Development of Tyrphostin Analogues to Study Inhibition of the *Mycobacterium tuberculosis* Pup Proteasome System**

Guido V. Janssen, [a, b] Susan Zhang, [c] Remco Merkx, [b] Christa Schiesswohl, [d] Champak Chatterjee, [d] K. Heran Darwin, [c] Paul P. Geurink, [a, b] Gerbrand J. van der Heden van Noort, [a, b] and Huib Ovaa [a, b]

Tuberculosis is a global health problem caused by infection with the *Mycobacterium tuberculosis* (Mtb) bacteria. Although antibiotic treatment has dramatically reduced the impact of tuberculosis on the population, the existence and spreading of drug resistant strains urgently demands the development of new drugs that target Mtb in a different manner than currently used antibiotics. The prokaryotic ubiquitin-like protein (Pup) proteasome system is an attractive target for new drug development as it is unique to Mtb and related bacterial genera. Using a Pup-based fluorogenic substrate, we screened for inhibitors of Dop, the Mtb depupylation protease, and identified i-OMe-Tyrphostin AG538 (1) and Tyrphostin AG538 (2). The hits were validated and determined to be fast-reversible, non-ATP competitive inhibitors. We synthesized > 25 analogs of 1 and 2 and show that several of the synthesized compounds also inhibit the depupylation actions of Dop on native substrate, FabD-Pup. Importantly, the pupylation activity of PafA, the sole Pup ligase in Mtb, was also inhibited by some of these compounds.

Introduction

Tuberculosis (TB), the disease resulting from infection with *Mycobacterium tuberculosis* (Mtb) is one of the leading causes of death, killing over a million people annually.\(^1\) Treatment of TB requires the use of one or more antibiotics that are taken daily for many months. This treatment can be accompanied by severe side effects such as hepatitis, dyspepsia, exanthema and arthralgia and are a major factor to poor adherence in TB treatment.\(^2\) As a result, multidrug-resistant tuberculosis (MDR-TB) has developed, that is resistant to both first line antibiotics isoniazid and rifampicin, and even totally drug-resistant tuberculosis has evolved that is resistant against all first- and second-line TB drugs.\(^3\) This poses a serious threat to human health and necessitates the development of new drugs to treat TB. These drugs ideally should have novel modes of action and/or inhibit targets different from those of currently used drugs, to minimize resistance. In addition, the targeted pathway should be specific for Mtb to circumvent the possibility of unwanted side effects in the human host.

An attractive target is the Mtb Pup-proteasome system (PPS) as it is essential for Mtb to cause lethal infections in animals.\(^4\) Mtb belongs to one of the few bacterial orders that have proteasomes, which are large protein complexes that degrade proteins.\(^5\) In general, proteins are degraded by a proteasome when they are post-translationally modified with a small protein tag. In eukaryotes, ubiquitin (Ub), a 76-amino acid small protein, serves as tag whereas in prokaryotes and eukaryotes, there is no sequence homology among proteases are, for the most part, conserved between prokaryotes and eukaryotes;\(^6\) there is no sequence homology among the enzymes used for the (de)conjugation in the Ub and Pup systems. Compared to the complex eukaryotic ubiquitin proteasome system, where an estimated 750 conjugating and deconjugating enzymes regulate ubiquitination,\(^7,8\) the Pup-proteasome system (PPS) is fairly simple as only two enzymes so far are identified in the (de)pupylation cascade (Figure 1A). Proteasome accessory factor A (PafA), is the sole ligase of Pup

---

[a] Dr. G. V. Janssen, Dr. P. P. Geurink, Dr. G. J. van der Heden van Noort, Prof. Dr. H. Ovaa\(^*\)
Oncode Institute and Department of Cell and Chemical Biology
Leiden University Medical Center
Eindhoovenweg 20, 2333 ZC Leiden (The Netherlands)
E-mail: gvanderheden@lumc.nl

[b] Dr. G. V. Janssen, Dr. R. Merkx, Dr. P. P. Geurink, Dr. G. J. van der Heden van Noort, Prof. Dr. H. Ovaa\(^*\)
Division of Cell Biology, Netherlands Cancer Institute
Plesmanlaan 121, 1066 CX Amsterdam (The Netherlands)

[c] S. Zhang, Prof. Dr. K. H. Darwin
Department of Microbiology
New York University School of Medicine
430 E. 29th Street, New York, New York 10016 (USA)

[d] Dr. C. Schiesswohl, Dr. C. Chatterjee
Department of Chemistry, University of Washington
Seattle, Washington 98195 (USA)

[\(^{[1]}\) In memory of Huib Ovaa, whose passion for science will remain an inspiration.

