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

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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 depupylating 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 *Mtb*, a 64-amino acid prokaryotic ubiquitin-like protein (Pup), is responsible for substrate recognition by a bacterial proteasome.[6]

Although the enzymatic activities of the proteasome core proteases are, for the most part, conserved between prokaryotes and eukaryotes,[7] 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,[6b] 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

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[+] *In memory of Huib Ovaa, whose passion for science will remain an inspiration.*

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

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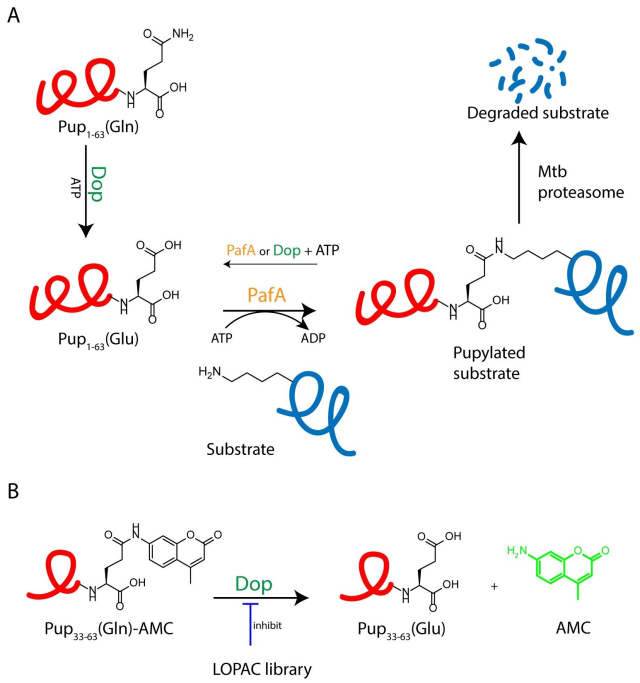

Figure 1. A) Schematic representation of the main enzymatic functions in the Pup-proteasome system in Mtb. B) Fluorogenic assay to monitor Dop activity and screen for inhibitors.

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.[8] 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 Pi.[9] 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.[9a] Although Dop and PafA have high sequence and structural similarity[9a,10] deamidation by Dop uses ATP as cofactor but is not dependent on the hydrolysis of ATP, in contrast to PafA ligase activity.[11] Both Dop[9a,12] and PafA[13] can also depupylate substrates,[14] although this function is less efficient then their respective primary deamidase and ligase function. Unlike the pupylation process, the precise mechanisms of de- and trans-pupylation (and deamidation) are incompletely understood.[9a,10a,11b,c,13a,15] Although Ub and Pup serve the same function, there is no sequence homology among the enzymes involved and therefore components of the PPS are attractive targets for the development of selective TB drugs. Currently, only pan-protease inhibitor (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) is reported as inhibitor for the Pup ligase PafA and binds covalently to a serine (Ser119 in *Mtb* H37Rv PafA), thereby disturbing Pup recognition.[16] No inhibitors for Dop are reported. In our efforts to find inhibitors to specifically target accessory factors of the *Mtb* PPS in the search for new drugs against TB, and to develop tools to study the mechanisms of action of these enzymes, we have previously developed a fluorogenic assay reagent to probe the activity of Dop *in vitro* that allows high-throughput screening to identify inhibitors (Figure 1B).[17] This reagent is based on synthetically prepared, truncated Pup (Pup_{33-63}) with a terminal Glu attached to 7-amino-4-methyl coumarin (AMC) via its γ-carboxylate. Cleavage of the fluorogenic Glu-AMC amide bond by Dop releases AMC as amine, which then yields a fluorescent signal. Here we describe the use of this assay reagent in a high-throughput screen to identify small-molecule inhibitors for Dop. Interestingly, several Dop-inhibiting compounds were identified that also inhibited both the conjugating and deconjugating activities of PafA.

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).[17] We identified several hits in this screen, including I-OMe-Tyrphostin AG 538 (**1**) and Tyrphostin 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,[18] while phosphoinotiside kinase PI5P4Kα is inhibited via competition with ATP,[19] and oxidative stress-sensitive Ca^{2+}-preamble channel TRPA1 and TPRM2 activated by H_2O_2 are inhibited by means of hydroxyl radical scavenging.[20] Compounds from the Tyrphostin cluster are known as pan-assay interference compounds (PAINS)[21] 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.[22,23]

Mode of action

To validate the initial screening hits, we determined the IC_{50} 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 IC_{50} values of the inhibitors (Supporting Information Figure S2).

