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Letter

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Arrival of Imidazo[2,1-*b*]thiazole-5-carboxamides: Potent Anti-tuberculosis Agents That Target QcrB

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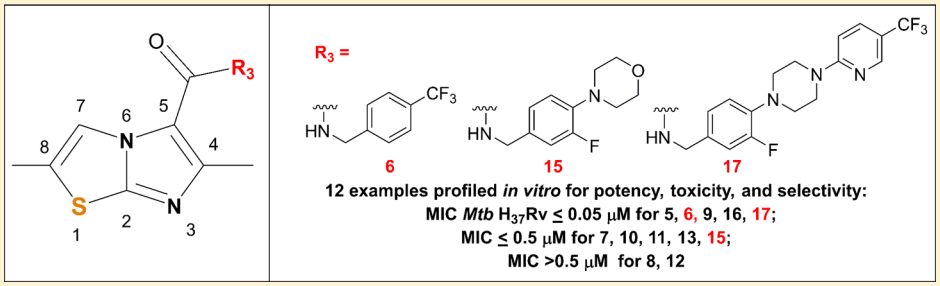
Supporting Information



ABSTRACT: Increasing interest in the potent anti-tuberculosis activity and the novel target (QcrB) of imidazo[1,2-*a*]pyridine-3-carboxamides encouraged extended structure−activity relationship studies of additional scaffolds. This study reports on the in vitro profiling of the imidazo[2,1-*b*]thiazole-5-carboxamides as a new promising class of anti-tuberculosis compounds endowed with nanomolar potency against replicating and drug-resistant *Mycobacterium tuberculosis* (*Mtb*) as well as low toxicity to VERO cells. Compounds **6**, **16**, and **17** had MIC values <10 nM and toxicity >100 μM. On-target selectivity of this series was confirmed by cross-resistance of specific QcrB mutants as well as the hypersusceptibility of a mutant with a functional gene deletion of the alternative cytochrome *bd* oxidase. Additionally, to demonstrate selectivity, three analogues (**6**, **15**, **17**) were broadly screened against a diverse set of eight strains of bacteria, including both Gram-positive and Gram-negative as well as six disease-causing non-tuberculosis mycobacteria. Finally, compounds **16** and **17** were found to be active in macrophages infected with *Mtb*.

KEYWORDS: *imidazothiazoles, antituberculosis, SAR, respiration target*

The heroic efforts, sacrifice, and steadfast tenacity of Waksman, Schatz, Feldman, Hinshaw, Domagk, Rosdahl, Hanngrenn, Crofton, Hotchkiss, and Lehmann have been responsible for many of the tuberculosis (TB) therapeutics in use today.[1] These agents have saved an estimated 43 million lives from 2000 to 2014.[2] Yet, TB remains a global health threat with 1.5 million deaths in 2014 and one-third of the world's population being infected with latent TB.[2] The modern-day global TB challenge is greater than ever because 5% of all TB cases are estimated to be multidrug-resistant strains of *Mycobacterium tuberculosis Mtb* (MDR-TB),[3] which do not respond well to current chemotherapy.[4] In 2014, there were an estimated 480,000 new cases of drug-resistant TB with extensively drug-resistant TB (XDR-TB) accounting for 9% of the cases.[3] With only two recently approved TB treatments[4] (bedaquiline and delamanid) during the past 40 years, heroic efforts in research are again needed to stop TB.

Our interest in developing agents to inhibit *Mtb*, the causative strain of tuberculosis, reflects our early work on the syntheses and biological evaluation of mycobactins, iron-binding mycobactial siderophores.[5,6] Retro-fragment-screening of mycobactin S[7] and T[8] components revealed that oxazolines and oxazoles, small-molecule heterocycles, are submicromolar inhibitors of *Mtb*.[9] Since that initial discovery, our extended efforts to find other small-molecule heterocyclic scaffolds led to the first disclosure of the imidazo[1,2-*a*]pyridine-3-carboxamides[10,11] and various other 5,6-heteroaromatic systems as potential anti-TB agents.[12] The imidazo[1,2-*a*]pyridine-3-

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carboxamides have shown notable therapeutic potential, being endowed with very potent antituberculosis activity,[13−17] a wide range of pharmacokinetic properties attenuated by structure,[14,16,18] impressive in vivo murine efficacy,[16,18,19] and a novel mechanism of action in targeting QcrB, a subunit of the menaquinol cytochrome *c* oxidoreductase (bc_1 complex), which is part of the bc_1-aa_3-type cytochrome *c* oxidase complex driving oxygen-dependent respiration.[17,18,20,21] Subsequently, various additional efforts have revealed other QcrB active structures demonstrating its potential as a drug target.[21,22]

