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Structure guided generation of thieno[3,2-*d*]pyrimidin-4-amine *Mycobacterium tuberculosis* *bd* oxidase inhibitors†

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Cytochrome *bd* oxidase (Cyt-*bd*) is an attractive drug target in *Mycobacterium tuberculosis*, especially in the context of developing a drug combination targeting energy metabolism. However, currently few synthetically assessable scaffolds target Cyt-*bd*. Herein, we report that thieno[3,2-*d*]pyrimidin-4-amines inhibit Cyt-*bd*, and report an initial structure-activity-relationship (SAR) of 13 compounds in three mycobacterial strains: *Mycobacterium bovis* BCG, *Mycobacterium tuberculosis* H37Rv and *Mycobacterium tuberculosis* clinical isolate N0145 in an established ATP depletion assay with or without the cytochrome *bcc:aa₃* (QcrB) inhibitor Q203. All compounds displayed activity against *M. bovis* BCG and the *M. tuberculosis* clinical isolate strain N0145 with ATP IC₅₀ values from 6 to 54 μM in the presence of Q203 only, as expected from a Cyt-*bd* inhibitor. All derivatives were much less potent against *M. tuberculosis* H37Rv compared to N0145 (IC₅₀'s from 24 to >100 μM and 9–52 μM, respectively), an observation that may be attributed to the higher expression of the Cyt-*bd*-encoding genes in the laboratory-adapted *M. tuberculosis* H37Rv strain. *N*-(4-(*tert*-butyl)phenethyl)thieno[3,2-*d*]pyrimidin-4-amine (**19**) was the most active compound with ATP IC₅₀ values from 6 to 18 μM against all strains in the presence of Q203, making it a good chemical probe for interrogation the function of the mycobacterial Cyt-*bd* under various physiological conditions.

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1. Introduction

Tuberculosis (TB) is a public health crisis. According to the 2018 World Health Organization (WHO) report, 10 million people became ill with TB, and 1.3 million died in 2017.¹ TB is one of the top 10 causes of death worldwide.¹ TB infection is caused by *Mycobacterium tuberculosis* (Mtb), a primarily aerobic bacterium. Infection is spread from person to person by aerosol transmission, usually *via* an infected individual's cough or sneeze. Initial symptoms are mild and often go unnoticed. Most troubling is the continued rise in drug resistant TB. It is estimated that between 483 000 and 639 000 people have developed TB that was resistant to rifampicin.¹ Thus, there is an urgent need for the development of antimicrobials and treatment options that can swiftly eliminate Mtb infections.

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Various bacteria can make adenosine triphosphate (ATP) by two methods: substrate-level phosphorylation of fermentable carbon sources or oxidative phosphorylation.² During infection, TB demonstrates remarkable competence in its ability to adapt to environmental stresses. Of the investigated attributes, the mechanism of adapting to oxygen levels has been appreciably studied. High-density mutagenesis and deletion studies have shown that Mtb cannot sufficiently produce ATP by substrate-level phosphorylation alone, and oxidative phosphorylation is strictly required for growth.^{3,4}

Small-molecule inhibitors have been synthesized that have various targets in the oxidative phosphorylation pathway. Clofazimine **1** (Fig. 1), a repurposed anti-leprosy drug, targets NDH-2 and probably additional respiratory cytochromes.^{5,6} The first line TB drug pyrazinamide incapacitates the proton motive force that maintains an electrochemical gradient across the membrane (Fig. 1).⁷ The diarylquinoline bedaquiline **2** targets ATP synthase^{8,9} whereas Q203 (**3**) inhibits the cytochrome *bcc:aa₃* complex (Fig. 1).¹⁰ Q203 belongs to the imidazopyridine class of compounds and entered phase 2 clinical trials in July of 2018.^{11,12} We have demonstrated that a potential clinical limitation of Q203 is that the compound is unable to inhibit oxygen respiration and is bacteriostatic due to a functional redundancy between