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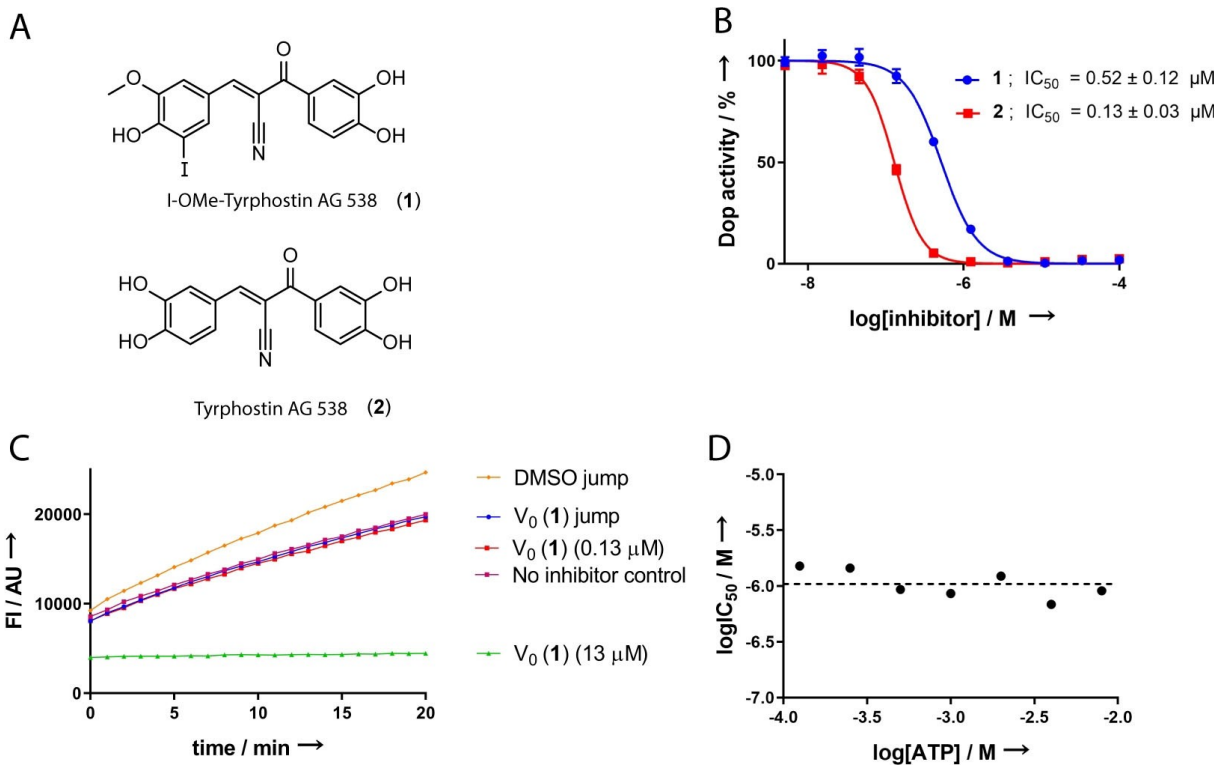

Figure 2. A) chemical structures for compounds **1** and **2**; B) IC_{50} curves for compounds **1** and **2**; C) jump-dilution assay, D) IC_{50} dependency at varying ATP concentrations.

Next, we tested if **1** is inhibiting Dop in an irreversible and covalent manner by determining IC_{50} values after different incubation times. If **1** is an irreversible covalent binder, lower IC_{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_{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),[24] where Dop was pre-incubated for 30 minutes with a concentration of 25 times the IC_{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_M of ATP for Dop (0.32 ± 0.03 mM, Supporting Information Figure S4). Subsequently, the IC_{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_{50} and log[ATP] was expected.[19,24b] Because the IC_{50} did not significantly increase with higher ATP concentration, we concluded that **1** is not an ATP competitive inhibitor. PAIN compounds can also inhibit their targets by means of aggregation, where the larger molecular aggregate of the inhibitor blocks enzyme activity.[25] To assess whether **1** inhibits Dop via aggregation we measured IC_{50} values of **1** at a routine and a 10-fold increased Dop concentration. If in such an assay a large potency shift (increase of IC_{50} at the higher enzyme concentration) is observed this might be contributed to non-competitive inhibition by potential aggregation of the inhibitor.[26] No significant change was found in IC_{50} values for **1** (IC_{50} = 0.26 ± 0.06 µM for 30 nM Dop; IC_{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 $Pup_{(33-63)}$-Glu(AMC) assay by measuring the IC_{50} values.