Although imidazo[2,1-*b*]thiazole-5-carboxamides bear strong resemblance to the imidazo[1,2-*a*]pyridine-3-carboxamides, few studies indicate they have any notable anti-TB activity[23−26] and no structure−activity relationship (SAR) campaigns have yet led to compounds with nanomolar potency.[27] We hypothesized that trends observed within the SAR of imidazo[1,2-*a*]pyridine-3-carboxamides may be used to generate more potent imidazo[2,1-*b*]thiazole-5-carboxamides. As such, six noteworthy imidazo[1,2-*a*]pyridine-3-carboxamides (**IAP1−6**; Figure 1)

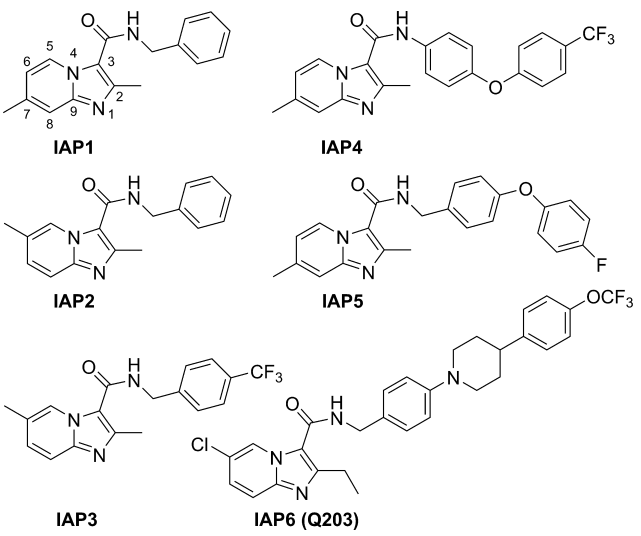

Figure 1. Previously reported benchmark imidazo[1,2-*a*]pyridine-3-carboxamides (**IAP1−6**) selected for SAR comparison.

selected from the literature were chosen as benchmarks for comparison between the two series. Simple benzyl amides (**IAP1** and **IAP2**) are well profiled and reported to have excellent potency against replicating and drug-resistant *Mtb* in addition to good pharmacokinetics.[10−15] **IAP3** was shown to have good in vivo exposure and murine efficacy when dosed at 300 and 500 mg/kg.[18] It was also used for the generation of resistant mutants, which established QcrB as the target of this class.[18] **IAP4** and **IAP5** first demonstrated that large lipophilic groups (like biaryl ethers) can increase potency dramatically yet with an overall preference for benzyl amides over anilines.[14] Finally, **IAP6**, known as **Q203**,[16,17] is the culmination of a massive SAR effort with particular emphasis on optimizing macrophage activity.[16] This compound is very lipophilic (ClogP = 7.6), resulting in very long half-lives ($t_{1/2}$ = 22 h at 10 mg/kg), is extremely potent (0.1−26 nM, Table 1), targets QcrB, and has shown in vivo murine efficacy at a low dose (0.4−10 mg/kg).[17] Thus, guided by these representative imidazo[1,2-*a*]pyridine-3-carboxamides, we prepared a focused panel of 12 imidazo[2,1-*b*]thiazole-5-carboxamides to discern any SAR trends. We evaluated their in vitro potency against the

Table 1. In Vitro Evaluation of Compounds 5−17, Rifampicin (RMP), and Isoniazid (INH) against Replicating *Mtb* H_{37}Rv (μM) and against Nonreplicating *Mtb* H_{37}Rv (μM) and Toxicity to VERO Cells (IC_{50} in μM)