[**\(^{[2]}\) A previous version of this manuscript has been deposited on a preprint server (https://www.biorxiv.org/content/10.1101/796359v1).

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202100333

© 2021 The Authors. ChemBioChem published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution-Non-Commercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
and catalyzes formation of the isopeptide bond between the γ-carboxylate of the C-terminal glutamate (Glu) of Pup and an ε-amino group of a substrate lysine. In this process, the C-terminal Glu of Pup is first phosphorylated by PafA, followed by nucleophilic attack of a substrate lysine ε-amino group to the activated carboxylic acid. As a result, PafA turns over ATP to ADP and P1. In species where Pup is translated with a C-terminal glutamine (Gln), a deamidation step is needed to convert the terminal Gln to Glu by Dop (deamidase of Pup) before it can be ligated to a substrate by PafA. Although Dop and PafA have high sequence and structural similarity, deamidation by Dop uses ATP as cofactor but is not dependent on the hydrolysis of ATP, in contrast to PafA ligase activity. Both Dop and PafA can also depupylate substrates, although this function is less efficient than their respective primary deamidase and ligase function. Unlike the pupylation process, the precise mechanisms of de- and trans-pupylation (and deamidation) are incompletely understood.

Figure 1. A) Schematic representation of the main enzymatic functions in the Pup-proteasome system in Mtb. B) Fluorogenic assay to monitor Dop activity and screen for inhibitors.

Results and Discussion

Screen for Dop inhibitors

We screened a library of 1280 pharmaceutically active compounds (LOPAC-library) for Dop inhibition using the fluorogenic Dop substrate, Pup(33-63)-Glu(AMC). We identified several hits in this screening, including I-OMe-Tyrphostin AG 538 (1) and Tyrophostin AG538 (2) as most potent inhibitors (Figure 2A). Both molecules consist of a benzylidene 2-cyanoacetophenone backbone in which the aromatic rings on both sides carry various substituents. Compounds 1 and 2 are known to act as inhibitors for several kinases with various modes of inhibition. IGF-1 Receptor Kinase is inhibited via competition with the substrate, while phosphoinositide kinase PI5P4K is inhibited via competition with ATP and oxidative stress-sensitive Ca2+-preamble channel TRPA1 and TRPM2 activated by H2O2 are inhibited by means of hydroxyl radical scavenging. Compounds from the Tyrophostin cluster are known as pan-assay interference compounds (PAINS) giving false positives in screens, mostly caused by the (DTT-mediated) redox-cycling potential of the catechol part of these compounds. By preparing analogues, where we systematically modify the Tyrphostin core while omitting the catechol part, we were able to study the mode of action of the initial hits. Several of these analogues of compound 1 and 2 (as described below), show moderate to good inhibition, excluding these compounds to collectively be considered as PAINS. In order to further prevent false positives due to redox-cycling we used cysteine instead of DTT as the reducing agent in our screen and follow-up assays in addition to using 0.01 % of the detergent CHAPS in the assay buffers to minimize aggregate formation.

Mode of action

To validate the initial screening hits, we determined the IC50 values for 1 and 2 (Figure 2A) and found values of 0.52 ± 0.12 μM and 0.13 ± 0.03 μM for 1 and 2, respectively (Figure 2B, Supporting Information Figure S1). We also show limited autofluorescence or interference of the compounds with AMC fluorescence in the assay at values in the range of the IC50 values of the inhibitors (Supporting Information Figure S2).
Next, we tested if 1 is inhibiting Dop in an irreversible and covalent manner by determining IC\textsubscript{50} values after different incubation times. If 1 is an irreversible covalent binder, lower IC\textsubscript{50} values would be expected when increasing incubation times, since 1 has more time to form a covalent bond with Dop.

