobic patches [5]. As described for other members of the AAA+ family [27], the recognition of these proteins by ClpC1 is likely different from the recognition and degradation of tightly folded proteins, such as GFPssra [27]. In order to possibly discern hits that act specifically on substrate recognition by ClpC1, we replaced GFPssra, a very stable and folded protein by casein, an intrinsically disordered protein. The assay employed FITC-casein as the proteolytic target. When FITC-casein is intact, FITC fluorescence is quenched. Protease-catalyzed hydrolysis of FITC-casein relieves this quenching, yielding highly fluorescent dye-labeled peptides. The excellent quality of this secondary screening setup is reflected by the Z-factor parameter that averaged 0.76 (Fig 4c).

3.6 ClpX Assay

Although ClpX and ClpC1 are both able to associate with ClpP2, the two complexes are functionally and structurally quite different. While ClpC1 has two AAA+ ATPase domains, ClpX contains only one. In addition their N-terminal domains are not conserved. In order to evaluate the specificity of the hits for the two ATPases, we tested the activity of the inhibitors towards ClpX [8]. ClpX assay used as substrate its standard substrate GFPssra, whose hydrolysis was measured as described for the Clp1 assay (fig 4d).

3.7 Further Analysis of Hits

While some of the compounds displayed low toxicity against a mammalian cell line, *in vitro* none of the compounds was able to inhibit growth of *Mtb*H37Rv strain (see table 1). Characterization of the hits revealed that a striking and alarming feature of the large majority of the hits was the lack of a specific inhibition profile (Table 1). Indeed, while the ATPase assay and peptidase assay report on very different targets and enzymatic activities, several of the hits were positive in both assays. We were aware that broad activity in distinct assays is a feature of pan-assay interference compounds (PAINS), promiscuous compounds that are often positive in several HTS efforts. There is a growing interest in both assay interference and promiscuous enzymatic inhibition, because the further investigation of such nonspecific compounds, in some cases all the way to clinical assays, represents a major problem in drug development [28-30]. Methods to identify PAINS include knowledge-based methods, for example, checking if the compounds are frequent hits in previous and unrelated assays, coupled to further orthogonal assays or controls. Analyzing GSK database we verified that 15 of the hits obtained in our assay were frequent hits in several GSK assays. While there are multiple causes for such promiscuous behavior, (aggregation, chelation, sample impurities, etc), the most frequent and easy to test is the non-selective reactivity of the hits with proteins by redox or singlet oxygen production with the addition of a reductant, likely DTT or TECP [31]. Accordingly, when the effect of reducing agents (DTT 10 mM) on the inhibition of the some of the hits was tested their activity was abolished. Therefore, cysteine reactivity or singlet oxygen formation was responsible for the compound's activity and explains the apparent lack of selectivity in the secondary assays (Fig 5a). For this reason, our selection strategy focused on eliminating hits with potential promiscuous behavior, either based on previous scientific data or an absence of activity in the presence of DTT. As shown in table 1, from the 19 compounds selected only 2 compounds fulfilled these conditions. Of the 2 hits, one displayed clear characteristics of an ATPase inhibitor with an IC₅₀ of 49 μ M (GSK hit 18), while the other, though showing activity against GFPssra and FITC-casein degradation, did not display clear inhibition in any of the secondary assays (GSK hit 17) suggesting a novel mechanism of action.

4. Conclusions

Because no specific substrates for ClpC1 were known when this work was initiated, we selected, GFPssra as a substrate. Using GFPssra offered the advantage of potentially allowing the identification of inhibitors that could act by disturbing substrate unfolding, but came at the expense of a requirement for high amounts of protein in the assay. Inherently protein degradation by the ATPase-ClpP complex is not a very sensitive assay. In prior