			MIC *Mtb* H_{37}Rv in assay and media		
compound ID	ClogP[a]	VERO toxicity	MABA GAS	MABA 7H12	LORA
5	3.58	>100	0.91	0.52	>100
IAP1[b]	3.60	>100	0.79	2.3	54
IAP2[c]	3.60	>100	0.11	0.05	59
6	4.46	>100	0.006	0.061	19
IAP3[d]	4.48	>100	0.004	0.008	18
7	4.46	59	0.095	0.46	6.4
8	4.46	73	>1 (67%)	>1 (68%)	>100
9	4.61	36	0.021	0.24	16
10	4.61	47	0.077	0.46	22
11	6.35	>100	0.310	0.41	>100
12	6.38	>100	>1 (61%)	>1 (48%)	>100
IAP4[e]	6.40	>100	0.2	0.78	>100
13	5.82	14	0.017	0.048	26
IAP5[e]	5.84	9	0.10	0.2	25
14	3.04	98	>1 (76%)	0.463	57
15	3.35	>100	0.398	0.227	>100
16	6.85	>100	0.0009	0.007	1
IAP6[f] **(Q203)**	7.62	>100	0.0001	0.026	4
17	5.43	>100	0.004	0.004	29
RMP	3.71	>100	0.021	0.050	0.2
INH	−0.67	>100	0.22	0.37	>100

[a]ClogP calculated by ChemBioDraw Ultra 14 from PerkinElmer. [b]**IAP1** has published MIC values from 0.2 to 3.4 μM depending on the assay conditions.[10,11,13,14] [c]**IAP2** has published MIC values from 0.006 to 0.11 μM depending on the assay conditions.[12,15] [d]**IAP3** has a published MIC value of 0.03 μM when screened in the Middlebrook 7H9 medium.[18] [e]**IAP4** and **IAP5** have published MIC_{99} values of 0.7 and 0.004 μM, respectively, when screened in a modified Middlebrook 7H9 medium.[14] [f]**IAP6** (**Q203**) has a published MIC_{80} value of 0.004 μM[16] and an MIC_{50} of 0.0027 μM. Minimum inhibitory concentrations (MICs) were determined against *Mtb* grown in various media: the glycerol−alanine−salts (GAS) and Middlebrook 7H12 by MABA assay[28] percent inhibition at 1 μM denoted in parentheses. Activity in nonreplicating conditions was determined by the LORA assay.[28] Toxicity, expressed as the concentration to inhibit 50% of cell growth, was determined using VERO cell based assays.[29]

replicating *Mtb* laboratory strain, H37Rv, in two different types of media using the Microplate Alamar Blue Assay (MABA),[28] against nonreplicating *Mtb* in the low oxygen recovery assay (LORA),[28] and against drug-resistant *Mtb*,[29] as well as their toxicity against African Green Monkey kidney epithelial (VERO) cells.[29] Additionally, we evaluated the selectivity of a subset of compounds against strains of Gram-positive and Gram-negative organisms and against a panel of QcrB mutants[20] to determine whether these compounds inhibit the same target as the imidazo[1,2-*a*]pyridine-3-carboxamides.

The syntheses of these select imidazo[2,1-*b*]thiazole-5-carboxamides (Scheme 1) involved amide bond formation between the corresponding imidazo[2,1-*b*]thiazole-5-carboxylic acid (**3**) and either the appropriate commercially available amines (**4a−l**) or synthetically accessible amine (**4m**, Scheme 2). The synthesis of acid **3** required only slight modification of our published procedure for the preparation of various 5,6-heteroaromatic acids,[10−12] namely, reaction of 2-amino-5-methylthiazole (**1**) with 2-chloroacetoacetic acid ethyl ester

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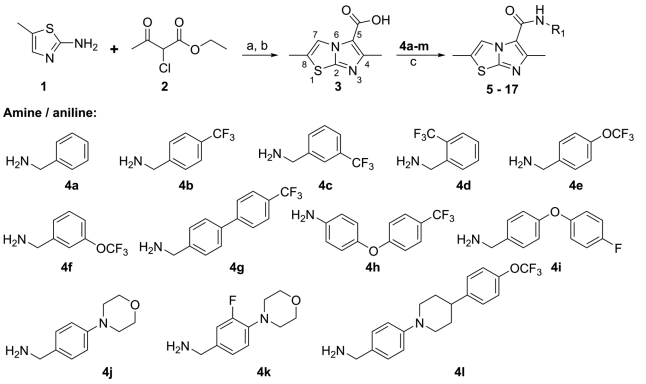
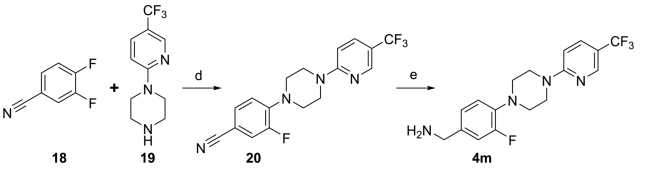
Scheme 1. Straightforward Syntheses of Imdiazo[2,1-*b*]thiazoles (5−17)[a]



[a]Reagents: (a) $NaHCO_3$, DME, 60 °C, 32 h; (b) 1, NaOH, EtOH, 50 °C, 24 h; 2, HCl; (c) EDC-HCl, DMAP, amine/aniline (**4a−4m**), CH_3CN, 16 h.

