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(54) Title: BORTEZOMIB AS AN INHIBITOR OF MYCOBACTERIAL CASEINOLYTIC PROTEASE (CLP) FOR TREATMENT OF TUBERCULOSIS

(57) Abstract: Methods and compositions for the treatment of *Mycobacterium tuberculosis* comprising Bortezomib, CEP-18770 (Delanzomib), Ixazomib (MLN-2238), Ixazomib citrate (MLN-9708), MG-262 and related compounds, analogs and derivatives are disclosed herein.



BORTEZOMIB AS AN INHIBITOR OF MYCOBACTERIAL CASEINOLYTIC PROTEASE (CLP) FOR TREATMENT OF TUBERCULOSIS

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/112,158, filed on February 4, 2015. This application also claims the benefit of U.S. Provisional Application No. 62/113,067, filed on February 6, 2015. The entire teachings of both applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] *Mycobacterium tuberculosis* (Mtb) is becoming a global health emergency that is rapidly worsening due to antibiotic resistance, resulting in nearly 2 million deaths annually, making it one of the leading causes of infectious disease mortality. It has been estimated that a third of all humans are infected with latent *Mycobacterium tuberculosis* (Mtb). Mtb has become increasingly resistant to available antibiotics, therefore, identifying new targets for drug development (*i.e.*, enzymes that are essential for viability of Mtb) and developing selective inhibitors of their function is essential.

SUMMARY OF THE INVENTION

[0003] The invention relates to a novel whole-cell active anti-mycobacterial molecule Bortezomib, an inhibitor of mycobacterial caseinolytic protease (Clp) for the treatment of tuberculosis. The said drug is identified as a human 26S proteasome drug and a potent inhibitor of ClpP1P2 in mycobacteria activity by high throughput screen. Bortezomib blocks degradation of caseinolytic protease substrate WhiB1 and further supports to exert its antibacterial activity via modulation of ClpP1P2. The invention demonstrates the feasibility of target mechanism-based whole cell screens, provides chemical validation of ClpP1P2 as target, and identifies a drug in clinical use as a new lead compound for tuberculosis.

[0004] Described herein are methods of treating tuberculosis, for example *Mycobacterium tuberculosis*, comprising administering to a subject (e.g., a human, such as a patient) in need thereof an effective amount of a caseinolytic protease inhibitor, such as a Bortezomib, CEP-18770 (delanzomib), MLN-2238, MLN-9708, MG-262, or any derivative thereof. In some embodiments, the caseinolytic protease is ClpP1P2. In some embodiments, the caseinolytic protease inhibitor MLN-9708. In some embodiments, the caseinolytic

protease inhibitor is administered in combination with an aminoglycoside. The aminoglycoside can be amikacin, streptomycin, or a combination thereof.

[0005] Also described are methods of inhibiting mycobacterial caseinolytic protease in a patient suffering from tuberculosis comprising administering to a patient in need thereof an effective amount of Bortezomib, CEP-18770 (delanzomib), MLN-2238, MLN-9708, MG-262, or any derivative thereof. In some embodiments, an aminoglycoside, such as amikacin, streptomycin, or a combination thereof, is also administered to the patient.

[0006] Also described are methods of blocking degradation of the substrate of ClpP121 comprising contacting ClpP1P2 with Bortezomib, CEP-18770 (delanzomib), MLN-2238, MLN-9708, MG-262, and all derivatives thereof. In some embodiments, the substrate is WhiB1.

[0007] Also described are methods of increasing sensitivity of *Mycobacterium* bacteria to an aminoglycoside in a subject who has *Mycobacterium tuberculosis* comprising administering Bortezomib, CEP-18770 (delanzomib), MLN-2238, MLN-9708, MG-262, or any derivative thereof. In some embodiments, the aminoglycoside can be amikacin, streptomycin, or a combination thereof.

[0008] Also described are methods of inhibiting mycobacterial caseinolytic protease activity comprising contacting mycobacterial caseinolytic protease with Bortezomib, CEP-18770 (delanzomib), MLN-2238, MLN-9708, MG-262, or any derivative thereof.

[0009] In some embodiments of the methods described herein, the methods further comprise administering to the patient one or more aminoglycoside(s). In some embodiments of the methods described herein, the methods further contacting ClpP1P2 with one or more aminoglycoside(s). In some embodiments, the aminoglycoside is amikacin, streptomycin, or a combination thereof. Other examples of aminoglycosides include gentamicin and tobramycin. In some embodiments, the aminoglycoside is a mistranslation-inducing aminoglycoside.

[0010] Also described herein are compositions for the treatment of *Mycobacterium tuberculosis* comprising an aminoglycoside and at least one caseinolytic protease inhibitor, e.g., a compound from the group consisting of Bortezomib, CEP-18770 (delanzomib), MLN-2238, MLN-9708, MG-262, or any derivative thereof. In some embodiments, the aminoglycoside can be amikacin, streptomycin, or a combination thereof.

[0011] The caseinolytic protease inhibitor and the aminoglycoside can be administered in appropriate dosages by any appropriate mode of administration. In some embodiments, streptomycin is administered daily as a single intramuscular injection 15 mg/kg (maximum 1 g). In some embodiments, the period of drug treatment of tuberculosis is a minimum of 1 year and up to 2 years. In some embodiments, amakacin, is administered 15 mg/kg (maximum 1 g) IM or IV every 24 hours for up to 2 years. In some embodiments, the MLN-9708 is administered orally.

[0012] In one embodiment, the dosage for Bortezomib is 1.3 mg/m². In one embodiment, it is administered as a bolus intravenous injection or subcutaneously. In one embodiment, it is administered twice weekly for two weeks (days 1, 4, 8, and 11) followed by a ten day rest period (days 12 through 21). The three week period can be considered a treatment cycle. In one embodiment, a minimum of 72 hours elapses between consecutive doses of bortezomib.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

[0014] The foregoing will be apparent from the following more particular description of example embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating embodiments of the present invention.

[0015] FIGs. 1A-C. Reporter strains and assays. FIG 1A. Reporter assay principle. Under undisturbed conditions, ClpP1P2 protease recognizes and degrades SsrA-tagged (YALAA) (SEQ ID NO: 1) GFP protein resulting in a low fluorescence level. In the presence of a ClpP1P2 inhibitor, GFP is not degraded. Its accumulation results in an increase in fluorescence. FIG. 1B. *M. smegmatis* pTet-GFP-SsrA / pTet-GFP and assay activities. SsrA-tagged GFP (or untagged GFP) expression has been placed under the control of an anhydrotetracycline (ATc) inducible promoter (pTet). In the absence of ATc induction, the fluorescence signal remains basal with both SsrA-tagged and untagged GFP. In the presence of ATc, fluorescence is low in Smeg-pTet-GFP-SsrA due to GFP degradation, whereas the fluorescence increases in cultures expressing untagged GFP. FIG 1C. *M. smegmatis* p38-mRFP-SsrA / p38-mRFP and assay activities. Similarly, SsrA-tagged RFP (or untagged

RFP) expression has been placed under the control of a constitutive promoter (p38). The fluorescence signal is low in Smeg-pTet-RFP-SsrA culture due to RFP degradation whereas it increases in culture expressing untagged RFP. Shown is the average of three independent experiments with error bars representing standard deviation. RFU, relative fluorescence unit.

[0016] FIG. 2. Scatter plot of primary hits from HTS. A library of half a million compounds was screened at a single concentration of 10 μ M for inhibitors of ClpP1P2 activity using *M. smegmatis* carrying GFP-SsrA under the control of pTet (FIG. 1B). Compounds mean fluorescence is represented by the red line. A threshold of two times the standard deviation from the mean (2SD, pink line) was used as a cut-off for hit selection. RFU, relative fluorescence units.

[0017] FIG. 3. Screening cascade and work-up of hits.

[0018] FIGs. 4A-B. Growth inhibition activity of Bortezomib in bacteria with decreased and increased ClpP1P2 levels. FIG 4A. ClpP1P2 under-expression in *M. smegmatis* pTet(chromosome)-ClpP1P2. Bortezomib growth inhibition was assessed in a *M. smegmatis* strain in which the expression of chromosomal ClpP1 and ClpP2 genes was placed under the control of a pTet promoter. Low, 1 μ M, concentration of ATc inducer resulted in lower level of ClpP2 expression as compared to wild type *M. smegmatis* culture, as confirmed on Western blot depicted on the right. On the left, the effect of lower ClpP1P2 level on BZ susceptibility is shown. FIG 4B. ClpP1P2 over-expression in *M. smegmatis* pTet-ClpP1P2. This strain carries, in addition to a chromosomal copy, an episomal copy of ClpP1P2 placed under the control of an ATc inducible pTet promoter. High, 50 μ M, concentration of inducer resulted in higher level of ClpP2 as compared to un-induced control, as confirmed on the Western blot depicted on the right. On the left, the effect of increased ClpP1P2 level on BZ susceptibility is shown. Anti-RpoB probing was carried out to confirm equal protein loading. Shown in the growth inhibition experiments are the averages of at three independent experiments with error bars representing standard deviation. The Western blots were carried three times showing the same results. One representative example is shown.

[0019] FIG. 5. Combination of Bortezomib and antibiotics. *M. smegmatis* wild type was treated with sub-inhibitory concentrations of Bortezomib (BZ, 1.5 μ M), amikacin (AK, 0.06 μ M), chloramphenicol (CM, 0.75 μ M), ciprofloxacin (CIPRO, 0.6 μ M) or rifampicin (RIF, 0.03 μ M), independently or in combination as indicated. After 24 hours, growth was

assessed via OD600 measurement. Shown is the average of three independent experiments with error bars representing standard deviation.

[0020] FIGs. 6A-C. Effect of Bortezomib on the level of the caseinolytic protease substrate WhiB1. FIG. 6A. Reporter strain principle. WhiB1 is a substrate of ClpP1P2. Under undisturbed conditions GFP-WhiB1 is recognized, and degraded, resulting in a basal level of fluorescence. In presence of an inhibitor of ClpP1P2 degradation is reduced, GFP-WhiB1 accumulates, resulting in an increase in fluorescence. FIG. 6B. WhiB1-GFP has been placed under the control of the pTet promoter and introduced episomally in *M. smegmatis*. *M. smegmatis* pTet-GFP-WhiB1 was exposed to increasing concentrations of BZ for 6 hours, in presence or absence of the inducer (ATc), upon which fluorescence was measured. FIG. 6C *M. smegmatis* pTet-GFP was used as a control, demonstrating WhiB1 dependence of fluorescence increase in B. Shown is the average of at three independent experiments with error bars representing standard deviation. RFU, relative fluorescence units.

[0021] FIGs. 7A-C. Correlation between ClpP1P2-dependent proteolytic- and growth inhibition potencies of structural derivatives of Bortezomib FIG. 7A. Structures of BZ and derivatives. FIG. 7B. Inhibition of ClpP1P2 proteolytic activity. *M. smegmatis* p38-mRFP-SsrA was used as the reporter strain. FIG. 7C. Growth inhibition of *M. smegmatis* WT. Compounds were assessed in a dose-response manner up to 100µM. Shown is the average of three independent experiments with error bars representing standard deviation. The same experiments were carried out in *M. bovis* p38-mRFP-SsrA (for RFU) and *M. bovis* WT (for growth inhibition) resulting in the same pattern observed for *M. smegmatis*: BZ and CEP-18770 showed high RFU increase and strongest growth inhibition, MLN-2238 and MG-262 showed medium strong, and BZ-al and MG-132 showed no responses in both assays (data not shown). RFU, relative fluorescence units.

[0022] FIG. 8. Modeling of Bortezomib into one of the 7 ClpP1 catalytic sites of ClpP1P2. ClpP1 is shown in red ribbon and ClpP2 in blue ribbon. The binding site residues are shown with grey carbon in thin stick while the catalytic triad Ser98-His123-Asp172 is shown in thick stick. Bortezomib is shown with plum carbon in thick stick. Hydrogen bonds between Bortezomib and ClpP1P2 are shown in purple dashed lines. The boronic acid of Bortezomib is covalently attached to the catalytic Serine. The boron is shown in green.

DETAILED DESCRIPTION OF THE INVENTION

[0023] A description of example embodiments of the invention follows.

[0024] Recent experimental evidence strongly suggests that proteasome inhibitors may indeed be beneficial in certain pathologies, such as in cancer, asthma, brain infarct, autoimmune encephalomyelitis and other infections.

[0025] It has been shown that Mtb expresses a proteasome core consisting of typical four heptameric rings stacked in a cylinder. Hence, proteasome inhibitors might be useful in the treatment of tuberculosis. However, the extensive conservation of proteasome structures militates against species selectivity of proteasome inhibitors.

[0026] Bortezomib, (VELCADE(R)), is the first approved therapeutic known to act as a potent and specific proteasome inhibitor. The present invention is directed to Bortezomib, a novel anti-tuberculosis lead compound and an inhibitor of mycobacterial caseinolytic protease (Clp), which blocks degradation of caseinolytic protease substrate WhiB1 and exerts its antibacterial activity via modulation of ClpP1P2. There is a significant need for new treatments for tuberculosis.

[0027] A novel type of antibacterial screen, a target mechanism-based whole cell screen, was developed to combine the advantages of target- and whole cell-based approaches. A mycobacterial reporter strain with a synthetic phenotype for caseinolytic protease (ClpP1P2) activity was engineered allowing detection of inhibitors of this enzyme inside intact bacilli. A high throughput screen identified Bortezomib, a human 26S proteasome drug, as a potent inhibitor of ClpP1P2 activity and bacterial growth. A battery of secondary assays was employed to demonstrate that Bortezomib exerts its antimicrobial activity indeed via inhibition of ClpP1P2: Down / up modulation of the intracellular protease level resulted in hyper / hypo sensitivity of the bacteria, the drug showed specific potentiation of translation error-inducing aminoglycosides, ClpP1P2-specific substrate WhiB1 accumulated upon exposure, and growth inhibition potencies of Bortezomib derivatives correlated with ClpP1P2 inhibition potencies. Furthermore, molecular modelling showed that the drug can bind into the catalytic sites of ClpP1P2. This work demonstrates the feasibility of target mechanism-based whole cell screens, provides chemical validation of ClpP1P2 as target, and identifies a drug in clinical use as a new lead compound for tuberculosis.