The core part of the Tyrphostin inhibitors contains a Michael acceptor moiety 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-

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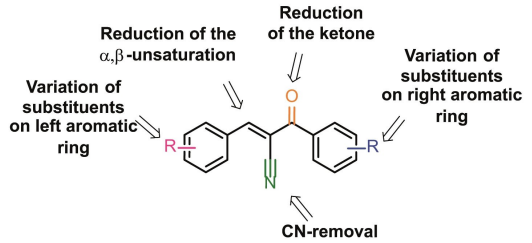
A



B

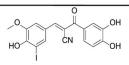
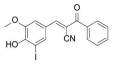
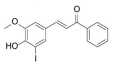
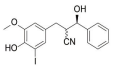
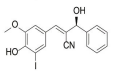
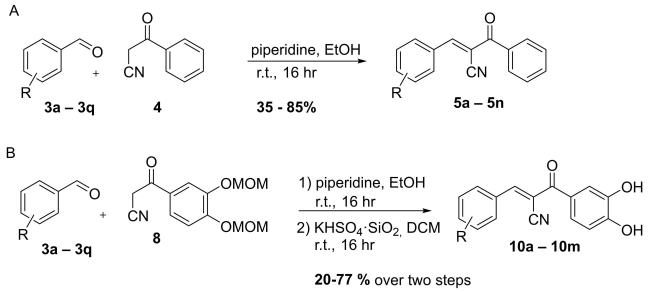
Entry	Compound	Structure	IC_{50} (μM)[a]
1	**1**		0.5±0.1
2	**5A**		5.4±0.5
3	**12**		29±10
4	**13**		57±8
5	**14**		>100

Figure 3. Focus areas for the SAR study. A) Modification of the Tyrphostin core. B) IC_{50} values for compounds **5A**, **12**–**14**. [a] The reported values are the means of one experiment ± SD run in triplicate.

reversible covalent inhibition to slow reversible covalent inhibition.[27] 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 analogous 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 R^2 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 R^2 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 then **1** (29 μM; Figure 3B, entry 2 and 3). Complete reduction of the Michael acceptor to alcohol **13** was achieved using $NaBH_4$ in methanol. This reduction rendered compound **13** even less potent (57 μM; entry 4). Additionally, we selectively reduced the carbonyl by applying Luche's reduction conditions ($NaBH_4/CeCl_3$) to obtain alcohol **14**, thereby reducing the α,β-unsaturated system, resulting in a complete loss of activity (entry 5). Although the full benzylidene 2-cyanoacetophenone backbone is not essential 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.

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 Kaufmann[28] 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 hydroxyl 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 $KHSO_4$ coated silicagel[29] 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 (R^1) where the right aromatic region (R^2) 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 R^2 ring. Further modification of this compound on the R^1 ring only further diminished the activity of such derivates. Removal of all substituents on the R^2 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, reintroduction of the 3,4-dihydroxyphenyl as R^1 (**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 R^1 was inactive (entry 8). The effect of the substitution pattern of the R^1-phenyl ring was further investigated by keeping the 3-methoxy-4-hydroxy pattern constant and varying the 5-position of R^1 (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 IC_{50} 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 R^2 phenyl ring (**5k**) was beneficial since the potency increased a 2-fold to



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

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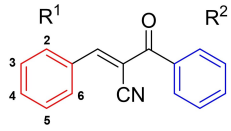
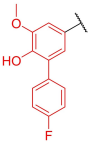
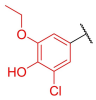
Table 1. IC_{50} values of the synthesized analogues on Dop activity.