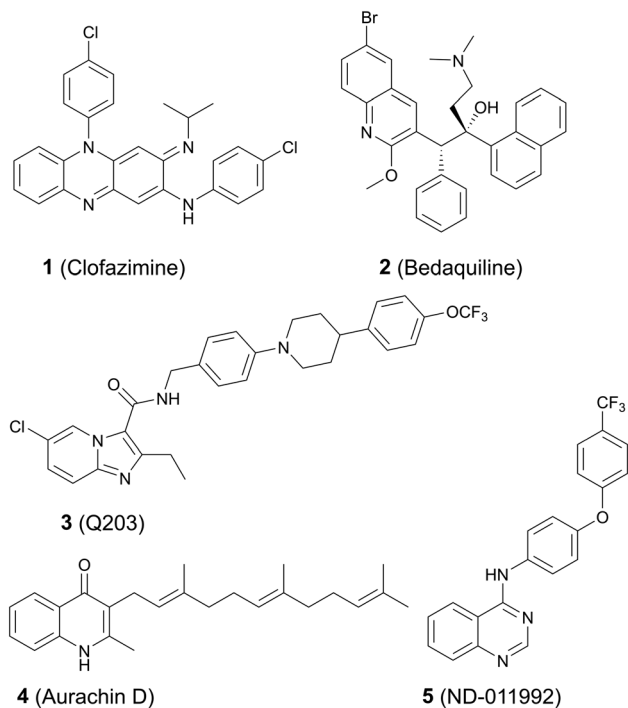


Fig. 1 Inhibitors of *M. tuberculosis* oxidative phosphorylation pathway.

the *Cyt-bcc:aa₃* and the cytochrome *bd* oxidase (*Cyt-bd*).¹⁰ Bactericidal agents are strongly desired. A neglected target in the oxidative phosphorylation pathway is the *Cyt-bd*, an oxidoreductase that is important for viability when the function of the *Cyt-bcc:aa₃* is compromised.² The *Cyt-bd* is over-expressed under low oxygen conditions that coincide with the entry into a reversible non-replicating, antibiotic tolerant state.^{13,14}

Knockouts and mutations of the cytochrome *bcc:aa₃* complex (*Cyt-bcc:aa₃*) show an upregulation of *Cyt-bd*,¹⁵ making *Cyt-bcc:aa₃* inhibitors themselves less effective. Although non-essential, the *Cyt-bd* is still required to maintain oxygen respiration and ATP homeostasis in concert with the *Cyt-bcc:aa₃*.¹⁶ In this manner, mycobacteria are protected against drug treatments by rerouting the electron flow to the *Cyt-bd*. Therefore, targeting cytochrome *bd* and adding a *Cyt-bd* inhibitor to a TB treatment regimen may be an effective means to kill replicating and non-replicating mycobacteria more effectively.

There are only two published *Mtb* *Cyt-bd* inhibitors, menaquinone analogue aurachin D¹⁷ (4) and ND-011992 (ref. 18) (5) (Fig. 1). Issues with aurachin D include not being able to permeate the TB cell wall effectively and having toxic off-target effects.¹⁷ However, aurachin D co-administered with Q203 shows almost identical bactericidal activity as that of Q203 within *Cyt-bd* knockout strains.¹⁷ As such, cytochrome *bd* oxidase is an attractive *Mtb* drug target particularly when used in combination with *Cyt-bcc:aa₃* inhibitors, as demonstrated by the bactericidal efficacy of the Q203/ND-011992 combination against replicating and antibiotic-tolerant non-replicating mycobacteria.¹⁸

2. Results and discussion

To discover new *Cyt-bd* oxidase inhibitors, we made use of a whole cell assay in *Mycobacterium bovis* BCG (BCG), followed by validation in *M. tuberculosis* H37Rv (H37Rv-Mtb) and *M. tuberculosis* clinical isolate N0145 (N0145-Mtb). This assay exploits the conditional essentiality of the *Cyt-bd* to maintain ATP homeostasis once *Cyt-bcc:aa₃* is selectively inhibited by Q203.¹⁸ Measurement of ATP depletion in the presence and absence of Q203 reveals whether a compound inhibits alone or synergizes with Q203. Compounds that deplete ATP in the presence of Q203 but not in its absence are putative *Cyt-bd* inhibitors. Next, we screened a small but diverse set of around 50 compounds selected from our long-standing antibacterial programs against *M. bovis* BCG to reveal active compounds like ND-011992.¹⁸ When screened in the presence of Q203, our screening revealed two thienopyrimidines—compound 6 (a thieno[2,3-*d*]pyrimidine-4-amine) and compound 7 (a thieno[3,2-*d*]pyrimidine-4-amine)—displaying divergent potency with $IC_{50} > 50 \mu M$ and $26 \mu M$, respectively (Fig. 2). Both 6 and 7 were inactive against BCG ($IC_{50} > 50 \mu M$ when tested the absence of Q203), suggesting that these compounds work by inhibition of *Cyt-bd*.