[0028] With 8.6 million new cases and 1.3 million deaths annually, tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a global infectious disease threat (1). Half a

million new cases of multidrug resistant patients each year compound the situation. There is an urgent medical need for new drugs with new mechanism of action to control drug resistant disease (2). After the failure of the genomics-driven, biochemical screening-based antibacterial drug discovery strategy employed during the previous decade, the field moved largely back to classical whole cell approaches. Although empirical whole cell strategies delivered several candidates and a new TB drug, they suffer from limited productivity due to their 'black box' nature (3-5). The lack of target knowledge prevents the use of structure based design during lead finding and optimization and can result in generation of compounds with mechanism-based toxicity. Another pitfall of whole cell-based drug discovery is that compounds might be optimized for targets that are only required under *in vitro* culture conditions but are dispensable *in vivo* (6-8). Using isolated biochemical targets in screening campaigns on the other hand often result in identification of potent enzyme inhibitors lacking antibacterial activity, due to their inability to penetrate cell membranes (9). Engineering compounds to penetrate bacterial cell envelopes turned out to be challenging because the physico-chemical and structural rules that govern permeability through bacterial cell walls remain highly complex. The situation is even more challenging for mycobacteria because they have a two-membrane system: an outer membrane made up of tightly packed mycolic acids and an inner, more standard plasma membrane. The mycobacterial double membrane system represents a formidable permeability barrier. This argues for a screening strategy that includes screening targets inside the mycobacterial cell, using the double-membrane barrier as a filter. This strategy enables selection of hits that are not only able to bind to their molecular target but are also able to access it (10).

[0029] Target- or pathway-based whole cell screens have therefore been developed combining the advantages of target- and cell-based approaches to identify enzyme inhibitors with antibacterial activity (11, 12). These screens employ pathway-selective sensitization via antisense RNA or conditional gene expression (11, 13-17) in which reduced expression of the targeted gene results in an increased sensitivity to inhibitors acting on that target. Abrahams et al. used tetracycline-regulatable promoter elements to generate mycobacterial strains that conditionally express pantothenate synthetase (*panC*) and subsequently screened for compounds that display greater potency against *PanC*-depleted TB bacteria (13). Antisense strategies have been employed to reduce the expression of the chromosome partitioning protein *ParA* in *M. smegmatis* and compounds with higher anti-mycobacterial activity have

been identified (18). Another type of pathway specific strategy makes use of strains that carry a reporter gene fused to a promoter that specifically responds to certain types of disturbances, such as 'cell wall synthesis stress' (19). The selective induction of the reporter signal enables screening for compounds that affect the pathway of interest. Applying this approach, Sequella screened a library with *M. tuberculosis* carrying the RV0341 gene promoter fused to a luciferase reporter gene. This screen identified SQ109 which is now evaluated in phase II clinical trials (20). A similar approach has led to the identification of thiophenes as a new class of anti-mycobacterials inhibiting mycolic acid biosynthesis (21). Both pathway-selective sensitization and stress-induced promoter assays provide means to identify hits that are whole cell active and pathway specific but may not provide information on the exact cellular target.

[0030] In this study we explore the feasibility of a novel type of target-based whole cell screen, a target mechanism-based whole cell approach in Mycobacterium. We selected the caseinolytic protease (ClpP1P2) as target and our aim was to identify whole cell active inhibitors of this enzyme, thus chemically validating ClpP1P2 as target for TB, and providing starting points for lead finding. Caseinolytic proteases are serine proteases found in a wide range of bacteria including *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* (12, 22, 23). In contrast to site-specific proteases, caseinolytic proteases form a degradative complex involved in removal of partially synthesized and misfolded proteins. In addition to these proteome housekeeping functions, caseinolytic proteases are also involved in adaptive processes by selectively removing specific regulatory functions (24). The transcription factor WhiB1 is the first regulatory function identified to be specifically degraded by mycobacterial caseinolytic protease (24). The caseinolytic protease complex is composed of catalytic protease subunits (ClpP) and regulatory subunits (ATPases). The regulatory subunits recognize substrates and provide the energy for unfolding of proteins that are to be degraded. The catalytic ClpP subunits form a degradative chamber in which proteolysis occurs. It was recently demonstrated that the proteolytic chamber of mycobacterial caseinolytic protease consists of two different subunits, ClpP1 and ClpP2, which are both essential for growth of *M. tuberculosis* in culture and in a mouse model of TB (25, 26). Importantly, these genetic studies also suggest that the ClpP1P2 protease core represents a vulnerable target with cidal potential: reduced protein levels resulted in growth arrest and cell death, suggesting that a small molecule inhibitor of ClpP1P2 should be able to inhibit intracellular proteolytic activity

to a degree that causes phenotypic consequences (25). The demonstration of genetic essentiality *in vitro* and *in vivo*, vulnerability, cidal potential, together with the demonstrated presence of ClpP1P2 in all clinical isolates, and - as protease - apparent druggability, makes mycobacterial caseinolytic protease an attractive target. Furthermore, genetic ClpP1P2 depletion experiments suggest that inhibitors may show synergy with mistranslation-inducing aminoglycosides, important second line drugs for TB, adding to the attractiveness of ClpP1P2 as target for tuberculosis drug development.

[0031] One function of caseinolytic proteases is the removal of aborted translation products. The tmRNA trans-translation system, a bacterial rescue system that frees ribosomes stuck during protein synthesis, tags partially synthesised proteins with a caseinolytic protease specific (SsrA) degradation peptide (27). SsrA-tagged proteins are recognized by the caseinolytic protease and degraded. We took advantage of this mechanism and used this caseinolytic protease-specific peptide degradation tag to develop a fluorescence-based synthetic phenotype to detect and measure intracellular ClpP1P2 inhibition. We carried out a high throughput screen, worked up the hit list with a series of secondary assays to demonstrate on-target whole cell activity and identified the first caseinolytic protease inhibitor with antibacterial whole cell activity.

[0032] Here, we developed a novel type of anti-mycobacterial screen attempting to combine the advantages of target- and whole cell approaches: a target-mechanism based whole cell screen. The degradative caseinolytic protease ClpP1P2 was selected as target and a reporter strain with a synthetic phenotype was engineered that allowed detection of inhibitors via intracellular accumulation of green fluorescent protein. A 500,000 compound library was screened and the human proteasome inhibitor BZ was found to be positive in two independent whole cell reporter assays measuring ClpP1P2 proteolytic activity. The compound showed growth inhibition and cidal activity in the screening strain *M. smegmatis* as well as in the tubercle bacillus *M. bovis* BCG, consistent with previous genetic depletion data of ClpP1P2. Six additional lines of evidence suggest that BZ exerts its antibacterial activity indeed via inhibition of the caseinolytic protease: i. Modulation of the intracellular ClpP1P2 level via genetic under- and over expression resulted in BZ hyper- and hypo sensitivity of the bacteria. ii. The drug potentiated the effect of aminoglycosides, phenocopying ClpP1P2 hypomorphs. iii. BZ exposure resulted in the accumulation of the ClpP1P2-specific substrate WhiB1. iv. Whole cell growth-inhibition potencies of BZ derivatives

correlated with inhibition potencies against ClpP1P2 activity. v. Replacement of the 'anti-protease' boronic acid warhead of BZ with an aldehyde resulted in an inactive compound in both the ClpP1P2 activity and growth inhibition assay. vi. Molecular modelling of Bortezomib and its boronic acid derivatives showed that they can be covalently attached to ClpP1P2 catalytic sites.

[0033] This work has several implications. Firstly, it demonstrates the feasibility of target mechanism-based whole cell screens as a new approach to anti-mycobacterial drug discovery. After the drug discovery community moved back to rather inefficient black box whole cell strategies, new avenues that reconnect antibacterial discovery with modern genome biology are urgently needed (5). Target-mechanism based screens might be a useful complement for other ongoing activities employing pathways screens with hyper-sensitized bacterial strains and pathway-stress specific promoters.

[0034] Secondly, we provide chemical validation of mycobacterial ClpP1P2 as target for tuberculosis. With BZ we show for the first time that a small molecule inhibitor of ClpP1P2 can indeed inhibit growth and kill mycobacteria, demonstrating pharmacologically the vulnerability and cidal potential of ClpP1P2. It should be noted that lactone derivatives with anti-mycobacterial properties have been proposed to act through inhibition of the caseinolytic protease (lactone 4 and 7 in (38, 39)). However, these compounds displayed a dramatic disconnect between biochemical (mM) and growth inhibitory (μ M) potency, suggesting that the antibacterial activity of these compounds is off-target, i.e. unrelated to the weak, biochemically observed, anti-ClpP1P2 activity. Indeed, lactones were neither positive in our ClpP1P2 activity reporter strain nor our ClpP1P2 under-expressing, hypersensitized strain (data not shown). It is interesting to note that several molecules, including acyldepsipeptides and cyclomarin have been identified that appear to increase the promiscuity of the caseinolytic protease complex and thus allow unspecific degradation of proteins (40, 41). Furthermore, lassomycin was recently identified to stimulate ATPase activity of a regulatory subunit of the caseinolytic protease while uncoupling it from the proteolytic activity of the complex (42). Our discovery of the first ClpP1P2-targeting whole cell active inhibitor adds to the growing list of caseinolytic protease modulators and shows that this proteolytic degradation machine represents an attractive multi-mechanism, multi-target complex for chemotherapeutic intervention.

[0035] Thirdly, we identified BZ, a human proteasome-targeting anti-cancer drug in clinical use, as a new lead compound for tuberculosis. Interestingly, mycobacteria are one of the few prokaryotes possessing a mammalian-like proteasome (43). Whereas this function is dispensable (non-essential) in *M. smegmatis* (44), the proteasome is essential in the tubercle bacillus (45-47). This might explain why we see stronger antibacterial potency of BZ in *M. bovis* BCG when compared with *M. smegmatis*: In the tubercle bacillus BZ might inhibit both ClpP1P2 and the tubercle bacillus' proteasome. Indeed BZ has been used in biochemical studies of the *M. tuberculosis* proteasome (48-50).

[0036] BZ is given intravenously and has a short half-life (34, 35). In addition to an unfavourable route of administration and poor pharmacokinetics, high costs and significant adverse effects including peripheral neuropathy, neutropenia and cytopenia (34, 35), obviously limit its direct use for tuberculosis. Second generation proteasome inhibitors, including orally bioavailable prodrugs are in development. It is worthwhile to mention that we tested one of the boronic acid ester prodrugs, MLL-9708 (51, 52), and found the compound to be active in both our ClpP1P2 reporter and growth inhibition assays (data not shown), indicating that mycobacteria can hydrolyse this prodrug to its biologically active boronic acid component. This suggest that introduction of oral bioavailability in a TB lead optimization program might be achievable. Considering the availability of in vitro assays for potency determination, the tools required for introducing selectivity, and the structural differences between the human proteasome and the mycobacterial caseinolytic protease (26, 32, 53, 54), BZ optimization appears to be an attractive opportunity.

[0037] In conclusion, our work demonstrates feasibility of target mechanism-based whole cell screens for anti-mycobacterial drug discovery, provides chemical validation of ClpP1P2 as target for TB, and identifies with BZ a new lead compound.

[0038] Material and methods

[0039] Bacterial strains, culture media and chemicals. *M. smegmatis* (ATCC 700084 / mc2155), *M. bovis* BCG (ATCC35734), wild-type strains and derived GFP and mRFP reporter strains were maintained in Middlebrook 7H9 media (Difco) supplemented with 0.5 % (v/v) glycerol, 0.05% (v/v) Tween 80 and 10 % (v/v) Middlebrook ADC (Albumin-Dextrose-Catalase) (Difco). When appropriate, hygromycin B (Roche) and anhydrotetracycline (Acros Organic) was added. Enumeration of bacteria was performed by plating on Middlebrook 7h10 (Difco) agar plates containing 0.5% (v/v) glycerol, and 10 %

(v/v) Middlebrook OADC (Oleic acid-Albumin-Dextrose-Catalase) (Difco). Antibiotics were purchased from Sigma-Aldrich. Stock solutions of the compounds were prepared in 90% DMSO. The Experimental Therapeutic Centre chemical library was collected from various providers.

[0040] GFP and mRFP plasmids constructs and reporter strains. pTet-GFP plasmid comprise the wild-type allele of the GFP gene cloned downstream of the tetracycline inducible pTet. GFP was amplified from GFPmut3 wildtype DNA via PCR and subsequently recombined into the pTet vector using gateway recombination (Clontech) as previously described (55). The fusions GFP-SsrA was generated via amplification from the same template using primers pair

GGGGACAAGTTTGTACAAAAAAGCAGGCTGAAGGAGATATACATATGGCTAGCA
AAGGAGAAGAAC (SEQ ID NO: 2) and

GGGGACCACTTTGTACAAGAAAGCTGGGTCGGCAGCGAGAGCG TAGTCG (SEQ
ID NO: 3) and cloned into the same vectors to generate pTet-GFP-SsrA. pTET-GFP and pTet-GFP-SsrA, plasmids were electroporated separately into WT *M. smegmatis* to generate Smeg-pTet-GFP, Smeg-pTet-GFP-SsrA, strains respectively. pGMEH-p38-mRFP plasmid carries mCherry RFP cloned downstream of the p38 strong mycobacterial promoter (56). pGMEH-p38-mRFP-SsrAec carries the same construct including the *E. coli* SsrA tag fused to mRFP gene. Both plasmids were obtained from Addgene (#27058 and 27059) and electroporated into WT *M. smegmatis* to generate Smeg-p38-mRFP and Smeg-p38-mRFP-SsrA, respectively.

[0041] pTet-GFP-SsrA assay optimization and high throughput primary screen. Smeg-pTet-GFP and Smeg-pTet-GFP-SsrA pre-cultures were harvested at mid-log phase and diluted to OD₆₀₀ 0.2 in complete 7H9 media. Anhydrotetracycline was added when appropriate and the bacterial suspension was distributed in flat-bottom, dark, medium-binding 384-well plate (30 μ L/well) (Greiner bio-one) and incubated at 37°C for 3 hours. Fluorescence signal (RFU) was measured using Synergy H1 microplate reader (Biotek) (excitation λ = 485 nm, emission λ = 520nm) with a 90 seconds shaking step prior to reading. OD normalization between each strain was verified by bacterial enumeration as mentioned above. DMSO tolerance was assessed by growing the strains in presence of increasing concentration of DMSO in 7H9 media and measuring effect on RFU as well as plating the cells and determining CFU. Following optimization of the primary screen assay, a high

throughput format was validated following the same procedure. Prior to screening the complete 503 879 compound library, The Pharmakon1600 (1600 compounds) library was used in a validation run to assess the performances indicators of the assay under high throughput conditions. We used Topotecan (Sigma-Aldrich) as a positive control for the inhibitor screen. Each compound was screened in duplicate at a final concentration of 10 μ M. GFP signal was measured on a Safire II microplate reader (Tecan) with the same parameters as described above. Hits were defined as compounds that induce a GFP signal response higher than a cut-off value defined by the mean+2*SD and were submitted to a re-test in an identical assay. Auto-fluorescence of re-test positive hits was measured by dispensing 10 μ M of each compounds (in 90% DMSO) in 384-well plate and measuring fluorescence signal with the same signal acquisition parameters as the primary screen. Auto-fluorescent compounds were filtered out. We next evaluated the GFP dose-response profile for all inhibitor hits and determined their respective GFP IC₅₀. Briefly, all selected hits were tested in 3-fold serial dilution GFP assay at a maximum concentration of 100 μ M in a 96 well plate format of the GFP assay using M200 Pro plate reader (Tecan).