studies, micromolar concentrations of ClpP monomer and associated ATPase [8,10,21,32] together with almost stoichiometric concentrations of substrate are used repeatedly and this obviously imposes a limit on the amount of test compound required to produce noticeable inhibition. Moreover, as we approach the micro molar range for a compound IC₅₀, the role of interferences, PAINS or sample impurities become relevant. In this context, a further test is extremely relevant to exclude false positives. Here we adapted to HTS four new secondary assays to follow the GFPssra ClpC1P1P2 assay. Given the excellent Z' value obtained and the easy adaptation to the HTS format, each could *per se* be independently used as a screening tool. While these secondary assays were initially meant to map the specific target of the hits obtained, they were extremely helpful in the exclusion of several nonspecific compounds whose activity was later associated with non-selective reactivity by redox or single oxygen reactions. For two of the hits obtained, this non-selective activity derives not from the test compound but from the salt in the compound mixture (Au³⁺ and Ni²⁺). As the recent example of acamprosate shows, where biological activity derived from the Ca⁺² and not the drug, care must be taken with compound formulation, and effects of supposedly inert compounds on the assay [33].

The hits that passed our filters did not display activity against *Mtb* but represent 2 new chemical entities with no aparent similarities with previously identified AAA+ or ClpP inhibitors [34]. Further studies are however required to fully understand their mode of action. Indeed, while GSK18 is a novel ClpC1 ATPase inhibitor, GSK17 did not display clear inhibition for ATPase or peptidase activities therefore suggesting a novel mechanism of action. We plan to further characterize these compounds identifying their binding site and testing their activity against other AAA+ ATPases and bacteria. The identification of these 2 new inhibitors demonstrates the success of our approach and it is extremely likely that by increasing the screening library other molecules with interesting characteristics will be found. It is noteworthy that a similar approach, recently published using a similar assay against the ClpXP degradation machine of *S. aureus*, did as we speculate result in molecules able to reduce the organism's virulence [10]. Also, recently *Liu et al* identified using *in silico* screening a group of pyrroles able to inhibit ClpP1P2 with interesting anti *Mtb* growth properties [35].

Acknowledgment

The research behind these results received support from Tres Cantos GSK Open Lab Foundation and R01GM51923 from NIH to AG. The authors gratefully acknowledge Paco de Dios for technical assistance. HF is a Tres Cantos GSK Open Lab Foundation COFUND recipient.

References

- [1]. Raju RM, Unnikrishnan M, Rubin DHF, Krishnamoorthy V, Kandror O, Akopian TN, et al., Mycobacterium tuberculosis ClpP1 and ClpP2 Function Together in Protein Degradation and Are Required for Viability in vitro and During Infection, PLoS Pathog. 8 (2012) e1002511–8. doi: 10.1371/journal.ppat.1002511. [PubMed: 22359499]
- [2]. Akopian T, Kandror O, Raju RM, Unnikrishnan M, Rubin EJ, Goldberg AL, The active ClpP protease from M. tuberculosis is a complex composed of a heptameric ClpP1 and a ClpP2 ring, The EMBO Journal. 31 (2012) 1529–1541. doi:10.1038/emboj.2012.5. [PubMed: 22286948]