Pre-incubation of Dop with 1 for 5 or 60 minutes before addition of the substrate (Supporting Information Figure S3) did not change IC\textsubscript{50} values significantly (0.47 ± 0.05 μM for 5 min incubation vs. 0.71 ± 0.08 μM for 60 min incubation) indicating a non-covalent or at least reversible-covalent mode of inhibition. We then performed a ‘jump-dilution’ assay (Figure 2C), where Dop was pre-incubated for 30 minutes with a concentration of 25 times the IC\textsubscript{50} value for 1, followed by a 100-fold dilution and reassessment of the activity. Complete restoration of Dop activity was observed after dilution, suggesting that there was a fast re-equilibration of the Dop-1 complex and that 1 is a fast-reversible inhibitor. We also tested if 1 could be inhibiting Dop by competing with the binding of ATP, as ATP binding as co-factor is essential for Dop activity. Therefore, we first determined the K\textsubscript{M} of ATP for Dop (0.32 ± 0.03 mM, Supporting Information Figure S4). Subsequently, the IC\textsubscript{50} value was determined in the presence of eight different concentrations of ATP ranging from 0.1–6.4 mM (Figure 2D). In the case of an ATP competitive inhibitor, a positive relationship between the IC\textsubscript{50} and log[ATP] was expected. No significant change was found in IC\textsubscript{50} values for 1 (IC\textsubscript{50} = 0.26 ± 0.06 μM for 30 nM Dop; IC\textsubscript{50} = 0.70 ± 0.03 μM for 300 nM Dop), pointing towards 1 most likely not inhibiting Dop via aggregation (Supporting Information Figure S5).

**Derivatizing Tyrphostins as Dop inhibitors**

With 1 validated as a fast-reversible non-ATP competitive inhibitor for Dop, we used it as lead compound in the subsequent structure activity relationship (SAR) study focusing on modification of the benzylidene 2-cyanoacetophenone core and variation of substituents on the aromatic rings (Figure 3A). In total, a number of 27 compound analogues were prepared and evaluated for their potency to inhibit the activity of Dop using the Pup\textsubscript{(33-63)}-Glu(AMC) assay by measuring the IC\textsubscript{50} values. The core part of the Tyrphostin inhibitors contains a Michael acceptor moity in the form of an α,β-unsaturated ketone functionalized with a cyano group on the 3-position. Such cyanoacrylamide groups were previously shown to modulate the mode of action of the corresponding acrylamide from non-

---

Figure 2. A) chemical structures for compounds 1 and 2; B) IC\textsubscript{50} curves for compounds 1 and 2; C) jump-dilution assay, D) IC\textsubscript{50} dependency at varying ATP concentrations.
reversible covalent inhibition to slow reversible covalent inhibition.²² Because the inhibition of Dop by 1 was determined to be fast reversible, we deem it unlikely that 1 binds reversible covalent with a nucleophilic residue of Dop. Hence, we do not expect the Michael acceptor to be a crucial structural element for inhibition and therefore prepared a series of analogues in which the Michael acceptor was systematically modified to verify this hypothesis (Figure 3B). We synthesized a series of compounds lacking the catechol group on the R² phenyl ring compared to 1, since they can be easily prepared using a one-step Knoevenagel condensation between an aldehyde and benzylidene 2-cyanoacetophenone. Compound 5a lacking the catechol group as the R² phenyl ring compared to 1, showed a 10-fold decrease in potency (5.4 μM, entry 2) indicating that these OH-groups are not crucial for inhibition but are important for the potency. Compound 12, that is based on compound 5a, but lacks the nitrile moiety on the Michael acceptor, was still active but 60-fold less potent than compound 1, indicating that these OH-groups are not crucial for inhibition. The various forms of selective reduction greatly influenced the potency of these compounds. A possible explanation could be that the rigidity of the unsaturated system is keeping the geometry and flexibility of the molecule optimal for inhibition.

Scheme 1. Synthetic routes for the synthesis of compounds 5 and 10.

Next we focused our attention on varying the substituents on the aromatic ring systems (Table 1). The benzylidene 2-cyanoacetophenone core (5, Scheme 1) was conveniently prepared through a Knoevenagel condensation reported by Kaufmann²⁸ involving treatment of differently substituted benzaldehydes (3) and benzylidene 2-cyanoacetophenone (4) with piperidine in ethanol to give the desired compounds in 35–85% yield (Scheme 1).