[0042] Constitutive p38-mRFP secondary assay. All 89 selected hits from the primary screen results were re-ordered and subjected to secondary screening assays from fresh-powder stocks (90% DMSO) using *M. smegmatis* strains carrying a constitutive mRFP reporter system. We first proceeded with optimization and validation of the secondary assay as described above. We then proceeded to re-screen selected hits using Smeg-p38-mRFP-SsrA. Pre-culture were harvested at mid-log phase, diluted to OD₆₀₀ 0.2 in complete 7H9 media and dispensed into 96-well plates (200 μ L/well) in presence of compounds. Smeg-p38-mRFP-SsrA alone was used as negative control whereas Smeg-p38-mRFP was used as positive control. Fluorescence signal acquisition was carried after 3 hours incubation using M200 Pro plate reader (Tecan). Red fluorescence was acquired under excitation/emission at λ = 587/630nm.

[0043] Turbidity-based growth inhibition assay. An inhibition assay was performed on selected hits to assess their inhibition potency. *M. smegmatis* strain WT pre-cultures were harvested at mid-log phase and diluted to OD₆₀₀ 0.05 in complete 7H9 media. Bacterial suspensions were then dispensed in 96-well plate (200 μ L/well, *M. smegmatis*) or in 24 well plates (1ml/well, BCG) with the indicated compound concentration and incubated for 24 hours (*M. smegmatis*) or 5 days (BCG) at 37°C under shaking (100 rpm). Cells were

manually resuspended and OD was measured at 600nm on M200Pro plate reader (Tecan). Positive control used ciprofloxacin at an MIC90 concentration of 0.6 μ M.

[0044] Aminglycosides potentiation assay. *M. smegmatis* WT (inoculum 0.01 OD600 in 1mL of 7H9) was treated with sub-inhibitory concentrations of Bortezomib (BZ, 1.5 μ M), Amikacin (AK, 0.06 μ M), Chloramphenicol (CM, 0.75 μ M), Ciprofloxacin (CIPRO, 0.6 μ M) or Rifampicin (RIF, 0.03 μ M), independently or in combination where indicated. After 24 hours, growth was assessed via OD600 measurement and growth inhibition was determined.

[0045] pTet-GFP-WhiB1 assay. *M. smegmatis* pTet-GFP-WhiB1 pre-culture was harvested at mid-log phase, diluted to OD600 0.2 in complete 7H9 media and dispensed into 96-well plates (200 μ L/well) in presence of ATc and BZ were indicated. *M. smegmatis* pTet-GFP was used as a control and assessed in similar conditions. Cells were incubated for 6 hours, manually resuspended and fluorescence signal was acquired as described above.

[0046] Protein purification and immunoblotting. Total protein lysates were prepared from equivalent cell numbers using bead beating. After probing with primary antibody, visualization was performed using HRP-conjugated secondary antibodies (Invitrogen), and detection was performed using Western Lighting Plus-ECL (Perkin Elmer) according the manufacturer's protocol. In all cases, blots were probed with monoclonal anti-RpoB (Abcam) to ensure equivalent loading of samples.

[0047] Clp under- and over expression and GFP-WhiB1 strains. The engineering of *M. smegmatis* pTet(Chromosome)-ClpP1P2 in which the native promoter of ClpP1P2 has been replaced by a tetracycline inducible promoter, and of *M. smegmatis* carrying an episomal copy of ClpP1P2 under the control of a tetracycline-inducible promoter (*M. smegmatis* pTet-ClpP1P2) in which the overexpression of ClpP1P2 can be induced by ATc have been previously described in (25). *M. smegmatis* pTet-GFP-WhiB1 carrying an episomal copy of WhiB1 gene fused to GFP and placed under the control of the pTet promoter has been described elsewhere (24).

[0048] Molecular Modeling. The *M. tuberculosis* ClpP1P2 X-ray structure of 4U0G was downloaded from the Protein Data Bank (54). Addition of hydrogens atoms, setting of protonation and tautomer states and hydrogen bond network optimization was done using the Protein Preparation Wizard in Maestro (Schrodinger Suite version 2014-2, Schrödinger, LLC: New York, NY, 2014). ClpP1P2 has 14 catalytic sites. The catalytic triad of the 7 ClpP1 sites consist of residues Ser98-His123-Asp172 and those of the 7 ClpP2 sites are Ser110-His135-

Asp186. The conformation of Bortezomib from the Yeast 20S Proteasome X-ray structure 4FWD (32) was manually positioned in the ClpP1P2 catalytic sites in an orientation that allowed hydrogen bonding between both amide NH donors and both amide carbonyls of Bortezomib and protein backbone residues. These are Gly69, Ile71 and Leu126 of the ClpP1 sites and Gly81, Phe83 and Ser138 of the ClpP2 sites. The boronic acid was covalently attached to the catalytic serine, Ser98 in ClpP1 and Ser110 in ClpP2. One oxygen of the boronic acid group occupied the oxyanion hole hydrogen bonding with the backbone NH of Gly69 and Met99 in ClpP1 and Gly81 and Ala111 in ClpP2. The other boronic acid oxygen formed a salt bridge with the catalytic histidine His123 of ClpP1 and His135 of ClpP2. This complex was minimized using the OPLS2005 force field, GBSA salvation model and 500 steps of Polak-Ribiere-Conjugate-Gradient method with MacroModel default settings (Schrodinger Suite version 2014-2, Schrödinger, LLC: New York, NY, 2014). All residues more than 12Å from Bortezomib were constrained during the minimization. Delanzomib, MG-262 and Ixazomib (MLN-2238; Millennium) were also modelled into ClpP1P2 using the conformation of Bortezomib as a template and the complexes were minimized.

[0049] Bortezomib, Delanzomib (CEP-18770, orally bioavailable), Ixazomib citrate (MLN-9708; Millennium) and Ixazomib (MLN2238; orally bioavailable), and MG262 are novel lead compounds for treatment of Tuberculosis. Further, each of these compounds, is a novel lead/target couple with ClpP1P2 for the initiation of medicinal chemistry. In addition, these compounds represent novel inhibitors of the target with whole-cell activity. Disclosed herein is the structure-activity relationship for these compounds as illustrated in the figures of this application, as well as their whole cell activity and target specificity. The means of assessing the target inhibition within the cell (via a target-based whole-cell assay) described herein can be employed to identify and develop new molecules that will be specific for bacteria (ClpP1P2 being the target) and not for human cells (proteasome).

[0050] As used herein, the terms “administering” and “introducing” are used interchangeably and refer to the placement of a modulator of ClpP1P2 protease into a subject by a method or route which results in at least partial localization of such agents at a desired site, such as a site of a *M. tuberculosis* bacterium, such that a desired effect(s) is produced.

[0051] As used herein, the terms “treat,” “treatment,” “treating,” or “amelioration” refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of a condition associated with a disease or disorder.

The term “treating” includes reducing or alleviating at least one adverse effect or symptom of a condition, disease or disorder associated with *M. tuberculosis* infection. Treatment is generally “effective” if one or more symptoms or clinical markers are reduced. Alternatively, treatment is “effective” if the progression of *M. tuberculosis* is reduced or halted. That is, “treatment” includes not just the improvement of symptoms, but also a cessation of at least slowing of progress or worsening of symptoms that would be expected in absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of the *M. tuberculosis*, stabilized (i.e., not worsening) state of *M. tuberculosis*, delay or slowing of disorder progression, amelioration or palliation of *M. tuberculosis*, and remission (whether partial or total), whether detectable or undetectable. The term “treatment” of *M. tuberculosis* also includes providing relief from the symptoms or side effects of *M. tuberculosis*.

[0052] As used herein, “pharmaceutically acceptable” refers to those compounds, compositions, and/or dosage forms which are suitable for administration to humans and animals without excessive toxicity, irritation, allergic response, or other problem or complications.

[0053] Also provided are methods of treating or preventing a *Mycobacterium tuberculosis* (Mtb) infection in a subject with or at risk of developing a Mtb infection. Subjects (e.g., humans, such as patients, or animals) with Mtb include subjects diagnosed with an Mtb infection. A subject at risk includes a subject with a known exposure or with a potential exposure to a Mtb source.

[0054] The phrase “pharmaceutically acceptable carrier” as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent, media, encapsulating material, manufacturing aid (e.g., lubricant, talc magnesium, calcium or zinc stearate, or steric acid), or solvent encapsulating material, involved in maintaining the stability, solubility, or activity of, a bispecific or multispecific polypeptide agent. Each carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient.

[0055] The terms “decrease”, “reduced”, “reduction”, “decrease” or “inhibit” are all used herein generally to mean a decrease by a statistically significant amount. For example, they can mean a decrease by at least about 5%-10% as compared to a reference level, for example a decrease by at least about 20%, or at least about 30%, or at least about 40%, or at least

about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% decrease or any decrease between 10-90% as compared to a reference level.

[0056] Bortezomib is a compound having the formula [(1R)-3-methyl-1-[[[(2S)-1-oxo-3-phenyl-2-[(pyrazinylcarbonyl) amino]propyl]amino]butyl]boronic acid.

[0057] Delanzomib (CEP-18770) is a compound having the formula ((R)-1-((2S,3R)-3-Hydroxy-2-(6-phenylpicolinamido)butanamido)-3-methylbutyl)boronic acid, also known as[(1R)-1-[[[(2S,3R)-3-hydroxy-2-[(6-phenylpyridine-2-carbonyl)amino]butanoyl]amino]-3-methylbutyl]boronic acid.

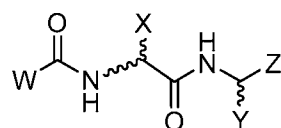
[0058] Ixazomib (MLN-2238) is a compound having the formula (R)-(1-(2-(2,5-dichlorobenzamido)acetamido)-3-methylbutyl)boronic acid.

[0059] MLN-9708 is a compound having the formula 4-(carboxymethyl)-2-((R)-1-(2-(2,5-dichlorobenzamido)acetamido)-3-methylbutyl)-6-oxo-1,3,2-dioxaborinane-4-carboxylic acid.

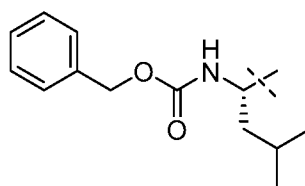
[0060] MG-262 is a compound having the formula [(1R)-3-methyl-1-[[[(2S)-4-methyl-2-[[[(2S)-4-methyl-2-(phenylmethoxycarbonylamino)pentanoyl]amino]pentanoyl]amino]butyl]boronic acid.

[0061] MG-132 is a compound having the formula benzyl (S)-4-methyl-1-((S)-4-methyl-1-((S)-4-methyl-1-oxopentan-2-ylamino)-1-oxopentan-2-ylamino)-1-oxopentan-2-ylcarbamate.

[0062] As used herein and unless otherwise indicated, the term “bortezomib and derivatives thereof” refers to a compound of formula



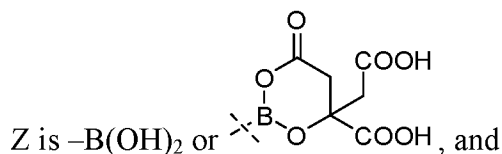
or a physiologically acceptable salt thereof,
wherein



W is (C₆-C₁₂)aryl, (5-12 atom)heteroaryl, or with halo or (C₆-C₁₂)aryl;

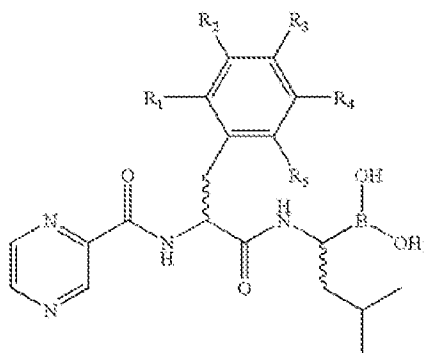
X is (C₆-C₁₂)aryl, (C₁ to C₆)alkyl, hydroxyl, (5-12 atom)heteroaryl or H, further wherein alkyl and aryl are optionally substituted with H, deuterium, straight chained, branched or

cycloalkyl (including (C₁ to C₆)alkyl), (C₁ to C₆)alkoxyl, (C₂-C₂₀)alkenyl, or (C₂-C₂₀)alkynyl, (C₆-C₁₂)aryl, CO-(C₁-C₂₀)alkyl, CO-(C₂-C₂₀)alkenyl, CO-(C₂-C₂₀)alkynyl, (C₆-C₁₂)aryl, (5-12 atom) heteroaryl, CO-(C₆-C₁₂)aryl, or CO-(5-12 atom)heteroaryl, CO-alkoxyalkyl, CO-aryloxyalkyl, sulfonyl, (C₁ to C₆)alkylsulfonyl, (C₆-C₁₂)arylsulfonyl, aralkylsulfonyl, halo or -CF₃, each optionally substituted with halo, -CF₃ or (C₁-C₆)alkyl; Y is (C₁ to C₆)alkyl; and



wherein --- represents a point of attachment between two atoms.

[0063] In another embodiment, the term “bortezomib and derivatives thereof” refers to a compound of formula:

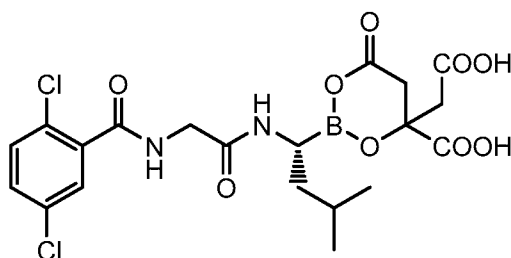


or physiologically acceptable salt thereof,

wherein

R₁, R₂, R₃, R₄, and R₅ each are independently H, deuterium, straight chained, branched or cycloalkyl (including (C₁ to C₆)alkyl), (C₁ to C₆)alkoxyl, (C₂-C₂₀)alkenyl, or (C₂-C₂₀)alkynyl, (C₆-C₁₂)aryl, CO-(C₁-C₂₀)alkyl, CO-(C₂-C₂₀)alkenyl, CO-(C₂-C₂₀)alkynyl, (C₆-C₁₂)aryl, (5-12 atom) heteroaryl, CO-(C₆-C₁₂)aryl, or CO-(5-12 atom)heteroaryl, CO-alkoxyalkyl, CO-aryloxyalkyl, sulfonyl, (C₁ to C₆)alkylsulfonyl, (C₆-C₁₂)arylsulfonyl, aralkylsulfonyl, halo or -CF₃, optionally substituted with halo or (C₁-C₆)alkyl.