When searching literature around the thienopyrimidine scaffold, we discovered an abundance of references including patent applications for use as pesticides,¹⁹ anti-cancer compounds,^{20,21} worm infections²² and autoimmune diseases.²³ Interestingly, only one group has published on thienopyrimidines as anti-TB agents.^{24,25} This group identified a thieno[2,3-*d*]pyrimidine-4-amine, CWHM-728 (8, Fig. 3), through iterative screening of commercially available compounds against *Mycobacterium smegmatis*. CWHM-728 (8) was found to have an IC_{50} value of $3.2 \mu M$ against *M. tuberculosis* Erdman strain. Through diligent SAR studies, they developed a much more potent analogue, CWHM-1023 (9, Fig. 3), possessing an IC_{50} value of $0.083 \mu M$ against *M. tuberculosis* Erdman strain. They also determined that mutations within the *QcrB* gene confer resistance, suggesting *Cyt-bcc:aa₃* as the primarily target of these compounds.

The precedence of “hit to lead” development of the thieno[2,3-*d*]pyrimidine-4-amines combined with the structural simplicity of compound 7 makes this core an attractive scaffold to explore structure–activity–relationship (SAR) studies. Herein, we report our initial findings on

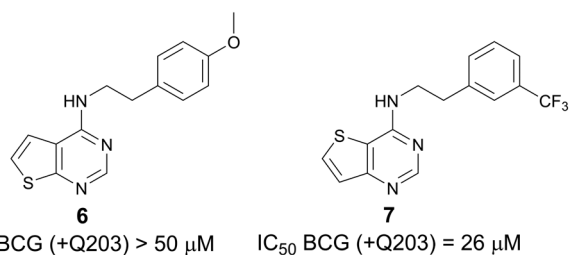


Fig. 2 Thienopyrimidines 6 and hit 7 were identified by ATP depletion within *Mycobacterium bovis* BCG.

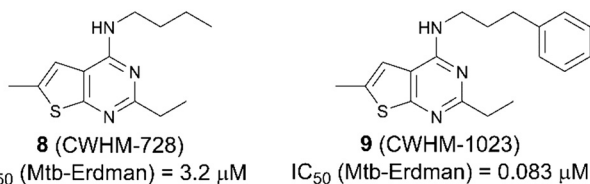


Fig. 3 Thieno[2,3-*d*]pyrimidine-4-amines **8** and **9** identified as novel Cyt-*bc*₁:*aa*₃ inhibitors.

thieno[3,2-*d*]pyrimidine-4-amines that inhibit mycobacteria Cyt-*bd* over Cyt-*bcc*:*aa*₃ within replicating BCG, replicating Mtb-H37Rv and clinical N0145-Mtb strains.