Entry	Comp	R^1	R^2	IC_{50} [μM][a]	Entry	Comp	R^1	R^2	IC_{50} [μM][a]
1	**5a**			5.4 ± 0.5	14	**5n**			> 100
2	**5b**			> 100	15	**10a**			> 100
3	**5c**			> 100	16	**10b**			4.1 ± 0.1
4	**5d**			> 100	17	**10c**			96 ± 10
5	**5e**			32 ± 10	18	**10d**			> 100
6	**5f**			23 ± 5	19	**10e**			> 100
7	**5g**			26 ± 5	20	**10f**			41 ± 1
8	**5h**			> 100	21	**10g**			> 100
9	**5i**			> 100	22	**10h**			1.7 ± 0.05
10	**5j**			72 ± 6	23	**10i**			2.7 ± 0.2
11	**5k**[b]			28 ± 2	24	**10j**			13 ± 2
12	**5l**			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.

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

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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-hydroxygroup (**10d**) again resulted in a completely inactive compound (entry 18). This once 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** (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-hydroxyl 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-hydroxyl group on the R^1-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 R^2-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** (IC_{50}: 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** (IC_{50}: 0.52 μM) incompletely inhibited Dop, whereas **10k** (IC_{50}: 0.29 μM) showed full inhibition. Compound **5a** with an IC_{50} of 5.4 μM in the fluorogenic assay, failed to inhibit depupylation of FabD-Pup.

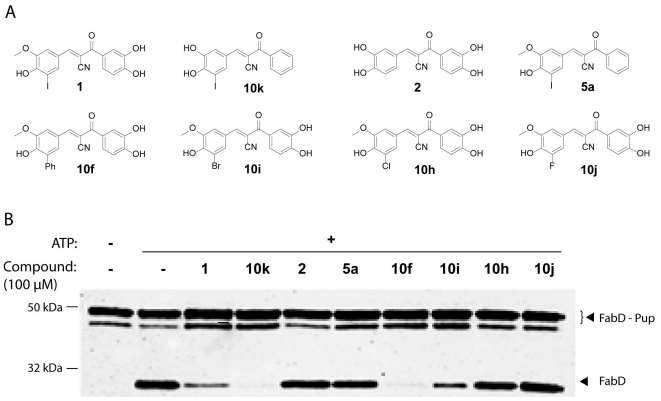

Figure 4. Depupylation of FabD-Pup by Dop. A) Structures of the eight selected compounds. B) Effect of the inhibitors (100 μM) on the depupylation reaction of FabD-Pup by Dop. Proteins were analyzed by immunoblotting using antibodies against *Mtb* FabD-His_6.[30] Note that FabD can be pupylated on any one of four lysines, resulting in two slightly different migration patterns.[31]

Remarkably, **10f** was not very potent in the fluorogenic assay (IC_{50}: 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 IC_{50} assay are not fully understood, but could potentially be attributed to the different nature of the used substrates. The IC_{50} 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 Pup_{Gln} to Pup_{Glu} or release AMC from $Pup_{(33\text{-}63)}$-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 tyrphostin 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.[32] However, compound 2 did not inhibit Dop in the depupylation of FabD-Pup, while it fully

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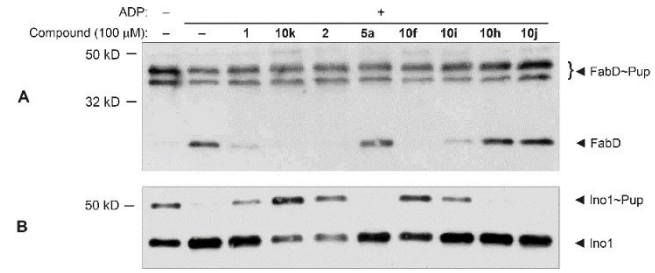

Figure 5. Inhibition of depupylation of *Mtb* proteasome substrates by PafA. A) Effect of the inhibitors (100 μM) on the depupylation of FabD-Pup by PafA. B) Effect of the inhibitors on the depupylation of Ino1-Pup by PafA. Note: Ino1 forms tetramers in which two molecules in each tetramer are pupylated; therefore, Ino1 and Ino1-Pup co-purify as a 2:2 complex.[15]

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_{Gln} to Pup_{Glu} before PafA ligates Pup_{Glu} to a substrate. The deletion of either gene product from *Mtb* results in the disappearance of the pupylome.[10a] 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 positioning 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 $Pup_{(33\text{-}63)}$-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.

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Conflict of Interest

The authors declare no conflict of interest.

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

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