[0064] In another embodiment, the term “bortezomib and derivatives thereof” refers to a compound, also referred to as MLN-9708, of formula:



[0065] All definitions of substituents set forth herein are further applicable to the use of the term in conjunction with another substituent.

[0066] The term “alkyl,” as used herein, refers to both a saturated aliphatic branched or straight-chain monovalent hydrocarbon radical having the specified number of carbon atoms. Thus, “(C₁-C₆) alkyl” means a radical having from 1-6 carbon atoms in a linear or branched arrangement. Examples of “(C₁-C₆) alkyl” include, for example, *n*-propyl, *i*-propyl, *n*-butyl, *i*-butyl, *sec*-butyl, *t*-butyl, *n*-pentyl, *n*-hexyl, 2-methylbutyl, 2-methylpentyl, 2-ethylbutyl, 3-methylpentyl, and 4-methylpentyl. Alkyl can be optionally substituted with halogen, -OH, oxo, (C₁-C₆)alkyl, (C₁-C₆)alkoxy, (C₁-C₆) alkoxy(C₁-C₄)alkyl, aryl, heteroaryl, heterocyclyl, cycloalkyl, carbocyclyl, nitro, cyano, amino, acylamino, or carbamyl, -C(O)O(C₁-C₁₀)alkyl, or -C(O)(C₁-C₁₀)alkyl.

[0067] The term “cycloalkyl,” as used herein, refers to saturated aliphatic cyclic hydrocarbon ring. Thus, “(C₃-C₈) cycloalkyl” means (3-8 membered) saturated aliphatic cyclic hydrocarbon ring. (C₃-C₈) cycloalkyl includes, but is not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, or cyclooctyl. Cycloalkyl can be optionally substituted in the same manner as alkyl, described above.

[0068] The term “alkenyl,” as used herein, refers to a straight-chain or branched alkyl group having one or more carbon-carbon double bonds. Thus, “(C₂-C₆) alkenyl” means a radical having 2-6 carbon atoms in a linear or branched arrangement having one or more double bonds. Examples of alkenyl groups include, but are not limited to, ethenyl, propenyl, butenyl, pentenyl, hexenyl, butadienyl, pentadienyl, hexadienyl groups, and the like. The one or more carbon-carbon double bonds can be internal (such as in 2-butene) or terminal (such as in 1-butene).

[0069] The term “alkynyl,” as used herein, refers to a straight-chain or branched alkyl group having one or more carbon-carbon triple bonds. Thus, “(C₂-C₆) alkynyl” means a radical having 2-6 carbon atoms in a linear or branched arrangement having one or more triple bonds. Examples of alkynyl groups include, but are not limited to, ethynyl, propynyl,

butynyl, pentynyl, and the like. The one or more carbon-carbon triple bonds can be internal (such as in 2-butyne) or terminal (such as in 1-butyne).

[0070] The term “alkoxy”, as used herein, refers to an “alkyl-O-” group, wherein alkyl is defined above. Examples of alkoxy group include methoxy or ethoxy groups.

[0071] The terms “halogen” or “halo,” as used herein, refer to fluorine, chlorine, bromine or iodine.

[0072] The term “aryl,” as used herein, refers to an aromatic monocyclic or polycyclic (e.g. bicyclic or tricyclic) carbocyclic ring system. Thus, “(C₆-C₁₈) aryl” is a 6-18 membered monocyclic or polycyclic system. Aryl systems include optionally substituted groups such as phenyl, biphenyl, naphthyl, phenanthryl, anthracenyl, pyrenyl, fluoranthyl or fluorenyl. An aryl can be optionally substituted. Examples of suitable substituents on an aryl include halogen, hydroxyl, (C₁-C₁₂) alkyl, (C₂-C₆) alkenyl, (C₂-C₆) alkynyl, (C₁-C₆) haloalkyl, (C₁-C₃) alkylamino, (C₁-C₃) dialkylamino (C₁-C₆) alkoxy, (C₆-C₁₈) aryloxy, (C₆-C₁₈) arylamino, (C₆-C₁₈) aryl, (C₆-C₁₈) haloaryl, (5-12 atom) heteroaryl, -NO₂, -CN, -OF₃ and oxo.

[0073] In some embodiments, a (C₆-C₁₈) aryl is phenyl, indenyl, naphthyl, azulenyl, heptalenyl, biphenyl, indacenyl, acenaphthylenyl, fluorenyl, phenalenyl, phenanthrenyl, anthracenyl, cyclopentacyclooctenyl or benzocyclooctenyl. In some embodiments, a (C₆-C₁₈) aryl is phenyl, naphthalene, anthracene, 1H-phenalene, tetracene, and pentacene.

[0074] The term “heteroaryl,” as used herein, refers aromatic groups containing one or more atoms is a heteroatom (O, S, or N). A heteroaryl group can be monocyclic or polycyclic, *e.g.*, a monocyclic heteroaryl ring fused to one or more carbocyclic aromatic groups or other monocyclic heteroaryl groups. The heteroaryl groups of this invention can also include ring systems substituted with one or more oxo moieties. Examples of heteroaryl groups include, but are not limited to, thiophenyl, pyridinyl, pyridazinyl, imidazolyl, pyrimidinyl, pyrazolyl, triazolyl, pyrazinyl, quinolyl, isoquinolyl, tetrazolyl, furyl, thienyl, isoxazolyl, thiazolyl, oxazolyl, isothiazolyl, pyrrolyl, quinolinyl, isoquinolinyl, indolyl, benzimidazolyl, benzofuranyl, cinnolinyl, indazolyl, indoliziny, phthalazinyl, pyridazinyl, triazinyl, isoindolyl, purinyl, oxadiazolyl, thiazolyl, thiadiazolyl, furazanyl, benzofurazanyl, benzothiophenyl, benzotriazolyl, benzothiazolyl, benzoxazolyl, quinazolinyl, quinoxaliny, naphthyridinyl, dihydroquinolyl, tetrahydroquinolyl, dihydroisoquinolyl, tetrahydroisoquinolyl, benzofuryl, furopyridinyl, pyrrolopyrimidinyl, and azaindolyl.

[0075] In other embodiments, a 5-20-membered heteroaryl group is pyridyl, 1-oxo-pyridyl, furanyl, benzo[1,3]dioxolyl, benzo[1,4]dioxinyl, thienyl, pyrrolyl, oxazolyl, imidazolyl, thiazolyl, a isoxazolyl, quinolinyl, pyrazolyl, isothiazolyl, pyridazinyl, pyrimidinyl, pyrazinyl, a triazinyl, triazolyl, thiadiazolyl, isoquinolinyl, indazolyl, benzoxazolyl, benzofuryl, indoliziny, imidazopyridyl, tetrazolyl, benzimidazolyl, benzothiazolyl, benzothiadiazolyl, benzoxadiazolyl, indolyl, tetrahydroindolyl, azaindolyl, imidazopyridyl, quinazolinyl, purinyl, pyrrolo[2,3]pyrimidinyl, pyrazolo[3,4]pyrimidinyl, imidazo[1,2-a]pyridyl, benzothienyl.

[0076] The term “haloalkyl,” as used herein, includes an alkyl substituted with one or more F, Cl, Br, or I, wherein alkyl is defined above.

[0077] The term “haloaryl,” as used herein, includes an aryl substituted with one or more F, Cl, Br, or I, wherein aryl is defined above.

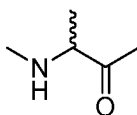
[0078] The term “hetero,” as used herein, refers to the replacement of at least one carbon atom member in a ring system with at least one heteroatom selected from N, S, or O. “Hetero” also refers to the replacement of at least one carbon atom member in a acyclic system. A hetero ring system or a hetero acyclic system may have 1, 2, or 3 carbon atom members replaced by a heteroatom.

[0079] The terms “heterocyclyl” or “heterocyclic,” as used herein, refer to a saturated or unsaturated group having a single ring or multiple condensed rings, from 1 to 10 carbon atoms and from 1 to 4 heteroatoms selected from nitrogen, sulfur or oxygen. In fused ring systems, one or more of the rings can be aryl or heteroaryl, provided that the point of attachment is at the heterocyclyl. Heterocyclyl can be unsubstituted or substituted in accordance with cycloalkyl.

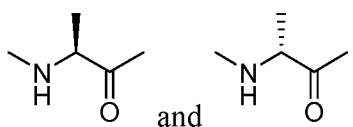
[0080] The term “oxo,” as used herein, refers to =O. When an oxo group is a substituent on a carbon atom, they form a carbonyl group (C(O)).

[0081] ---, as used herein, represents a point of attachment between two atoms.

[0082] ~~~, as used herein, represents both (R) and (S) stereochemical isomers. For example, the structure:



refers to:



EXAMPLE 1 - Reporter strain and assay development.

[0083] Previous work had shown that the caseinolytic protease is structurally and functionally conserved in the fast-growing and non-pathogenic mycobacterial model organism *M. smegmatis* (Raju, R. M., Unnikrishnan, M., Rubin, D. H., Krishnamoorthy, V., Kandrór, O., Akopian, T. N., Goldberg, A. L., and Rubin, E. J. (2012) "Mycobacterium tuberculosis ClpP1 and ClpP2 function together in protein degradation and are required for viability in vitro and during infection," *PLoS Pathog* 8, e1002511). We took advantage of this finding and engineered a *M. smegmatis* screening strain that allows detection of inhibitors of intracellular ClpP1P2 activity via accumulation of SsrA-tagged green fluorescent protein (GFP). The underlying principle is that in the undisturbed state ClpP1P2 degrades SsrA-GFP to background fluorescence levels. An inhibitor of ClpP1P2 activity would block degradation of tagged GFP resulting in a gain of signal (FIG. 1A). The engineered screening strain, *M. smegmatis* pTet-GFP-SsrA, carries an episomal SsrA-tagged GFP gene placed under the control of a tetracycline-inducible promoter (FIG. 1B). A strain carrying an untagged episomal GFP gene under the control of the same tetracycline-inducible promoter (*M. smegmatis* pTet-GFP) was used as a control for GFP expression and, as a ClpP1P2 small molecule inhibitor as a positive control for the assay was not available, to provide an estimated upper value of fluorescence signal upon complete inhibition of SsrA-GFP degradation in the screening strain (FIG. 1B).

[0084] We developed a reporter assay in 384-well plate format for High Throughput Screening (HTS). In the optimized assay format bacteria were seeded in 30 μ L at low density (OD₆₀₀=0.2, log phase) and incubated for 3 hours (to remain within one generation time) in presence or absence of 50 ng/mL of the inducer anhydrotetracycline (ATc) prior to GFP fluorescence signal measurement. FIG. 1B shows that after 3h induction with ATc, the screening strain *M. smegmatis* pTet-GFP-SsrA showed low level background fluorescence, whereas high fluorescence levels were detected in the strain expressing the untagged version

of GFP (*M. smegmatis* pTet-GFP). The assay was assessed in a pilot screen using a small collection of 1600 compounds (Pharmakon) to examine its robustness and reproducibility. The corresponding performance indicators were satisfactory with signal-to-noise ratio of 5.6+/-0.3, a Z' factor of 0.8+/-0.1, and a low hit rate of 0.5%.

[0085] High throughput screen: 1000 primary hits. A library of 503 879 compounds was screened at a single point concentration of 10 μ M. Performance indicators were again satisfactory with a signal-to-noise ratio of 3.8+/-1 and a Z' factor value of 0.8+/-0.1. Using a cut-off of two times the standard deviation from the mean value of all compounds, 1033 primary hits were identified (0.2% hit rate) (FIG. 2). Auto-fluorescent compounds were eliminated and non/low-fluorescent hits (209) were subjected to a 10 points dose-response assay. Compounds that showed any type of dose response and were available as powders (89) were characterized further (FIG. 3).

[0086] Secondary ClpP1P2 activity-based assay: 3 survivors. To exclude false positive hits due to interference with the tetracycline-dependent pTet-GFP-SsrA reporter system of the screening strain we employed a second reporter system for ClpP1P2 activity in which both the promoter and the reporter were different from the system used in the primary screen: the SsrA-tagged mCherry Red Fluorescent Protein (mRFP) gene placed under the control of a constitutive p38 promoter (*M. smegmatis* p38-mRFP-SsrA, FIG. 1C). A strain carrying an untagged version of the reporter protein (*M. smegmatis* p38-mRFP) was used again as a positive control for signal acquisition (FIG. 1C). Using this assay, and compound solutions newly prepared from powder stocks, three hits were selected that induced a significant and dose-dependent increase in mRFP fluorescence (#52, #96, #100, Table 1).

[0087] Growth inhibition activity of potential ClpP1P2 inhibitors: 1 survivor. To determine whether any of the three candidate ClpP1P2 inhibitors showed antibacterial activity we carried out 8 point growth inhibition assays with *M. smegmatis* and turbidity as readout. Table 1 shows that, whereas compounds #52 and #96 did not display any growth inhibition activity up to a concentration of 100 μ M, compound #100 showed an MIC₅₀ of 4 μ M, comparable to a ClpP1P2 IC₅₀ of 6 μ m (Table 1). Determination of cidal activity of #100 showed that MBC₉₀, the minimum bactericidal concentration that kills 90% of an initial inoculum, was 30 μ M.

[0088] The compounds were also tested for growth inhibition potency against the tubercle bacillus *M. bovis* BCG and again compound #100 showed clear growth inhibition

whereas the other two compounds did not inhibit growth up to 100 μ M (Table 1). It is interesting to note that #100 was more potent against the tubercle bacillus (MIC₅₀ = 0.3 μ M) compared to *M. smegmatis* (MIC₅₀ = 4 μ M, see discussion). Determination of cidal activity of #100 showed that MBC₉₀ for *M. bovis* BCG was with 0.75 μ M also accordingly lower.

[0089] The survivor: Bortezomib, a human proteasome inhibitor. The whole cell active, candidate ClpP1P2 protease inhibitor, compound #100 (Table 1) is the dipeptide-boronic acid Bortezomib (BZ, VELCADE, CYTOMIB). BZ is the first proteasome inhibitor approved by the US FDA for the treatment of newly diagnosed multiple myeloma and relapsed/refractory multiple myeloma and mantle cell lymphoma (28-30). The human proteasome, like bacterial caseinolytic protease, is a degradative protease complex involved in proteome housekeeping in man. The boronic acid warhead of BZ forms a covalent adduct to the catalytic hydroxyl group of threonine in the active site of the proteasome, resulting in enzyme dysfunction leading to cell-cycle arrest and apoptosis in cancer cells (31, 32).

EXAMPLE 2 - Growth inhibition activity of Bortezomib in bacteria with decreased and increased ClpP1P2 levels.