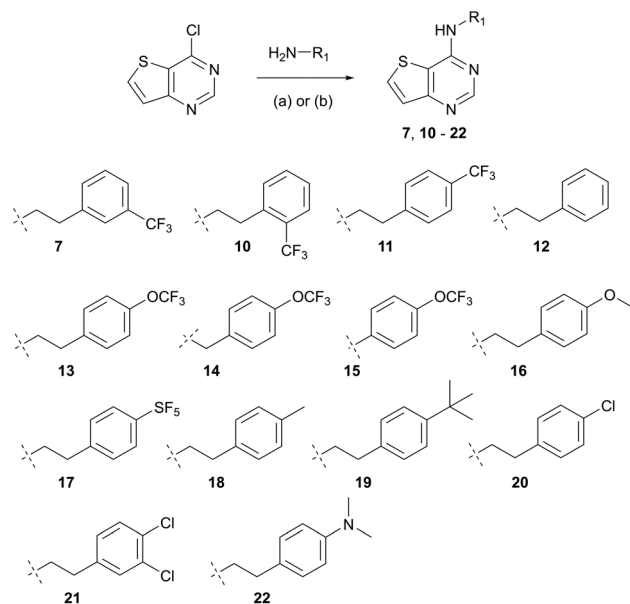
Our SAR efforts focused on probing three structural elements of hit **7**: 1) positioning of aryl substitution (2', 3' and 4' positions), 2) alteration of the pendant 2-(3-(trifluoromethyl)phenyl)ethan-1-amino moiety with other substitutions (*i.e.* H, halogen, CH₃, OCF₃, *etc.*), and 3) changing of the aliphatic chain length (*i.e.* anilino, benzyl, ethanyl).

Compounds were prepared in one step by classical S_NAr reactions of 4-chlorothieno[3,2-*d*]pyrimidine, desired amines and potassium carbonate at elevated temperature (Scheme 1).²⁶ Yields varied from 22 to 70%, after purification by recrystallization (see ESI†). We prepared thirteen analogues (**10–22**) that were screened in a whole-cell ATP Cyt-*bd* assay – in BCG with subsequent validation in H37Rv-Mtb and the clinical isolate N0145-Mtb—under replicating conditions with and without added Q203. The BCG strain was used to identify any general Cyt-*bd* inhibitor (when Q203 was added, “+Q203”). The laboratory adapted *M. tuberculosis* H37Rv strain over expresses Cyt-*bd* compared to clinical

isolates.⁸ As such, strains H37Rv and N0145 were used to identify and rank compounds that target *M. tuberculosis* Cyt-*bd* (when Q203 was added). Compounds that inhibited intracellular ATP level in mycobacteria (“-Q203”) but did not show better activity when Cyt-*bcc*:*aa*₃ was inhibited (“+Q203”) were presumed to not be targeting Cyt-*bd*, an example being the F₀F₁ ATP synthase inhibitor bedaquiline (BDQ)⁹ which is equipotent in the presence or absence of Q203. All screening data along with compound molecular mass and calculated clog *P* are listed in Table 1.

Separate placement of a trifluoromethyl group at the *ortho*, *meta*, or *para* positions of the peripheral phenyl group (compounds **10**, **7** and **11**, respectively) revealed that the *ortho*-CF₃ analogue (**10**) had good potency against BCG and N0145-Mtb (IC_{50} values of 12.4 and 15.5 μ M, respectively; when Q203 was added). The *ortho*-CF₃ (**10**) was slightly more potent than the screening hit *meta*-CF₃ (**7**) and *para*-CF₃ (**11**) compounds against those two Mtb strains. The *para*-CF₃ compound (**11**) had slightly improved potency against H37Rv-Mtb but the IC_{50} value was high at 63 μ M. Despite the slightly better potency at the *ortho*-position, there is greater availability of *para*-substituted phenyl analogues with which to probe the effect of substituents on SAR.