For the synthesis of compounds 10a–10m, bearing two hydroxy groups on the benzylidene 2-cyanoacetophenone part, we envisioned 3,4-dimethoxymethyl benzylidene 2-cyanoacetophenone 8 as the reaction partner for the Knoevenagel reaction, which proceeded smoothly with a range of benzaldehydes. Deprotection of the hydroxyl groups is conveniently achieved using a mild method that employs KHSO₄, coated silicagel²⁹ to afford the final compounds 10a–10m in good yield after purification. The first series of compounds focused on variation on the left aromatic region (R¹) where the right aromatic region (R²) was fixed as a phenyl in analogy to compound 5a (entries 1–14, Table 1). We already established that compound 5a loses potency 10-fold, compared to compound 1, by removing the 2 hydroxyl groups on the R² ring. Further modification of this compound on the R² ring only further diminished the activity of such derivate. Removal of all substituents on the R² ring completely abolished Dop inhibition (Table 1, 5b, entry 2) and restoration of the p-hydroxyl (5c) and the m-methoxy (5d) group also did not result in active compounds (entries 3 and 4). While on the other hand, re-introduction of the 3,4-dihydroxyphenyl as R² (5e), in analogy to compound 2, showed moderate activity (32 μM, entry 5). Introduction of a nitro group on 5c and 5d yielding 5f (23 μM, entry 6) and 5g (26 μM, entry 7) also rendered them active in the same order of magnitude as 5e. N,N-Dimethylphenyl (5h) as R¹ was inactive (entry 8). The effect of the substitution pattern of the R¹-phenyl ring was further investigated by keeping the 3-methoxy-4-hydroxy pattern constant and varying the 5-position of R¹ (entries 9–12). Adding a methoxy-group on the 5-position (5i) did not result in inhibition of Dop (entry 9), while a chloride (5j) resulted in an active compound with poor activity (72 μM, entry 10). Changing the chloride to a bromide (5l) increased the potency further to an IC₅₀ of 20 μM (entry 12) compared to 5j. Changing the 3-methoxy group to an ethoxy group and have an additional 3-methoxy on the R² phenyl ring (5k) was beneficial since the potency increased a 2-fold to
From the results obtained so far, we concluded that the hydroxyl group on the 4-position of $R_1$ is essential for inhibition, while the potency is dependent on the electronic properties of the groups around the 4-position. To test this hypothesis, we measured inhibition of Dop by compound 5m that is lacking the 4-hydroxyl group compared to 5l and 5n in which the $R_1$ substituents are shifted one place compared to 5a. Both compounds were inactive (entries 13 and 14), supporting our hypothesis. We continued by investigating the role of the 3,4,5-trisubstitution on $R_1$ with $R_2$ being 3,4-dihydroxyphenyl like in the original two hits (entries 13–25). This change of $R_2$ should deliver more potent compounds compared to the previous series. To further investigate the role of the substitution pattern on $R_1$, we started with compound 5a.

28 μM (entry 11) compared to 5j. From the results obtained so far, we concluded that the hydroxyl group on the 4-position of $R_1$ is essential for inhibition, while the potency is dependent on the electronic properties of the groups around the 4-position. To test this hypothesis, we measured inhibition of Dop by compound 5m that is lacking the 4-hydroxyl group compared to 5l and 5n in which the $R_1$ substituents are shifted one place compared to 5a. Both compounds were inactive (entries 13 and 14), supporting our hypothesis. We continued by investigating the role of the 3,4,5-trisubstitution on $R_1$ with $R_2$ being 3,4-dihydroxyphenyl like in the original two hits (entries 13–25). This change of $R_2$ should deliver more potent compounds compared to the previous series. To further investigate the role of the substitution pattern on $R_1$, we started with compound 5a.