- [3]. Hwang BJ, Park WJ, Chung CH, Goldberg AL, Escherichia coli contains a soluble ATP-dependent protease (Ti) distinct from protease La, Proc Natl Acad Sci USA. 84 (1987) 5550–5554. [PubMed: 3303028]
- [4]. Katayama-Fujimura Y, Gottesman S, Maurizi MR, A multiple-component, ATP-dependent protease from Escherichia coli, J Biol Chem 262 (1987) 4477–4485. [PubMed: 3549708]
- [5]. Li M, Kandror O, Akopian T, Dharkar P, Wlodawer A, Maurizi MR, et al., Structure and Functional Properties of the Active Form of the Proteolytic Complex, ClpP1P2, from Mycobacterium tuberculosis, J Biol Chem 291 (2016) 7465–7476. doi:10.1074/jbc.M115.700344. [PubMed: 26858247]
- [6]. Gao W, Kim J-Y, Anderson JR, Akopian T, Hong S, Jin Y-Y, et al., The cyclic peptide ecumicin targeting ClpC1 is active against Mycobacterium tuberculosis in vivo, Antimicrob. Agents Chemother 59 (2015) 880–889. doi:10.1128/AAC.04054-14. [PubMed: 25421483]
- [7]. Schmitt EK, Riwanto M, Sambandamurthy V, Roggo S, Miault C, Zwingelstein C, et al., The Natural Product Cyclomarin Kills Mycobacterium Tuberculosis by Targeting the ClpC1 Subunit of the Caseinolytic Protease, Angew. Chem. Int. Ed 50 (2011) 5889–5891. doi:10.1002/anie.201101740.
- [8]. Akopian T, Kandror O, Tsu C, Lai JH, Wu W, Liu Y, et al., Cleavage Specificity of Mycobacterium tuberculosis ClpP1P2 Protease and Identification of Novel Peptide Substrates and Boronate Inhibitors with Anti-bacterial Activity, J Biol Chem 290 (2015) 11008–11020. doi:10.1074/jbc.M114.625640. [PubMed: 25759383]
- [9]. Gaillot O, Pellegrini E, Bregenholt S, Nair S, Berche P, The ClpP serine protease is essential for the intracellular parasitism and virulence of Listeria monocytogenes, Mol Microbiol 35 (2000) 1286–1294. [PubMed: 10760131]
- [10]. Fetzter C, Korotkov VS, Thänert R, Lee KM, Neuenschwander M, von Kries JP, et al., A Chemical Disruptor of the ClpX Chaperone Complex Attenuates the Virulence of Multidrug-Resistant Staphylococcus aureus, Angew. Chem. Int. Ed. Engl 56 (2017) 15746–15750. doi:10.1002/anie.201708454. [PubMed: 28906057]
- [11]. Cole A, Wang Z, Coyaude E, Voisin V, Gronda M, Jitkova Y, et al., Inhibition of the Mitochondrial Protease ClpP as a Therapeutic Strategy for Human Acute Myeloid Leukemia, Cancer Cell. 27 (2015) 864–876. doi:10.1016/j.ccell.2015.05.004. [PubMed: 26058080]
- [12]. Bhaskaran S, Pharaoh G, Ranjit R, Murphy A, Matsuzaki S, Nair BC, et al., Loss of mitochondrial protease ClpP protects mice from diet-induced obesity and insulin resistance, EMBO Reports. (2018) e45009–17. [PubMed: 29420235]
- [13]. Compton CL, Schmitz KR, Sauer RT, Sello JK, Antibacterial Activity of and Resistance to Small Molecule Inhibitors of the ClpP Peptidase, ACS Chem. Biol 8 (2013) 2669–2677. doi:10.1021/cb400577b. [PubMed: 24047344]
- [14]. Smith DM, Fraga H, Reis C, Kafri G, Goldberg AL, ATP binds to pro-teasomal ATPases in pairs with distinct functional effects, implying an ordered reaction cycle, Cell. 144 (2011) 526–538. doi:10.1016/j.cell.2011.02.005. [PubMed: 21335235]
- [15]. Zimmerman M, Yurewicz E, Patel G, A new fluorogenic substrate for chymotrypsin, Analytical Biochemistry. 70 (1976) 258–262. [PubMed: 1259147]
- [16]. Hackl MW, Lakemeyer M, Dahmen M, Glaser M, Pahl A, Lorenz-Baath K, et al., Phenyl Esters Are Potent Inhibitors of Caseinolytic Protease P and Reveal a Stereogenic Switch for Deoligomerization, J. Am. Chem. Soc 137 (2015) 8475–8483. doi:10.1021/jacs.5b03084. [PubMed: 26083639]
- [17]. Brötz-Oesterhelt H, Sass P, Bacterial caseinolytic proteases as novel targets for antibacterial treatment, International Journal of Medical Microbiology. 304 (2014) 23–30. doi:10.1016/j.ijmm.2013.09.001. [PubMed: 24119566]
- [18]. Gersch M, Famulla K, Dahmen M, Gbl C, Malik I, Richter K, et al., AAA+ chaperones and acyldepsipeptides activate the ClpP protease via conformational control, Nat Commun 6 (2015) 6320. doi:10.1038/ncomms7320. [PubMed: 25695750]
- [19]. Lavey NP, Coker JA, Ruben EA, Duerfeldt AS, Sclerotiamide: The First Non-Peptide-Based Natural Product Activator of Bacterial Caseinolytic Protease P, J. Nat. Prod 79 (2016) 1193–1197. doi:10.1021/acs.jnatprod.5b01091. [PubMed: 26967980]