[0090] The identification of the proteasome (protease) inhibitor BZ as inhibitor in our cell-based ClpP1P2 proteolytic activity assay suggests that BZ might inhibit directly the catalytic protease subunits of the caseinolytic protease complex, ClpP1P2. To determine whether the growth inhibition effect of BZ is indeed due to interference with ClpP1P2 (and not some other, caseinolytic protease complex-related or -unrelated targets) we measured the effect of reducing and increasing intracellular ClpP1P2 levels on the growth inhibition activity of the compound. Reducing the level of ClpP1P2 is expected to increase sensitivity of the bacterium to the compound, whereas increasing the level is expected to decrease sensitivity of the cells.

[0091] To generate cultures of bacteria with lower and higher ClpP1P2 levels compared to the wild type, we employed two different *M. smegmatis* strains in which the ClpP1 and ClpP2 genes are under control of a tetracycline dependent promoter. To generate *M. smegmatis* with reduced ClpP1P2 level we employed *M. smegmatis* pTet(chromosome)-ClpP1P2 in which the expression of the native (*i.e.*, chromosomal) ClpP1P2 genes was placed under the control of a tetracycline-dependent promoter (25). In this strain the level of ClpP1P2 can be modulated as a function of added concentrations of the inducer ATc. The

Western blot analysis in FIG. 4A shows that under low (1 μ M) ATc concentration the ClpP1P2 protease level was indeed reduced. Comparative growth inhibition experiments of low-level ClpP1P2 culture with wild type bacteria depicted in FIG. 4A show that reduction of ClpP1P2 protein level indeed resulted in a pronounced hyper-sensitization of the bacteria to BZ: MIC₅₀ of BZ shifted down from 4 μ M to 0.5 μ M.

[0092] To generate *M. smegmatis* with increased ClpP1P2 level we employed *M. smegmatis* pTet-ClpP1P2, a strain which carried in addition to the wild type chromosomal ClpP1P2 genes an episomal copy of ClpP1P2 under control of the same tetracycline-inducible promoter mentioned above (25). Addition of an appropriate high (50 μ M) ATc concentration increased the level of ClpP1P2 as shown in the Western blot analysis in FIG. 4B. FIG. 4B also shows that increase of ClpP1P2 level de-sensitized the bacteria: BZ's MIC₅₀ shifted from 4 μ M to 20 μ M. Taken together, the ClpP1P2 under- and over expression results, showing an inverse correlation between candidate target level and antibacterial drug susceptibility, suggest that BZ exerts its growth inhibitory whole cell effect by targeting ClpP1P2.

[0093] In the growth inhibition experiment with *M. smegmatis* wild type only BZ/#100 showed an effect. Compounds #52 and #96, weakly positive in the reporter ClpP1P2 activity assays, did not show any antibacterial activity (Table 1). To determine whether antibacterial whole cell activity might be detectable in sensitized bacteria with reduced ClpP1P2 level, we determined the effect of compounds #52 and #96 against under-expressing cultures of *M. smegmatis* pTet(chromosome)-ClpP1P2. However, no effect on growth was observed up to concentration of 100 μ M (data not shown). This suggests that #52 and #96 do not act via ClpP1P2. Whether these two compounds act via other, non-proteolytic components of the caseinolytic protease complex such as the regulatory ATPases, or whether their apparent reporter activity in the ClpP1P2 fluorescence assays is an artefact remains to be determined.

EXAMPLE 3 - Combination of Bortezomib with aminoglycosides.

[0094] Genetic depletion experiments showed previously that bacteria with reduced ClpP1P2 level display increased sensitivity to the aminoglycosides amikacin and streptomycin, supporting the notion that mycobacterial ClpP1P2 is involved in the removal of mistranslated proteins as shown for other bacteria (25). This potentiation effect for protein synthesis inhibitors was specific for the mistranslation inducing aminoglycosides, e.g.,

amikacin and streptomycin, whereas no potentiation was observed for the ribosome stalling antibiotic chloramphenicol (33). If BZ is an authentic small molecule ClpP1P2 inhibitor the drug is expected to copy that phenotype. FIG. 5 shows that the predicted selective potentiation effect can be indeed observed. A combination of sub-inhibitory concentrations of BZ and amikacin or streptomycin caused complete growth inhibition whereas BZ had no potentiation effect on chloramphenicol. Taken together, the drug combination experiments show that BZ copies the selective aminoglycoside-hypersensitivity phenotype observed for ClpP1P2 under-expressing bacteria, consistent with BZ being an inhibitor of this protease.

EXAMPLE 4 - Effect of Bortezomib on the level of the caseinolytic protease substrate WhiB1.

[0095] Genetic experiments combined with quantitative proteomic and transcriptomic analyses identified recently the first specific protein substrate of ClpP1P2, the transcription factor WhiB1. Depletion of ClpP1P2 resulted in accumulation of this DNA binding protein (25). If BZ is an authentic ClpP1P2 inhibitor, exposure of the bacteria to the compound is expected to copy the effect of ClpP1P2 under-expression on WhiB1 and result in an increase of WhiB1 protein level. To determine the effect of BZ on the degradation of WhiB1 we employed again a *M. smegmatis* reporter strain expressing tetracycline-inducible GFP, but with WhiB1 as ClpP1P2-specific degradation 'tag' instead of the SsrA-tag used for the primary screen (*M. smegmatis* pTet-GFP-WhiB1, FIGS. 6A, 6B). FIG. 6B shows that BZ exposure increased fluorescence in a dose dependent manner, suggesting that the drug indeed inhibits ClpP1P2-dependent degradation of WhiB1 resulting in accumulation of GFP-WhiB1. FIG. 6C shows that this effect of BZ on the GFP signal was WhiB1-dependent: BZ did not affect the fluorescence of *M. smegmatis* culture carrying the same episomal pTet-GFP construct but with GFP lacking the WhiB1 tag. Our results that BZ blocks degradation of the caseinolytic protease substrate WhiB1 further supports the model that the drug exerts its antibacterial activity via modulation of ClpP1P2.

EXAMPLE 5 - Correlation between ClpP1P2-dependent proteolytic- and growth inhibition potencies of structural derivatives of Bortezomib.

[0096] Apparent dose-dependent ClpP1P2 protease inhibition in two strains carrying different ClpP1P2-activity-based SsrA reporter systems, expected susceptibility shifts in under- and over expressing ClpP1P2 strains, specific synergy with aminoglycosides and

accumulation of the ClpP1P2 substrate WhiB1 upon BZ exposure, are all supporting the notion that BZ inhibits ClpP1P2 and that it is via this interaction that the compound exerts its whole cell growth inhibitory activity.

[0097] A powerful independent method to show that a particular chemical scaffold exerts its whole cell growth inhibitory effect via modulation of a particular target is based on demonstrating a correlation between the two (whole cell vs enzyme) structure activity relationships. The concept is to identify structural derivatives of the scaffold that cover a range of enzyme inhibition activities (highly, medium and no potency) and determine whether the IC50s for the enzyme correlate with whole cell MIC50s. A positive correlation argues for an on-target effect. FIG. 7A shows three Bortezomib derivatives CEP-18770, MNL-2238 and MG-262. CEP-18770 and MNL-2238 are second generation proteasome inhibitors, and MG-262 is another boronate peptide showing activity against human proteasome (34-37). FIG. 7B shows that these three compounds show high (CEP-18770, same activity as BZ itself), and medium (MNL-2238, MG-262) inhibitory potencies in the cell-based fluorescent assay measuring ClpP1P2 proteolytic activity (*M. smegmatis* p38-mRFP-SsrA). FIG. 7C shows that the whole cell growth inhibitory activities of the compounds follow the same pattern: CEP-18770 shows the same potent growth inhibition as Bortezomib. The weaker inhibitors in the ClpP1P2 reporter assay, MNL-2238 and MG-262, also show weaker growth inhibition. The observed correlation between potency against ClpP1P2 and growth inhibition of BZ analogues suggests that the drug acts via the assumed target.

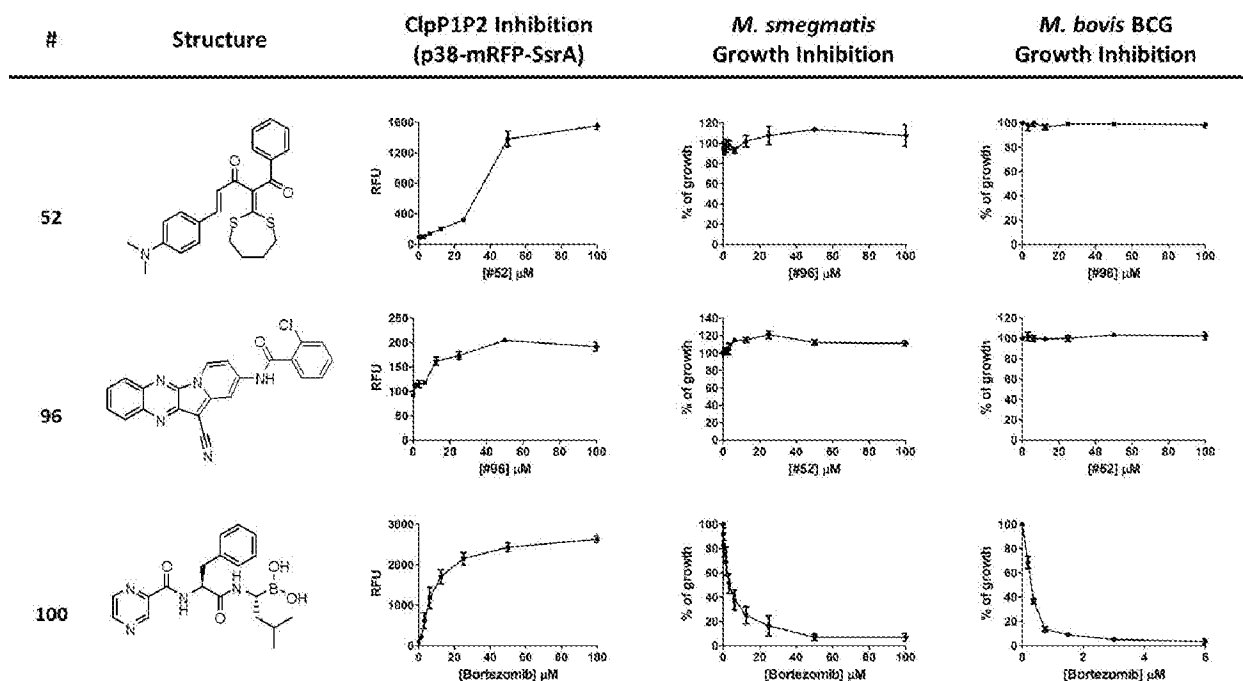
EXAMPLE 6 - Dependence of ClpP1P2 and growth inhibitory activity of Bortezomib on its boronic acid warhead.

[0098] The boronic acid warhead of the human proteasome inhibitors reacts covalently with the active site threonine hydroxyl moiety of the proteasome and is important for selectivity and potency of the compounds. For MG-262 for instance it has been shown that substitution of the boronic acid warhead with an aldehyde resulted in a 100-fold reduced activity against the proteasome (34, 35). Similarly, BZ was developed as a more potent analogue of its peptide aldehyde counterpart (36, 37). Assuming a similar, boronic acid-dependent mechanism for the inhibition of the mycobacterial ClpP1P2 serine proteases, the prediction is that removal of the warhead results in a simultaneous loss of activity of the

compound in both the cellular ClpP1P2 activity and the growth inhibition assay. FIG. 7A shows the aldehyde derivatives of BZ and MG-262, BZ-al and MG-132, respectively. FIG. 7B and 7C show that the substitution of boronic acid with aldehyde completely abrogated both enzyme- and growth-inhibition activity of the two compounds. These results show that the boronic acid warhead is essential for anti-ClpP1P2 proteolytic- and anti-bacterial activity, and indicate that Bortezomib inhibits ClpP1P2 via covalent modification of its active sites.

EXAMPLE 7 - Modeling of inhibitors with boronic acid warhead into *M. tuberculosis* ClpP1P2.

[0099] Both ClpP1 and ClpP2 are heptamers and each has 7 catalytic sites. The boronic acid based inhibitors (FIG. 7) were modelled into the ClpP1 and ClpP2 catalytic sites and covalently attached to the serine of the catalytic triad (Ser98 and Ser110 in ClpP1 and ClpP2, respectively). All the nitrogen and oxygen atoms of the inhibitor amide groups hydrogen bond with the protein backbone. One oxygen of the boronic acid occupies the oxyanion hole while the other forms a salt bridge to the catalytic histidine (His123 and His135 of ClpP1 and ClpP2, respectively). This is shown in FIG. 8 for Bortezomib modelled into one of the ClpP1 sites. The P1 site is hydrophobic in both ClpP1 and ClpP2 consistent with the hydrophobic side chain of the inhibitors. Neither ClpP1 nor ClpP2 has a proper P2 site and the P2 side chain of the inhibitors makes poor contacts with the protein. Bortezomib, CEP-18770, and MNL-2238 modelled well into both ClpP1 and ClpP2 sites. MG-262 has an extra residue compared to the other inhibitors and this compound did not model well due to the P3 side-chain clashing with the protein (data not shown). This is consistent with the observed enzyme inhibitory activities shown in FIG. 7B in which MG-262 was the least potent compound of the four.



[00100] **Table 1.** ClpP1P2 activity- and growth inhibition of prioritized hits. Shown are the structure, ClpP1P2 activity dose response in *M. smegmatis* p38-mRFP-SsrA and the growth inhibition dose response in *M. smegmatis* and *M. bovis* BCG. The experiments were carried out three times in showing the same results. One representative example is depicted. RFU, relative fluorescence units.

[00101] The teachings of all patents, published applications and references cited herein are incorporated by reference in their entirety.

[00102] While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

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CLAIMS

What is claimed is:

1. A method of treating *Mycobacterium tuberculosis* comprising administering to a patient in need thereof an effective amount of a caseinolytic protease inhibitor in combination with an aminoglycoside.
2. The method of Claim 1 wherein the caseinolytic protease inhibitor is selected from the group consisting of Bortezomib, CEP-18770 (Delanzomib), MLN-2238, MLN-9708, and MG-262.
3. The method of Claim 1 or 2 wherein the caseinolytic protease is ClpP1P2.
4. The method of Claim 1 or 2, wherein the aminoglycoside is amikacin, streptomycin, or a combination thereof.
5. The method of Claim 2, wherein the caseinolytic protease inhibitor is Bortezomib.
6. The method of Claim 2, wherein the caseinolytic protease inhibitor is CEP-18770.
7. A method of inhibiting mycobacterial caseinolytic protease in a patient suffering from tuberculosis comprising administering to the patient an effective amount of Bortezomib, CEP-18770 (delanzomib), MLN-2238, MLN-9708, or MG-262.
8. The method of Claim 7 further comprising administering to the patient an aminoglycoside.
9. A method of blocking degradation of the substrate of ClpP121 comprising contacting ClpP1P2 with Bortezomib, CEP-18770 (delanzomib), MLN-2238, MLN-9708, MG-262, or any derivative thereof.
10. The method of claim 9 wherein the substrate is WhiB1.
11. A composition for the treatment of *Mycobacterium tuberculosis* comprising an aminoglycoside and at least one compound selected from the group consisting of Bortezomib, CEP-18770 (delanzomib), MLN-2238, MLN-9708, and MG-262.