### Table 1. IC$_{50}$ values of the synthesized analogues on Dop activity.

| Entry | Comp | $R_1$ | $R_2$ | IC$_{50}$ [μM]$^{[a]}$ | Entry | Comp | $R_1$ | $R_2$ | IC$_{50}$ [μM]$^{[a]}$ |
|-------|------|-------|-------|----------------|-------|------|-------|-------|----------------|
| 1     | 5a   | HO    |      | 5.4 ± 0.5     | 14    | 5n   |       |       | > 100        |
| 2     | 5b   |       |      | > 100         | 15    | 10a  |       |       | > 100        |
| 3     | 5c   | HO    |      | > 100         | 16    | 10b  |       |       | 4.1 ± 0.1    |
| 4     | 5d   | HO    |      | > 100         | 17    | 10c  |       |       | 96 ± 10      |
| 5     | 5e   | HO    |      | 32 ± 10       | 18    | 10d  |       |       | > 100        |
| 6     | 5f   | HO    | NO$_2$| 23 ± 5        | 19    | 10e  |       |       | > 100        |
| 7     | 5g   | HO    | NO$_2$| 26 ± 5        | 20    | 10f  |       |       | 41 ± 1       |
| 8     | 5h   | N     |      | > 100         | 21    | 10g  |       |       | > 100        |
| 9     | 5i   | HO    |      | > 100         | 22    | 10h  |       |       | 1.7 ± 0.05   |
| 10    | 5j   |       |      | 72 ± 6        | 23    | 10i  |       |       | 2.7 ± 0.2    |
| 11    | 5k$^{[b]}$ | HO |      | 28 ± 2        | 24    | 10j  |       |       | 13 ± 2       |
| 12    | 5l   | HO    | Br    | 20 ± 3        | 25    | 10k  |       |       | 0.29 ± 0.01  |
| 13    | 5m   |       |      | > 100         |       |      |       |       |              |

[a] The reported values are the means of one experiment ± SD run in triplicate. [b] Obtained from a commercial supplier.
10a, which lacks the iodine moiety compared to 1. The presence of a group on the 5-position seemed to be crucial based on the observation that 10a was inactive (entry 13). The presence of an electron withdrawing nitro-group (10b) restored potency (4.1 μM, entry 15) and compared favorably to 5g (entry 7). Subsequent removal of the methoxy group on the 3-position (10c) again led to a significant loss of potency (96 μM, entry 15), while additional removal of the 4-hydroxy group (10d) again resulted in a completely inactive compound (entry 18). This one more indicates that both the 3-methoxy and 4-hydroxy are essential for potent inhibitors. We thus continued investigating the effect of different substituents on the 5-position (entries 19–24). Interestingly, when a methoxy group was placed on the 5-position (10e), this compound appeared to be inactive (entry 19), similar as for 5i. Electron withdrawing groups appear to be required on the 5-position. When we substituted the 5-position with a phenyl (10f) the compound was moderately active (41 μM, entry 20). It was, however, not beneficial for the potency to add a more electron poor 4-fluorophenyl on position 5 (10g, entry 21). To further investigate the electronic properties, we tested analogs containing a Cl, Br or F on the 5-position (10h–10j, entries 22–24). 10h and 10i were slightly less potent than the original hit (1.7 and 2.7 μM respectively) while fluorine (10j), resulted in further loss of potency (13 μM, entry 22). Apparently, subtle changes in acidity of the 4-hydroxy moiety by modification of the 5-substituent, have a great impact on the potency of the compounds. The hybrid compound (10k) based on both 1 and 2 that has the 3-methoxy moiety replaced for a hydroxy moiety, was the most potent compound from the synthesized analogs (0.29 μM, entry 25). In conclusion, the 4-hydroxy group on the R1-phenyl ring is essential for inhibition and the potency is most likely dependent on its acidity that is regulated by the 5-substituent. The two hydroxyl groups on the R1-phenyl ring increase the potency, but are not essential.