- [20]. Leung E, Datti A, Cossette M, Goodreid J, McCaw SE, Mah M, et al., Activators of Cylindrical Proteases as Antimicrobials: Identification and Development of Small Molecule Activators of ClpP Protease, *Chem Biol* 18 (2011) 1167–1178. doi:10.1016/j.chembiol.2011.07.023. [PubMed: 21944755]
- [21]. Schmitz KR, Sauer RT, Substrate delivery by the AAA+ ClpX and ClpC1 un-foldases activates the mycobacterial ClpP1P2 peptidase, *Mol Microbiol* 93 (2014) 617–628. doi:10.1111/mmi.12694. [PubMed: 24976069]
- [22]. Moreira W, Ngan GJY, Low JL, Poulsen A, Chia BCS, Ang MJY, et al., Target Mechanism-Based Whole-Cell Screening Identifies Bortezomib as an Inhibitor of Caseinolytic Protease in Mycobacteria, *mBio* 6 (2015) e00253–15–13. doi:10.1128/mBio.00253-15. [PubMed: 25944857]
- [23]. Powers JC, Kam CM, Isocoumarin inhibitors of serine peptidases, *Meth. Enzymol* 244 (1994) 442–457. [PubMed: 7845225]
- [24]. Ballell L, Bates RH, Young RJ, Alvarez-Gomez D, Alvarez-Ruiz E, Barroso V, et al., Fueling open-source drug discovery: 177 small-molecule leads against tuberculosis, *ChemMedChem* 8 (2013) 313–321. doi:10.1002/cmdc.201200428. [PubMed: 23307663]
- [25]. Rebollo-Lopez MJ, Lelièvre J, Alvarez-Gomez D, Castro-Pichel J, Martínez-Jiménez F, Papadatos G, et al., Release of 50 new, drug-like compounds and their computational target predictions for open source anti-tubercular drug discovery, *PLoS ONE*. 10 (2015) e0142293. doi: 10.1371/journal.pone.0142293. [PubMed: 26642067]
- [26]. Kar NP, Sikriwal D, Rath P, Choudhary RK, Batra JK, Mycobacterium tuberculosis ClpC1, *Febs J*. 275 (2008) 6149–6158. doi:10.1111/j.1742-4658.2008.06738.x. [PubMed: 19016865]
- [27]. Erbse AH, Wagner JN, Truscott KN, Spall SK, Kirstein J, Zeth K, et al., Conserved residues in the N-domain of the AAA+ chaperone ClpA regulate substrate recognition and unfolding, *Febs J*. 275 (2008) 1400–1410. doi:10.1111/j.1742-4658.2008.06304.x. [PubMed: 18279386]
- [28]. Baell JB, Holloway GA, New Substructure Filters for Removal of Pan Assay Interference Compounds (PAINS) from Screening Libraries and for Their Exclusion in Bioassays, *J. Med. Chem* 53 (2010) 2719–2740. doi:10.1021/jm901137j. [PubMed: 20131845]
- [29]. Dahlin JL, Walters MA, How to Triage PAINS-Full Research, ASSAY and Drug Development Technologies. 14 (2016) 168–174. doi:10.1089/adt.2015.674. [PubMed: 26496388]
- [30]. Erlanson DA, Learning from PAINful lessons, *J. Med. Chem* 58 (2015) 2088–2090. doi:10.1021/acs.jmedchem.5b00294. [PubMed: 25710486]
- [31]. Lee H, Torres J, Truong L, Chaudhuri R, Mittal A, Johnson ME, Reducing agents affect inhibitory activities of compounds: Results from multiple drug targets, *Analytical Biochemistry*. 423 (2012) 46–53. doi:10.1016/j.ab.2012.01.006. [PubMed: 22310499]
- [32]. Leodolter J, Warweg J, Weber-Ban E, The Mycobacterium tuberculosis ClpP1P2 Protease Interacts Asymmetrically with Its ATPase Partners ClpX and ClpC1, *PLoS ONE*. 10 (2015) e0125345. doi:10.1371/journal.pone.0125345. [PubMed: 25933022]
- [33]. Spanagel R, Vengeliene V, Jandeleit B, Fischer W-N, Grindstaff K, Zhang X, et al., Acamprosate produces its anti-relapse effects via calcium, *Neuropsychopharmacology*. 39 (2014) 783–791. doi:10.1038/npp.2013.264. [PubMed: 24081303]
- [34]. Chapman E, Maksim N, de la Cruz F, Clair J, Inhibitors of the AAA+ Chaperone p97, *Molecules*. 20 (2015) 3027–3049. doi:10.3390/molecules20023027. [PubMed: 25685910]
- [35]. Liu P, Yang Y, Ju Y, Tang Y, Sang Z, Chen L, et al., Design, synthesis and biological evaluation of novel pyrrole derivatives as potential ClpP1P2 inhibitor against Mycobacterium tuberculosis, *Bioorg. Chem* 80 (2018) 422–432. doi:10.1016/j.bioorg.2018.06.004. [PubMed: 30005200]
- [36]. Hill AP, Young RJ, Getting physical in drug discovery: a contemporary perspective on solubility and hydrophobicity, *Drug Discov. Today*. 15 (2010) 648–655. doi:10.1016/j.drudis.2010.05.016. [PubMed: 20570751]
- [37]. Veber DF, Johnson SR, Cheng H-Y, Smith BR, Ward KW, Kopple KD, Molecular Properties That Influence the Oral Bioavailability of Drug Candidates, *J. Med. Chem* 45 (2002) 2615–2623. doi: 10.1021/jm020017n. [PubMed: 12036371]
- [38]. Young RJ, Green DVS, Luscombe CN, Hill AP, Getting physical in drug discovery II: the impact of chromatographic hydrophobicity measurements and aromaticity, *Drug Discov. Today*. 16 (2011) 822–830. doi:10.1016/j.drudis.2011.06.001. [PubMed: 21704184]