12. A method of increasing sensitivity of *Mycobacterium bacteria* to an aminoglycoside in a patient suffering from *Mycobacterium tuberculosis* comprising administering Bortezomib, CEP-18770 (delanzomib), MLN-2238, MLN-9708, or MG-262.
13. The method of claim 12 wherein the aminoglycoside is amikacin, streptomycin, or a combination thereof.
14. The method of claim 12 wherein the aminoglycoside is a mistranslation-inducing aminoglycoside.
15. A method of inhibiting mycobacterial caseinolytic protease activity comprising contacting mycobacterial caseinolytic protease with Bortezomib, CEP-18770 (delanzomib), MLN-2238, MLN-9708, or MG-262.
16. A method of treating *Mycobacterium tuberculosis* comprising administering to a patient in need thereof an effective amount of MLN-9708.
17. The method of Claim 16, wherein the MLN-9708 is administered orally.
18. A method of treating *Mycobacterium tuberculosis* comprising administering to a patient in need thereof an effective amount of Bortezomib, CEP-18770 (delanzomib), MLN-2238, MLN-9708, or MG-262.

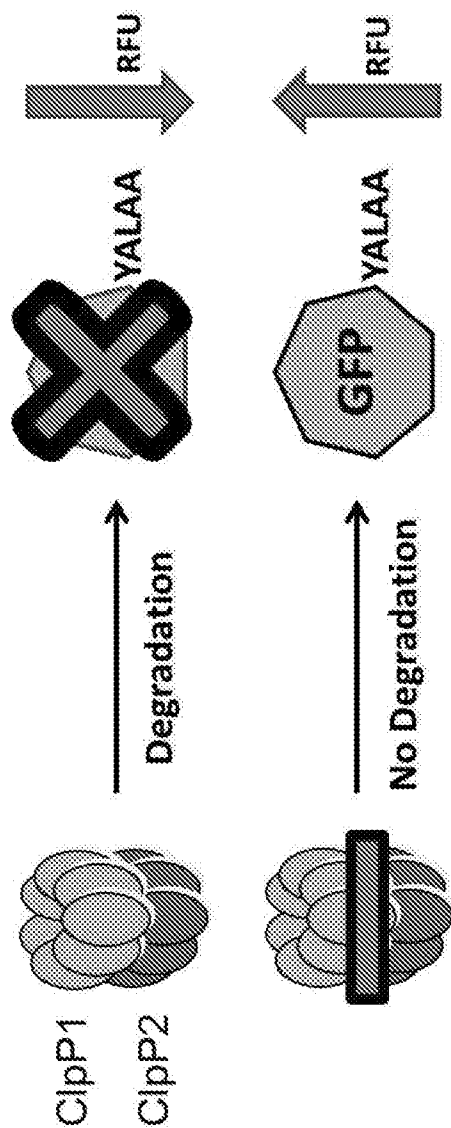


FIG. 1A

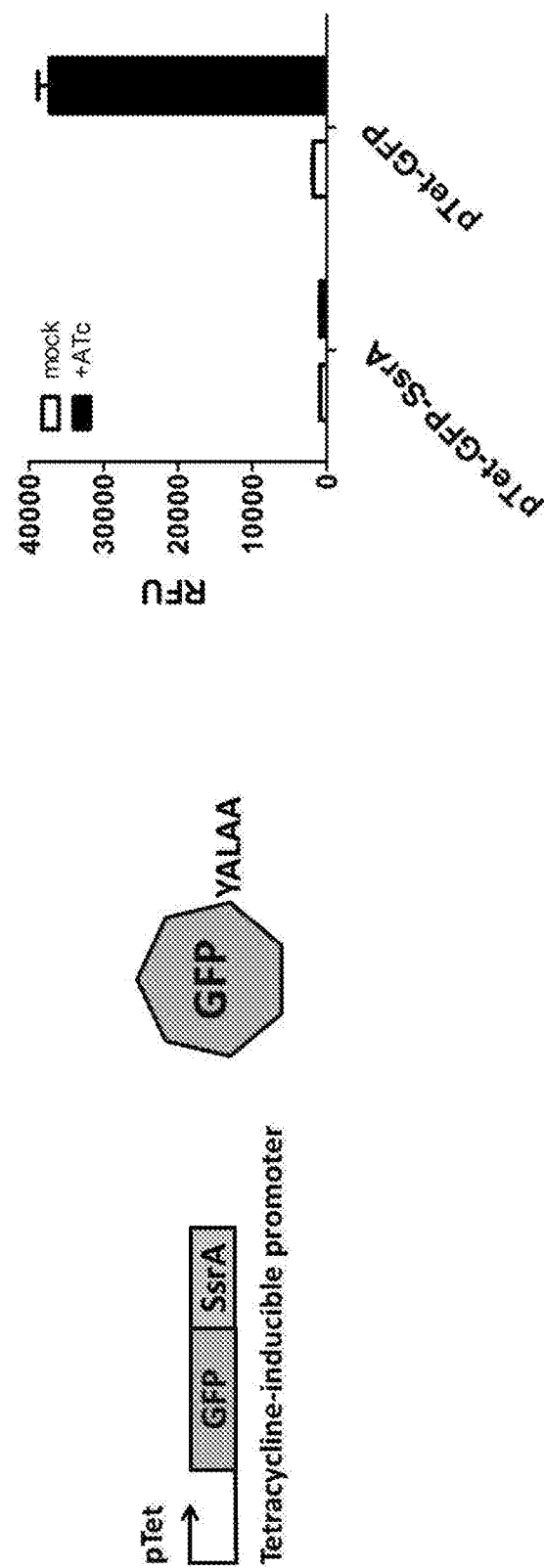


FIG. 1B

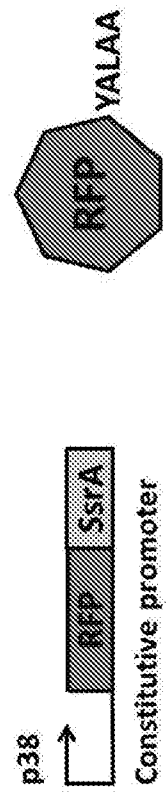
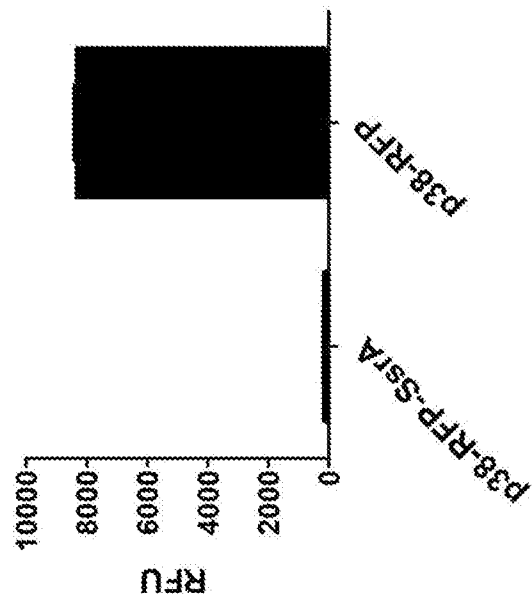