Considering the phenylethyl analogue (**12**) as electronically and sterically “neutral” we found this benchmark compound to have weak potency against the three strains (IC_{50} from 51 to >100 μ M, when Q203 added). However, functionalization at the *para* position of the phenyl group significantly improved potency as the *para*-OCF₃ compound (**13**) had lower IC_{50} values than the *para*-CF₃ compound (**11**) (IC_{50} from 11 to 25 μ M, in three strains when Q203 was added). In general, all electron withdrawing groups (SF₅, Cl, di-Cl) improved potency as compared to the unsubstituted phenylethyl (**12**) but none were more potent than the *para*-OCF₃ analogue (**13**). Electron donating groups gave diverse results as the *para*-methoxy (**16**) and *para*-dimethylamine (**22**) compounds had (weak) potency akin to the unsubstituted phenylethyl compound (**12**). The *para*-methyl analogue (**18**) had slightly better potency than **12** (IC_{50} from 41 to >100, in the presence of Q203) but not nearly as potent as the compounds with electron withdrawing groups (**7**, **10**, **11**, **13**, **17**, **20**, and **21**). Interestingly, the most potent compound was the 4-*para*-C(CH₃)₃ analogue (**19**) having IC_{50} values of 6 to 18 μ M against all three stains (with Q203 added). Structurally, **19** is most like the *para*-SF₅ analogue (**17**) based upon volume²⁷ but more lipophilic (clog *P* of 5.85 for **19** compared to 5.26 for **17**). The scope of active substituents suggests a pocket that can accommodate larger groups (SF₅, *t*-butyl, Cl) and these larger substituents had good potency. Conformational flexibility was probed through the synthesis and screening of the 4-(trifluoromethoxy) phenylethyl (**13**), 4-(trifluoromethoxy)benzyl (**14**) and 4-(trifluoromethoxy)aniline (**15**) analogues. The effect of the linker between the thienopyrimidine core and peripheral phenyl group was interesting in that the compound with the rotatable benzyl side chain (**14**) was less active than that with



Scheme 1 Syntheses of target thieno[3,2-*d*]pyrimidin-4-amines (**7**, **10–22**). Reagents and conditions: (a) K₂CO₃, DMSO, 100 °C, 12 h, or (b) HCl, THF : IPA (3 : 1), 70 °C, 12 h, yield: 22–70%.

Table 1 *In vitro* activity of thieno[3,2-*d*]pyrimidin-4-amines (**7**, **10**–**22**) and control compounds **3** and **5** against three mycobacterial strains (*M. bovis* BCG, *M. tuberculosis* H37Rv, *M. tuberculosis* clinical isolate N0145)

Compound	Mol wt	clog <i>P</i>	Replicating ATP IC ₅₀ (mM)					
			BCG		H37Rv		N0145	
			–Q203	+Q203	–Q203	+Q203	–Q203	+Q203
7	323.34	4.91	>50	25.6 ± 4.59	>100	61.7 ± 7.85	>50	23.4 ± 0.39
10	323.34	4.91	>50	12.4 ± 3.36	>100	68.3 ± 10.47	>50	17.4 ± 2.10
11	323.34	4.91	>50	33.1 ± 4.50	>100	63.2 ± 1.55	>50	24.9 ± 0.16
12	255.34	4.02	>50	43.3 ± 9.44	>100	>100	>50	51.5 ± 4.30
13	339.34	5.05	>50	12.6 ± 1.10	>100	24.4 ± 1.52	>50	10.6 ± 0.30
14	325.31	4.40	>50	38.1 ± 7.43	>100	99.8 ± 19.06	>50	36.1 ± 3.80
15	311.28	4.79	>50	22.9 ± 3.55	>100	66.7 ± 1.79	>50	22.8 ± 4.34
16	285.36	3.94	>50	42.8 ± 10.34	>100	>100	>50	45.0 ± 14.63
17	381.39	5.26	>50	27.1 ± 6.38	>100	>100	>50	18.9 ± 2.88
18	269.36	4.52	>50	40.4 ± 11.29	>100	108.3 ± 7.80	>50	37.4 ± 10.23
19	311.44	5.85	>50	5.8 ± 1.06	>100	18.9 ± 9.03	>50	8.5 ± 2.38
20	289.78	4.74	>50	30.4 ± 9.27	>100	83.5 ± 0.97	>50	31.8 ± 9.98
21	324.23	5.33	>50	25.6 ± 8.35	>100	109.3 ± 17.36	>50	35.3 ± 9.49
22	298.41	4.20	>50	38.9 ± 9.08	>100	>100	>50	41.5 ± 15.31
3 (BDQ)	555.52	7.25	0.17 ± 0.005	0.09 ± 0.0008	0.04 ± 0.004	0.07 ± 0.01	0.01 ± 0.0005	0.03 ± 0.003
5 (ND-011992)	381.36	6.69	>50	0.80 ± 0.07	>100	5.8 ± 1.18	>50	1.6 ± 0.69

IC₅₀ values were determined by ATP depletion in the presence and absence of Q203 (see, ESI† for assay details). clog *P* was calculated by PerkinElmer ChemDraw Professional 16.0. Bedaquiline (BDQ) and ND-011992 were used as positive control compounds. IC₅₀ values were determined using GraphPad Prism 9. The values reflected in the table represent the average and standard deviation, which were calculated from the IC₅₀ values of replicates from two experimental repeats.

aniline (**15**) and nearly 3-fold less active than the phenylethyl compound (**13**) (IC₅₀ values of 33 to 96 μM in all three strains, when Q203 was added).