Figure 1.

a) The GFPssra degradation rates by ClpP1P2 in association with the ClpC1 and 10 mM ATP were measured by following the decrease in GFPssra intrinsic fluorescence as the protein is hydrolyzed. As a small decrease in fluorescence, likely due to protein plate absorption, was observed in the absence of ATP the initial rates were corrected for the decrease seen when ATP was not included. b) Degradation of GFPssra (5 μM) as a function of indicated ATP concentration. Notice the rapid inhibition and short linearity observed with low concentrations of ATP as a consequence of ADP inhibition. c) ATP dependence for the rates of degradation of GFPssra by ClpP1P2 in association with the chaperone ClpC1. d) With high concentrations GFPssra fluorescence showed deviations to linearity. A concentration of GFPssra below the apparent K_M was used for further assays.

Figure 2.

a) GFPssra C1P1P2 assay. Briefly, in a Corning 3820 plate previously loaded with the test compounds, a mixture containing all the reaction components with the exception of ATP column was added. Columns 6 and 18 were used for positive (no test compound) and negative (no test compound and later no ATP added) controls respectively. Following a 5 minutes incubation, ATP was added to a final concentration of 10 mM to all columns except column 18. The plates were then briefly centrifuged and GFPssra fluorescence was measured at different times. Inhibitors, an example show in the scheme, lead to a reduction in the slope calculated using the different time points. b) GFPssra ClpC1P1P2 assay was tested against previously identified inhibitors of ClpP1P2 (DCC IC_{50} 80 μ M, black circles), and ClpC1 (ecumicin IC_{50} 4 μ M, black triangles).