FIG. 1C

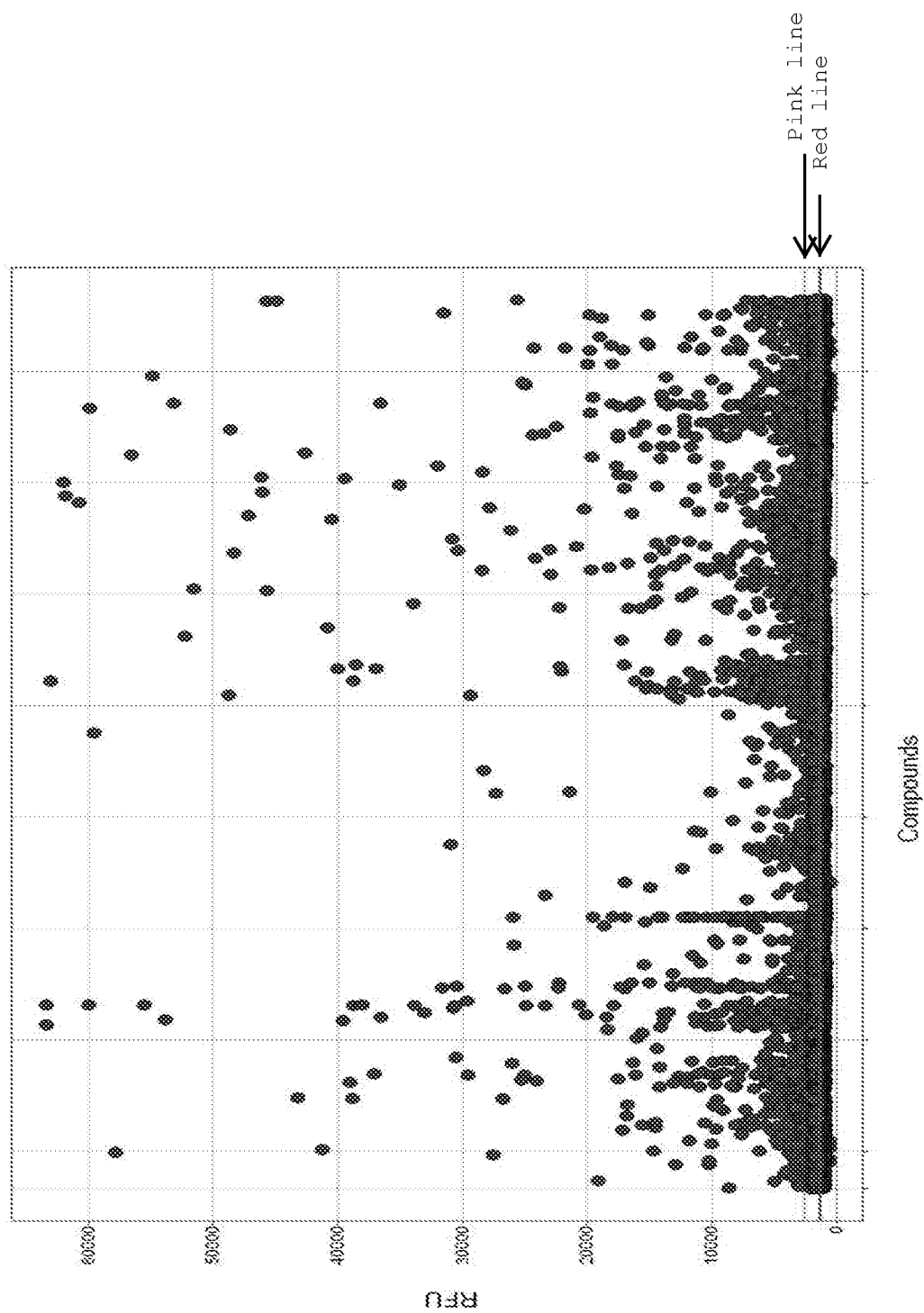


FIG. 2

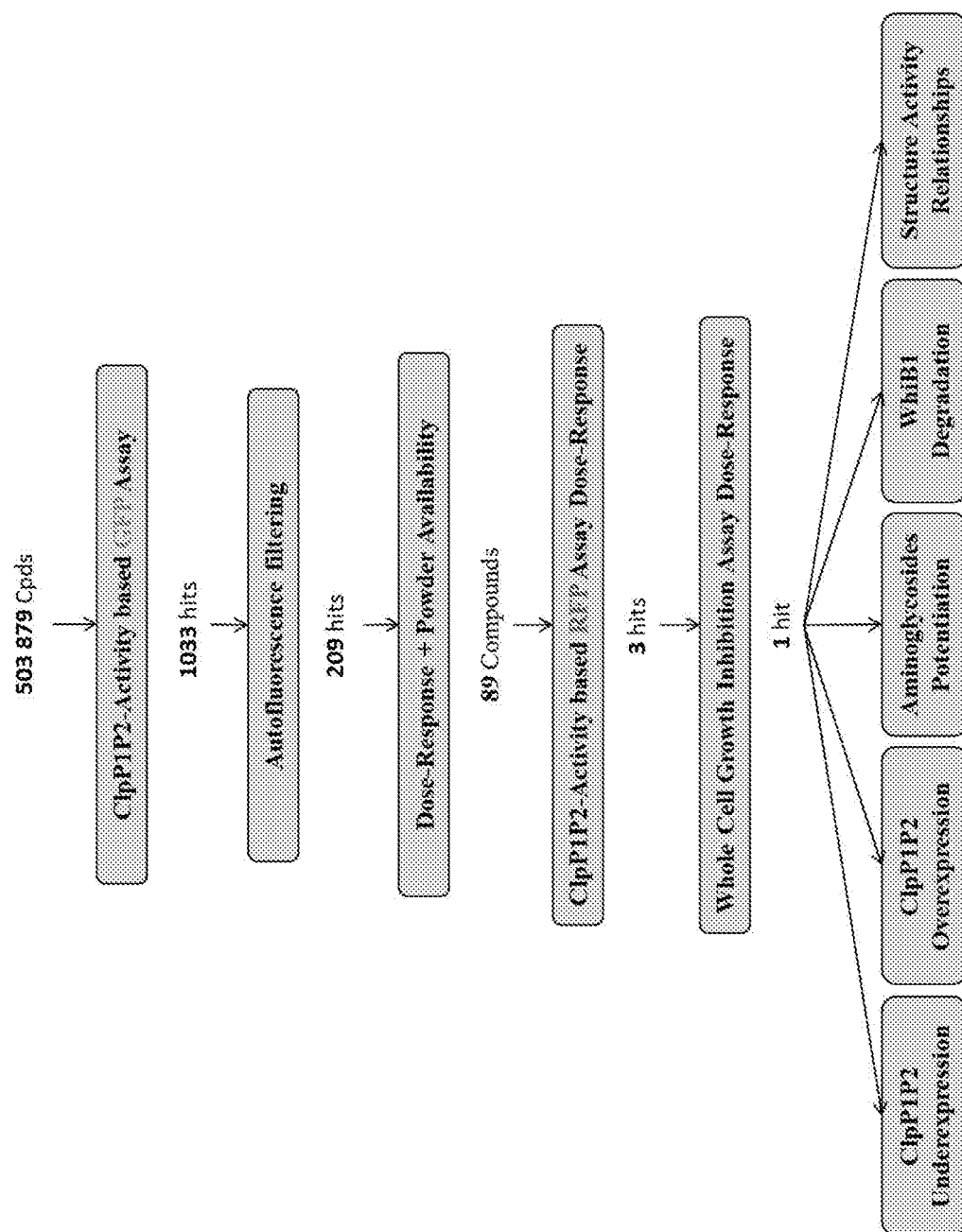


FIG. 3

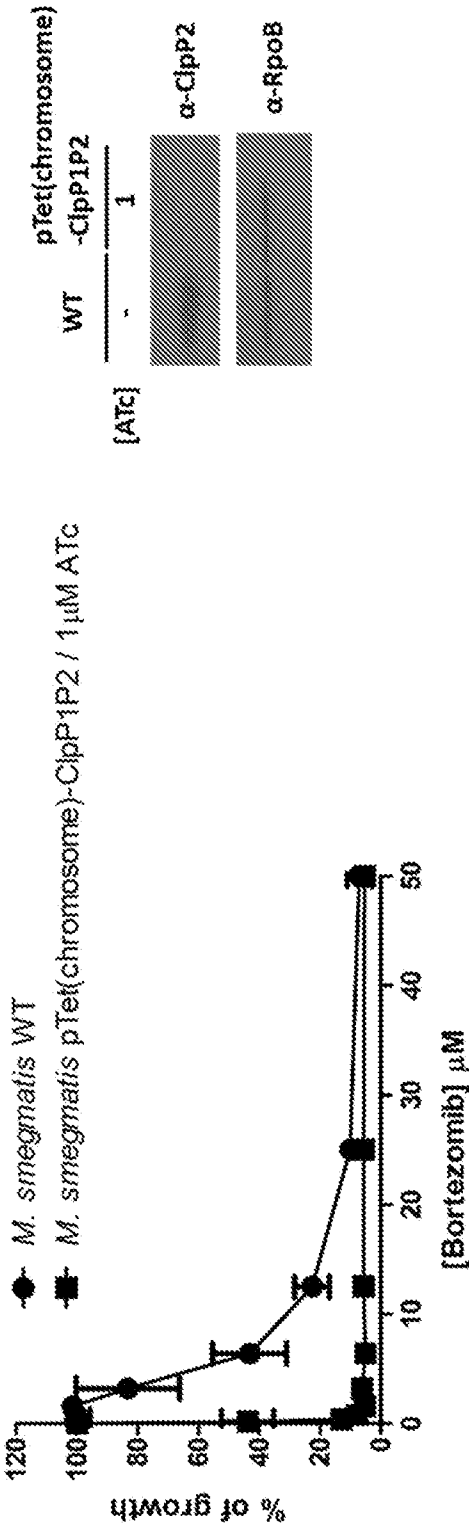


FIG. 4A

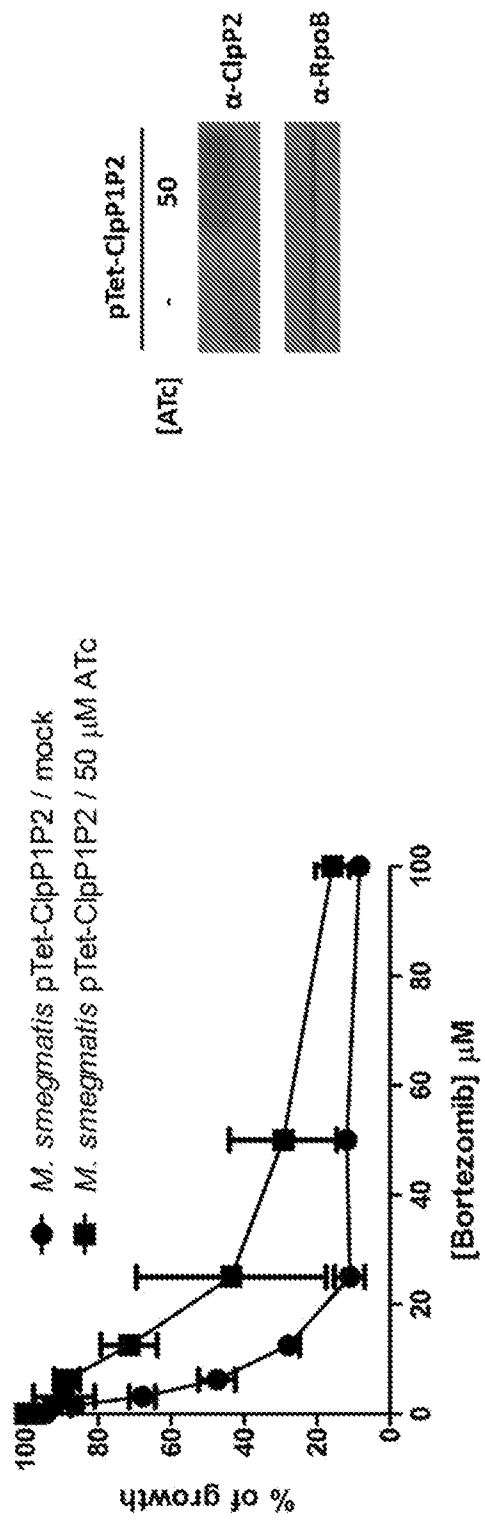


FIG. 4B

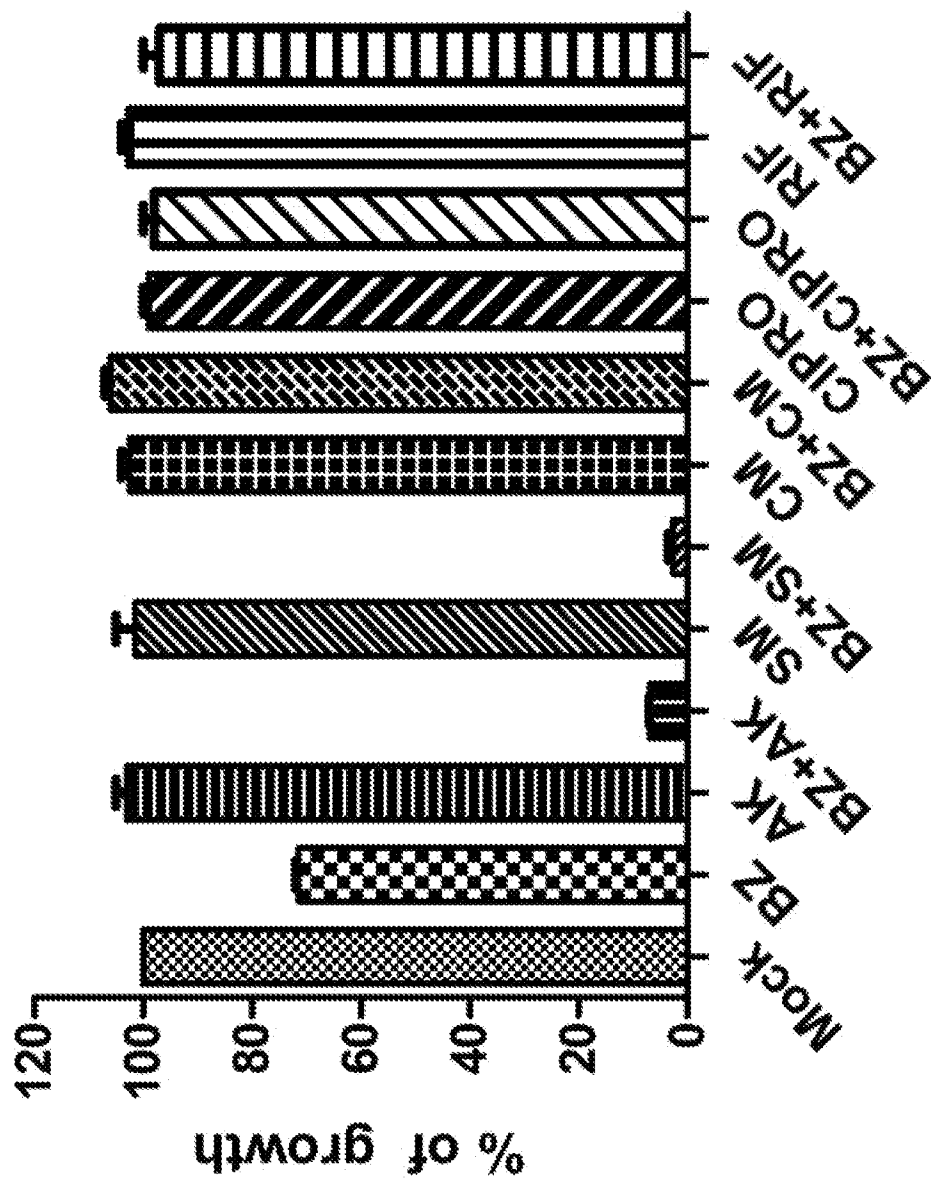


FIG. 5

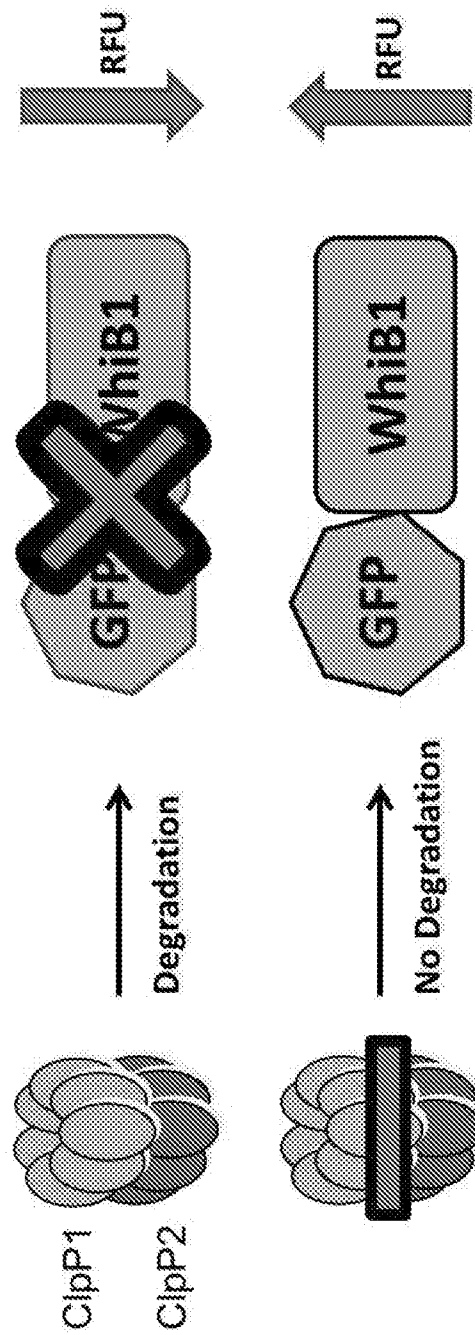


FIG. 6A

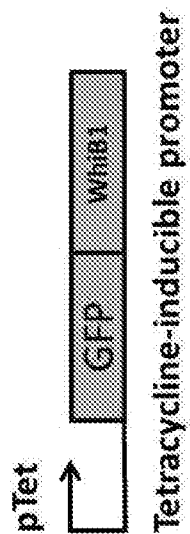
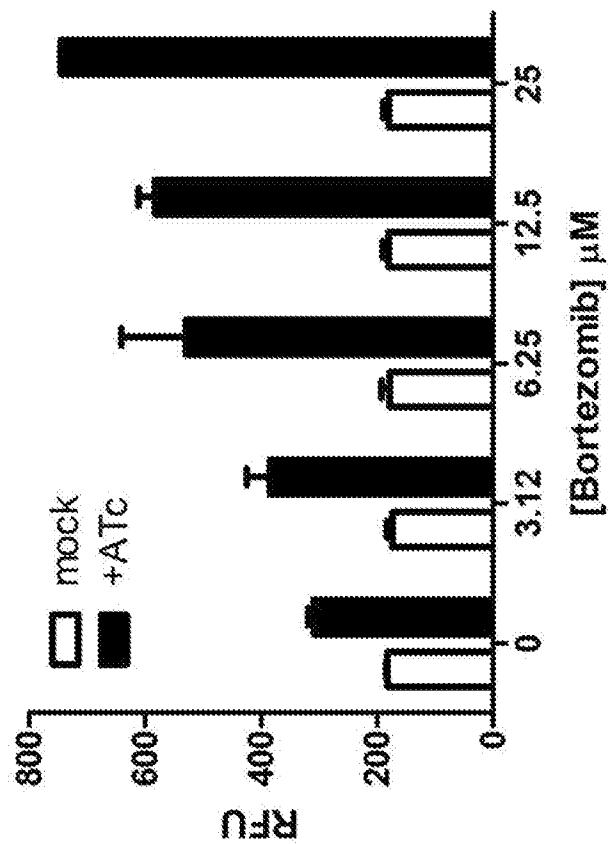


FIG. 6B

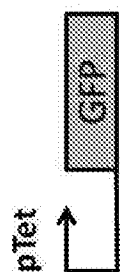
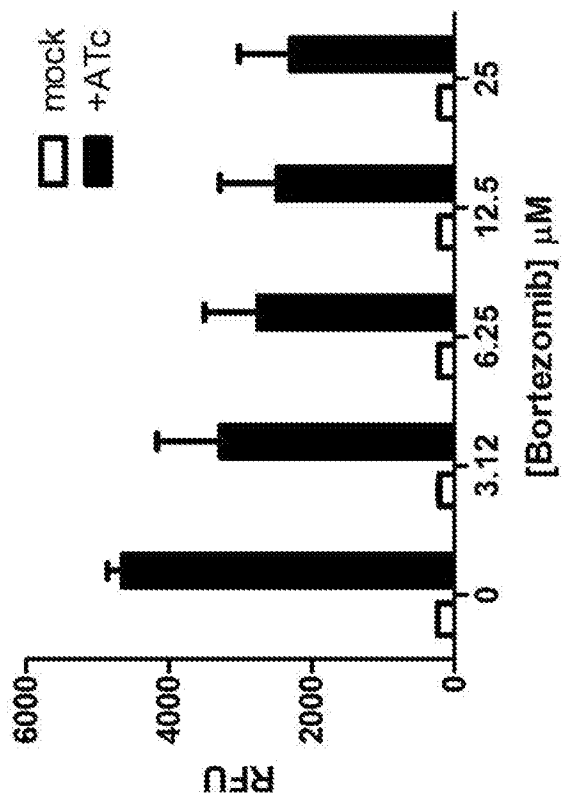