Testing the compounds on native Mtb proteasome substrates

After the identification of the important structural features of the inhibitors, we tested a selection of the synthesized inhibitors on their potency to inhibit Dop activity in depupylation of native substrates. We chose the depupylation reaction of a model pupylated substrate, malonyl coenzyme A (CoA)-acyl carrier protein transacylase (FabD-Pup). The depupylation reaction was largely complete within 1 hour and hence we selected a 30 minute time point as a suitable intermediate assay point to test the selected inhibitors (250 nM FabD-Pup and 10 nM Dop, see Supporting Information Figure S6). Interestingly, the degree of inhibition observed as shown in the assay using the fluorogenic Pup-AMC was not consistently reflected in the depupylation assays using a native pupylated substrate. Compound 2 (IC50: 0.13 μM) was identified as the most potent inhibitor, however, failed to inhibit depupylation of FabD-Pup by Dop (Figure 4B). On the other hand, compound 1 (IC50: 0.52 μM) incompletely inhibited Dop, whereas 10k (IC50: 0.29 μM) showed full inhibition. Compound 5a with an IC50 of 5.4 μM in the fluorogenic assay, failed to inhibit depupylation of FabD-Pup.

Remarkably, 10f was not very potent in the fluorogenic assay (IC50: 42 μM), but fully inhibited the depupylation of FabD-Pup. For the three compounds bearing a bromine (10i), chlorine (10h) or fluorine (10j) on the 5-position of the left ring, only 10i partially inhibited Dop. The differences found between this in vitro assay and the IC50 assay are not fully understood, but could potentially be attributed to the different nature of the used substrates. The IC50 assays make use of truncated Pup carrying a C-terminal hydrophobic fluorophore, whereas in the in vitro assays a full length Pup is linked via an isopeptide bond to the lysine of a FabD protein.

Recently, we described a depupylase activity for PafA.[13a] Interestingly, PafA cannot deamidate PupPam, or release AMC from PuP[33-63]-Glu(AMC) and appears to be more effective at depupylating inositol 1-phosphate synthetase (Ino1-Pup) than FabD-Pup. This observation suggests PafA and Dop may have different affinities for various native substrates. We therefore tested if the compounds could also inhibit the depupylation of either substrate by PafA (Figure 5A). Compounds 10k and 2 and 10f completely inhibited depupylation of FabD-Pup, while 1 and 10i partly inhibited the depupylation by PafA. Depupylation of Ino1-Pup was fully inhibited by 10k and 10f and partially inhibited by 1, 2 and 10i (Figure 5B).

Given that depupylation of native substrates by PafA was inhibited by some of the tyrophostin analogues we tested whether pupylation of FabD by PafA was likewise inhibited (Supporting Information Figure S7). Similar to depupylation by PafA the conjugation of Pup to FabD was completely inhibited by compounds 10k and 2 and 10f, while 1 and 10i partly inhibited the ligase activity of PafA. The remainder of the compounds showed no inhibition. Given the structural homology of the targeted proteins, it is not surprising that inhibitors for Dop also inhibit PafA.[13b] However, compound 2 did not inhibit Dop in the depupylation of FabD-Pup, while it fully
inhibits both PafA activities. Apparently, despite the structural homology, small differences in inhibitor structure allow inhibitors to act selectively on PafA. Both Dop and PafA are required for pupylation in Mtb; Dop must first deamidate Pup to Pup să before PafA ligates Pup să to a substrate. The deletion of either gene product from Mtb results in the disappearance of the pupylome. 

Because our screen identified inhibitors of the enzymes involved in pupylation as well as depupylation and do not compete with ATP, these molecules may affect the position-ing of the C-terminus of Pup and its substrate within the active sites. The fact that inhibitors for both enzymes in the PPS could not compete with ATP, these molecules may affect the position-ing of the C-terminus of Pup and its substrate within the active sites. The fact that inhibitors for both enzymes in the PPS could be found underscores the success of the first proof-of-principle high-throughput screen against Dop and PafA. It however is important to keep in mind that 1) in our Pup-AMC assay we made use of a truncated form of Pup as substrate and 2) the C-terminal adduct on Pup was not a lysine residue but a more bulky and hydrophobic coumarin fluorophore. Both modifications could potentially affect the recognition of the substrate by Dop and explain the discrepancy between the screening results and the in vitro experiments with native substrates. Nevertheless, our goals were to determine whether or not Dop could be targeted for inhibitor discovery as well as to identify molecules that may give new insight into the mechanism of catalysis by this highly unique protease.