Figure 3).

a) GSK representative library selection. Starting with the entire GSK collection a set of filters were applied. In a first set only clusters whose families contained more than 5 members were selected. Following this first selection, compounds with good properties based on GSKs Property Forecast Index (PFI) defined as $PFI = (\text{Chrom log D7.4}) + (\# \text{ of aromatic rings})$ were selected [36-38]. Next, as the TbBox was screened independently those compounds were excluded and finally we selected compounds with up to 2 analogs based on GSKs greedy diversity selection algorithm. b) HTS progression cascade leading to the 2 new ClpC1P1P2 inhibitors. Compounds derived from GSK representative and GSK TB Box libraries were screened in singlet shot at 40 μM . Compounds above the assay threshold (3 standard deviations corresponding to 12.3 % inhibition) were confirmed in duplicate. Following confirmation, only compounds above 50 % inhibition were moved forward. Hit characterization included toxicity and secondary assays – see table 1 for more details.

Figure 4).

a) Peptidase assay was based on the degradation of the peptide PKMamc designed according to ClpP1P2 cleavage preferences[8]. b) The ATPase assay is based on the decrease in NADH absorbance as a consequence of ATP regeneration by the PK/LDH enzymes. c) FITC-casein assay is based on the degradation of casein by the complex. d) The degradation of GFPssra by ClpXP1P2 complex was used to evaluate the specificity of the hits toward ClpC1 versus ClpX.

Figure 5.

a) DTT abolishes the activity of several of the hits obtained. Shown is the dose-response curve for the inhibitor, GSK2 in the GFPssra ClpC1P1P2 assay in the presence (squares) and in the absence (circles) of DTT 10 mM. b) GSK17 is a new compound with an undetermined mechanism of action. GSK18 was characterized as a new ATPase inhibitor based on the absence of activity against ClpP1P2 peptidase activity and an IC_{50} of $42 \pm 7 \mu M$ in the ATPase assay.

Table 1.

Analysis of hits was based on several criteria. For the goal of identifying potential anti-TB drugs the activity of the hits obtained against the *Mtb* H37Rv and a mammalian cell line (HepG2) was tested. From the 19 compounds tested none was active (active criteria was a minimum inhibitory concentration, MIC₉₀, below 50 µM) while several of the hits were found toxic to the HepG2 cell line (active criteria pIC₅₀ above 4). In secondary assays, hits were considered active when they displayed at least 20% inhibition on a given assay. Because several hits displayed activity in both the ATPase activity and peptidase assay, indicating a possible promiscuous behavior, the history of the hit in previous GSK screens was verified. Compounds with multiple activities in other assays were considered non-specific hits. In addition, the effect of DTT 10 mM in the activity of the hits was tested, and compounds whose IC₅₀ was not significantly affected were considered as active in reducing conditions. Only hits with no promiscuous activity and active in a reducing environment were further considered.

nr	Activity H37Rv	Toxicity Hep	Peptidase Assay	ATPase Assay	FTICasein Assay	MtbClpX Assay	Activity other GSK Assays	Active in Reducing conditions	Selected
GSK 1	×	✓	✓	✓	✓	✓	Yes	No	No
GSK 2	×	×	✓	×	✓	✓	Yes	No	No
GSK 3	×	✓	✓	×	✓	✓	Yes	-	No
GSK 4	×	✓	✓	✓	✓	✓	Yes	No	No
GSK 5	×	✓	✓	✓	✓	✓	Yes	No	No
GSK 6	×	✓	✓	✓	✓	✓	Yes	-	No
GSK 7	×	✓	✓	×	✓	✓	Yes	No	No
GSK 8	×	✓	✓	✓	✓	✓	Yes	No	No
GSK 9	×	×	✓	✓	✓	✓	Yes	Yes	No
GSK 10	×	✓	✓	✓	✓	✓	Yes	No	No
GSK 11	×	✓	✓	✓	✓	✓	Yes	No	No
GSK 12	×	✓	✓	×	✓	✓	Yes	No	No
GSK 13	×	×	✓	×	✓	✓	Yes	No	No
GSK 14	×	✓	✓	×	✓	✓	No	No	No
GSK 15	×	✓	✓	✓	✓	✓	Yes	No	No
GSK 16	×	✓	✓	✓	✓	✓	No	No	No
GSK 17	×	✓	×	×	✓	×	No	Yes	Yes
GSK 18	×	✓	×	✓	✓	×	No	Yes	Yes
GSK 19	×	✓	✓	×	✓		Yes	No	No