Since the increased level of Cyt-*bd* expression in H37Rv-Mtb implied a lower potency of the inhibitors, the extra effort of screening the clinical strain in tandem with more commonly used H37Rv-Mtb strain was justified. While it was possible that strain selectively (either BCG or Mtb) could have been revealed, instead, there was strong activity correlation between the BCG and N0145-Mtb strains for all the compounds screened. Finally, none of the compounds alone inhibited ATP, strongly suggesting that these compounds do target Cyt-*bd* since potency was only revealed when Cyt-*bcc*:*aa*₃ was selectively inhibited by Q203. The lack of ATP inhibition against Mtb (or BCG) greatly increases the challenge to discover Cyt-*bd* inhibitors and establishes combination drug therapy as a viable treatment option to use with such inhibitors.

General procedure for base promoted S_NAr for synthesis of compounds **7**, **11**, **13**, **17**, **19**, **22**

In a sealed vial, 4-chlorothieno[3,2-*d*]pyrimidine (100 mg, 0.57 mmol), desired amine (0.57 mmol) and K₂CO₃ (79 mg, 0.57 mmol) were dissolved in DMSO (4 mL). The reaction was heated to 100 °C for 12 h. The reaction mixture was concentrated to dryness and the residue was dissolved in CH₂Cl₂ and washed with 5% aqueous acetic acid solution (2×), water and brine. The organic phase was collected, dried over sodium sulfate, filtered, and concentrated *in vacuo*. Crude material obtained was purified by either silica gel column chromatography with a gradient of CH₂Cl₂:ethyl

acetate:solvent system (0 to 80%) or recrystallized from hot isopropanol or acetonitrile to afford the product.

General procedure for acid catalyzed S_NAr for synthesis of compounds **12**, **14**–**16**, **18**, **20**, **21**

In a sealed vial, 4-chlorothieno[3,2-*d*]pyrimidine (100 mg, 0.57 mmol) and desired amine (0.57 mmol) were dissolved in a 3:1 tetrahydrofuran: 2-propanol solution (8 mL). Next, a drop of 12 M HCl (~0.05 mL) was added and the solution was heated at 70 °C for 24 h. The reaction mixture was concentrated to dryness and the residue was dissolved in CH₂Cl₂ and washed with saturated aqueous NaHCO₃ solution, water, and brine. The organic phase was collected, dried over sodium sulfate, filtered, and concentrated *in vacuo*. Crude material obtained was purified by either silica gel column chromatography with a gradient of CH₂Cl₂:ethyl acetate solvent system (0 to 80%) or recrystallized from hot isopropanol or acetonitrile to afford the product.

3. Conclusions

Herein we described our preliminary SAR assessment of the thieno[3,2-*d*]pyrimidin-4-amines as Cyt-*bd* inhibitors. This is one of a few published examples of synthetically accessible compounds that can inhibit Cyt-*bd* in mycobacteria. While, the IC₅₀ values of the most potent compound (**19**) are merely good (6.2 μM *vs.* BCG and 7.3 μM *vs.* N0145-Mtb, when Q203 was added) this class of compounds can be used as a new tool to probe the mycobacterial oxidative phosphorylation pathway. Based upon this exploratory work, we will endeavour to design new thieno[3,2-*d*]pyrimidin-4-amines

with improved potency and acceptable pharmacokinetics to warrant *in vivo* evaluation.

Conflicts of interest

Hsiri Therapeutics has licensed this technology. M. J. M. and G. C. M. own equity in Hsiri. M. J. M. is CSO of Hsiri. G. C. M. and K. P. are consultants to Hsiri. Hsiri Therapeutics did not fund this study and was not involved in study design or interpretation.

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