FIG. 6C

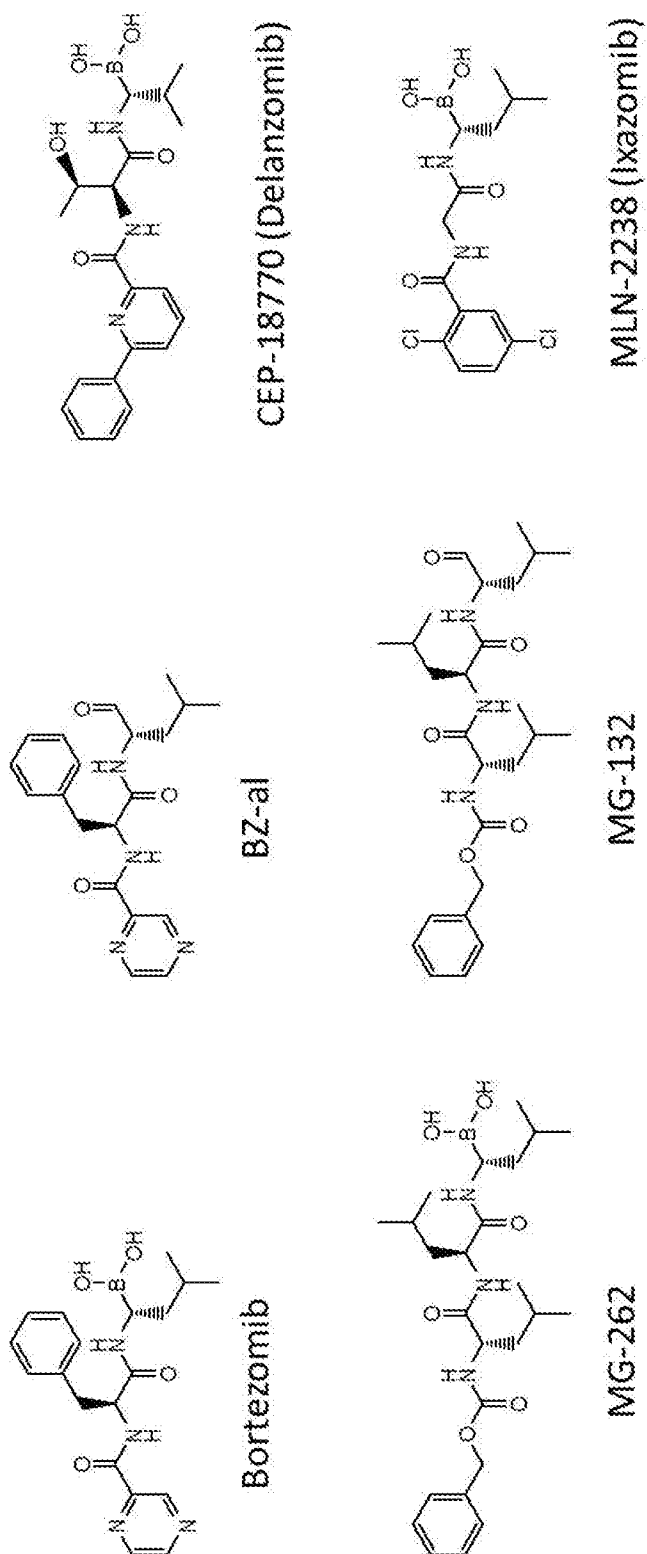


FIG. 7A

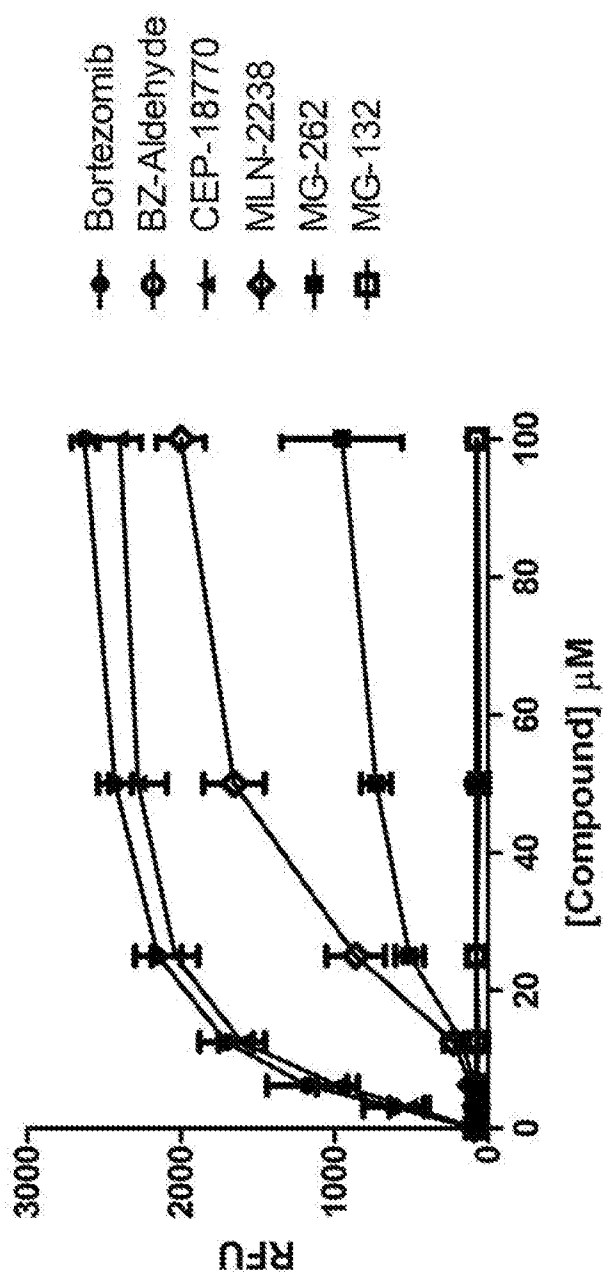


FIG. 7B

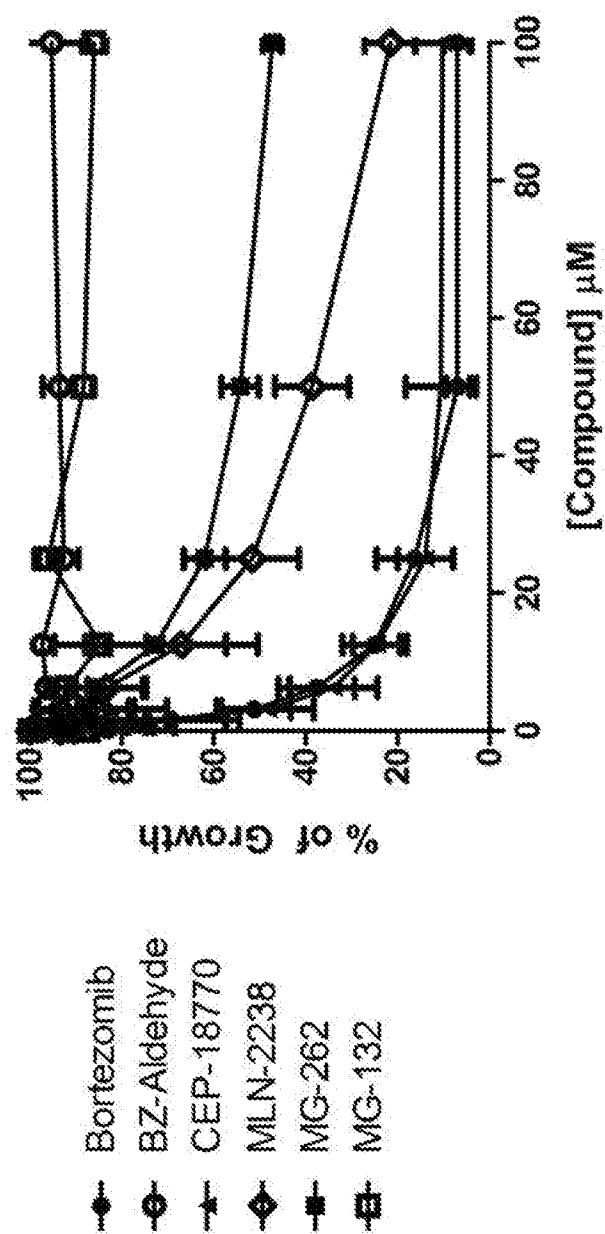


FIG. 7C

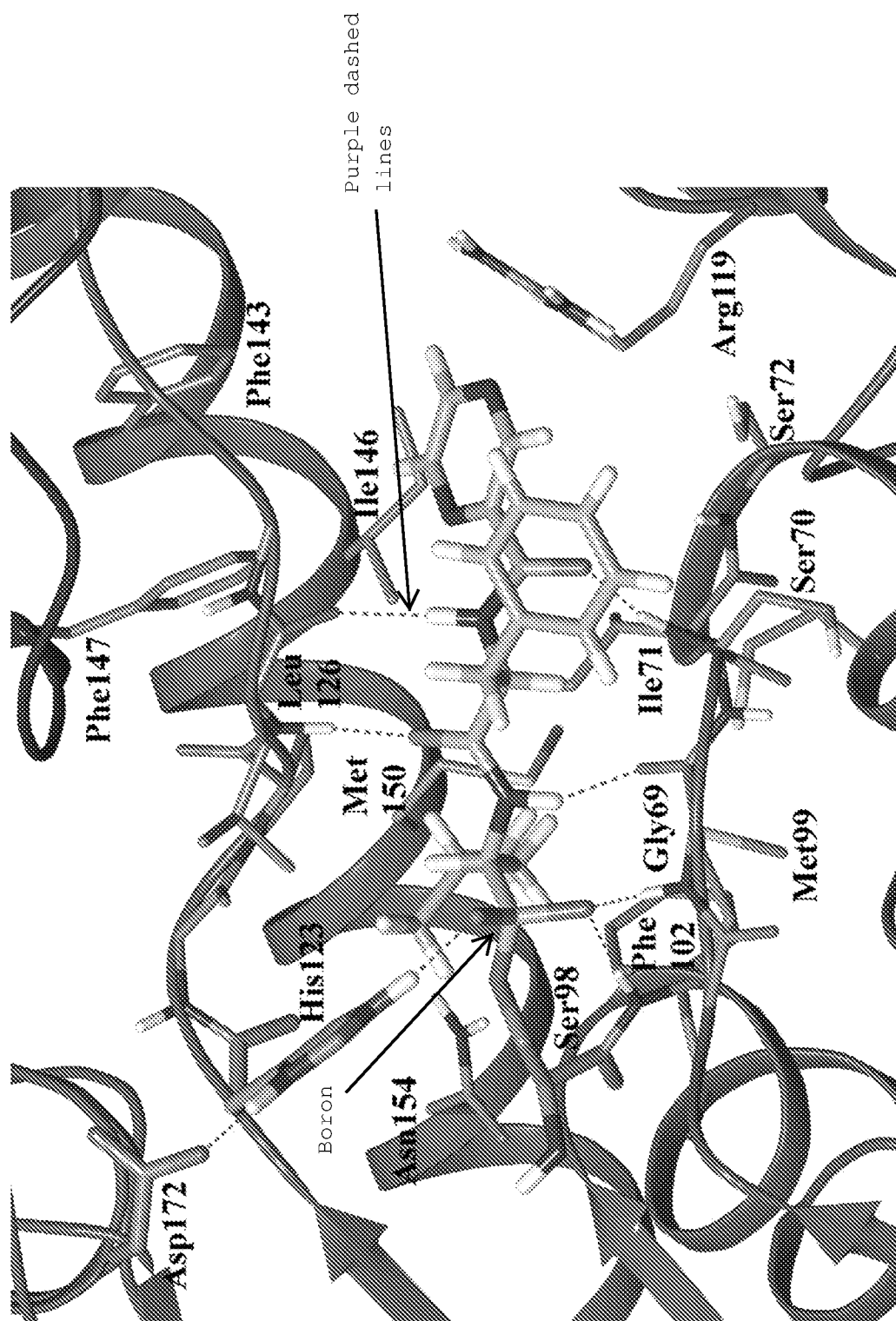


FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/016658

A. CLASSIFICATION OF SUBJECT MATTER

A61K 31/69 (2006.01) A61K 31/195 (2006.01) A61P 31/06 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DATABASES: MEDLINE, EPODOC, WPIAP, CAPLUS, BIOSIS, EMBASE.

KEYWORDS: Tuberculosis, caseinolytic, ClpP+, ClpAP+, protease, peptidase, proteinase, WhiB1, ClpP1P2, inhibit+, decreas+, reduc+, block, bortezomib, PS-341, LDP-341, velcade, neomib, bortecad, CEP_18770, delanzomib, MLN_2238, ixazomib, ninlaro, MLN-9708, MG-262, Z-Leu-Leu-Leu-B-OH2 OR boro-LLL, aminoglycoside?, amikacin, streptomycin, mycobacteri+ as well as synonyms and similar terms.

Applicant and/or Inventor searches of the patent and non-patent literature was performed using Patentscope (<http://www.wipo.int/patentscope/en/>), PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>), and in internal databases provided by IP Australia.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 6 April 2016	Date of mailing of the international search report 06 April 2016
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaaustralia.gov.au	Authorised officer Monica Graham AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262833179

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/US2016/016658
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/092103 A2 (SPECTRUM PHARMACEUTICALS, INC.) 31 July 2008 (see Example 1)	11
X	WO 2013/059622 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 25 April 2013 (see paragraph [0010], [0012], [0013], [0052] and claims)	1, 3 and 4
A	WO 2009/026579 A1 (CORNELL RESEARCH FOUNDATION, INC.) 26 February 2009 (see abstract and claims)	1-18
A	COMPTON, C.L. et al. "Antibacterial activity of and resistance to small molecule inhibitors of the ClpP peptidase." ACS Chem Biol. 2013 Dec 20;8(12):2669-77. Epub 2013 Oct 4. (see abstract and page 4 last paragraph – page 5 paragraph 2)	1-18
A	OLLINGER, J. et al. "Validation of the essential ClpP protease in Mycobacterium tuberculosis as a novel drug target" Journal of Bacteriology 2012; 194(3), 663-668. (see whole document)	1-18
A	HU, G. et al. "Structure of the Mycobacterium tuberculosis proteasome and mechanism of inhibition by a peptidyl boronate." Mol Microbiol. 2006 Mar;59(5):1417-28. (see whole document)	1-18
A	AHN, J.S. et al. "Poor prognostic significance of Mycobacterium tuberculosis infection during bortezomib-containing chemotherapy in patients with multiple myeloma." Blood Res. 2013 Mar;48(1):35-9. Epub 2013 Mar 25. (see whole document)	1-18
P,X	MOREIRA, W. et al. "Target mechanism-based whole-cell screening identifies bortezomib as an inhibitor of caseinolytic protease in mycobacteria." MBio. 2015 May 5; 6(3):e00253-15. (see whole document)	1-15 and 18
P,X	WO 2015/130922 A2 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA and THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY) 03 September 2015 (see claims 36-53)	7, 9, 15, 16 and 18

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/US2016/016658	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2008/092103 A2	31 July 2008	WO 2008092103 A2	31 Jul 2008
		US 2008181971 A1	31 Jul 2008
WO 2013/059622 A1	25 April 2013	WO 2013059622 A1	25 Apr 2013
		US 2014243255 A1	28 Aug 2014
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