Conclusions

In an effort to identify inhibitors of Dop, we used our fluorogenic Pup-AMC substrate to successfully screen a 1280-compound library from which compounds 1 and 2 were identified as hits. 1 was validated as a fast reversible and non-ATP competitive inhibitor for Dop and was used as starting point for a SAR-study that revealed the important structural features of the scaffold. Furthermore, we showed that newly prepared analogue compounds 10k and 10f were able to inhibit depupylation of FabD-Pup by Dop, as well as pupylation and depupylation of FabD and Ino1 by PafA in an in vitro setting. In addition, compound 2 seemed to be able to efficiently inhibit PafA activities whilst leaving Dop activity intact. Hence, we showed that high-throughput screening using our Pup-AMC substrate can be a valuable tool to find new compounds as potential therapeutic leads to inhibit both Dop and PafA synergistically, which could minimize the acquisition of drug resistance of these compounds by Mycobacterium tuberculosis.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Acknowledgements

K.H.D. was supported by NIH grant AI088075. This work was also supported by an Investigators in the Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund to K.H.D. C.C. was supported by NIH grant R01GM110430. G.J.v.d.H.v.N. acknowledges funding by an Off-Road grant (ZonMW) and a VIDI grant (NWO).

Conflict of Interest

The authors declare no conflict of interest.

Keywords: inhibitor screen · Mycobacterium tuberculosis · pup proteasome system · tuberculosis

[1] Global TB Report 2020, http://www.who.int/tb/publications/global report/en.
[2] N. Awofeso, Bull. W. H. O. 2008, 86, B-d.
[3] a) S. K. Sharma, A. Mohan, Indian J. Med. Res. 2004, 120, 354–376; b) L. P. Ormerod, Br. Med. Bull. 2005, 72, 74–79; c) D. Komander, M. Rape, Nature 2009, 460, 479–485.
[4] a) K. H. Darwin, S. Ehrt, J. C. Gutierrez-Ramos, N. Weich, C. F. Nathan, Science 2003, 302, 1963–1966; b) K. H. Darwin, G. Lin, Z. Chen, H. Li, C. F. Nathan, Mol. Microbiol. 2005, 55, 561–571; c) M. I. Samanovic, S. Tu, O. Novák, L. M. Iyer, F. E. McAllister, L. Aravind, S. P. Gygi, S. R. Hubbard, M. Strnad, K. H. Darwin, Mol. Cell 2015, 57, 984–994; d) S. Gandotra, D. Schnappinger, M. Monteleone, W. Hillen, S. Ehrt, Nat. Med. 2007, 13, 1515–1520.
[5] I. Dikic, Annu. Rev. Biochem. 2017, 86, 193–224.
[6] a) D. Komander, M. Rape, Annu. Rev. Biochem. 2012, 81, 203–229; b) K. N. Swatek, D. Komander, Cell Res. 2016, 26, 399–422.
[7] a) P. Zwickl, D. Ng, K. M. Woo, H. P. Klenk, A. L. Goldberg, J. Biol. Chem. 1999, 274, 26008–26014; b) J. B. Jastrab, K. H. Darwin, Annu. Rev. Biochem. 2015, 84, 69–109.
[8] M. Sutter, F. F. Damberger, F. Imkamp, F. H. Allain, E. Weber-Ban, J. Am. Chem. Soc. 2010, 132, 5610–5612.
[9] a) F. Striebel, F. Imkamp, M. Sutter, M. Steiner, A. Mamedov, E. Weber-Ban, Nat. Struct. Mol. Biol. 2009, 16, 647–651; b) E. Guth, M. Thommen, E. Weber-Ban, J. Biol. Chem. 2011, 286, 4412–4419.
[10] a) F. A. Cerda-Maira, M. J. Pearce, M. Fuortes, W. R. Bishai, S. R. Hubbard, K. H. Darwin, Mol. Microbiol. 2010, 77, 1123–1135; b) L. M. Iyer, A. M. Burnoughs, L. Aravind, Biol. Direct. 2008, 3, 45.
[11] a) F. Striebel, M. Hunkeler, H. Summer, E. Weber-Ban, EMBO J. 2010, 29, 1262–1271; b) K. E. Burns, F. E. McAllister, C. Schwerdtfeger, J. Minteris, F. Cerda-Maira, E. E. Noens, M. Wilmanns, S. R. Hubbard, F. Melandri, H.