Scheme 2. Synthesis of Novel (3-Fluoro-4-(4-(5-(trifluoromethyl)pyridin-2-yl)piperazin-1-yl)benzylamine (4m)[a]



[a]Reagents: (d) DMF, 110 °C, 32 h; (e) 1, Pd/C, H_2, HCl, MeOH, 4 h; 2, 4 N NaOH.

(**2**) followed by saponification. Additionally, we prepared a novel benzyl amine derivative (**4m**) by an S_NAR reaction between 3,4-difluorobenzonitrile **18** and commercially available aryl-piperazine (**19**) followed by nitrile reduction with palladium and hydrogen in the presence of HCl[30] (Scheme 2). Standard EDC-mediated coupling of acid **3** and the various amines (**4a−m**) resulted in the analogue panel (**5−17**, Figure 2) that was used for evaluation and comparison with representative imidazo[1,2-*a*]pyridine-3-carboxamides (**IAP1−6**).

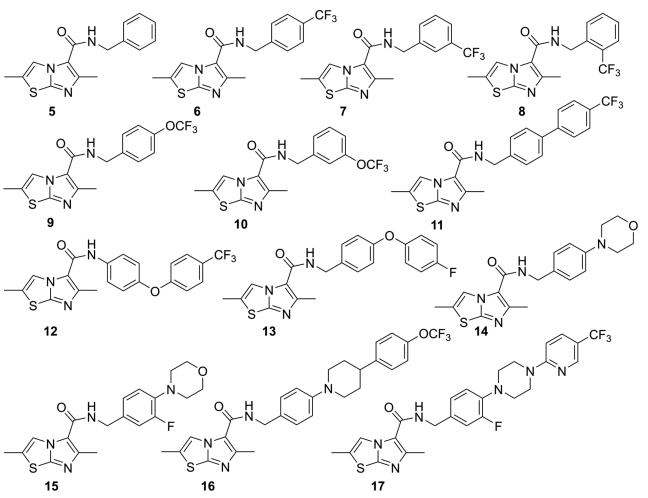


Figure 2. Imidazo[2,1-*b*]thiazole-5-carboxamides prepared for in vitro profiling.

Anti-tuberculosis assays revealed that two modifications were not well tolerated, namely, a trifluoromethyl group in the ortho-position (**8** compared to **6** and **7**) and biaryl ether aniline (**12**) compared to biaryl ether benzyl amine (**13**) (Table 1). Both of these compounds (**8** and **12**) had relatively poor potency (MIC > 1 μM) compared to the excellent potency of their SAR neighbors (MIC < 0.1 μM for **6**, **7**, and **13**, respectively). With the noted exception of analogues **8** and **12**, the other 10 compounds studied had excellent potency against replicating *Mtb* H37Rv with four compounds (**6**, **7**, **16**, and **17**) being as or more potent than rifampicin (RIF, MICs = 0.02−0.05) (Table 1). Two different media (GAS and 7H12) were used in our assays to avoid erroneous indications of potency due to carbon source dependency[31] and to give a wider MIC range for compound ranking when taking into account any effect from protein binding (the 7H12 assay medium[28] has added bovine serum albumin, see the Supporting Information). In our past experience with the imidazo[1,2-*a*]pyridine series, we saw a correlation between improved potency with larger lipophilic benzyl amides as noted by others.[14,16,38] However, it was encouraging to see that the trend was not as apparent with the imidazo[2,1-*b*]thiazole-5-carboxamides, as smaller more polar compounds were found to have impressive potency. For instance, compound **6** (MICs = 0.006−0.061 μM, MW 353.1, ClogP of 4.5) had potency similar to that of compound **16** (MICs = 0.0009−0.007 μM, MW 528.2, ClogP of 6.9) despite having a much lower molecular weight and ClogP. Although nanomolar potency is encouraging, compounds with properties that incorporate the medicinal chemist's "rule of five"[32−34] rubric are often more attractive drug leads.

In direct comparison to the benchmark imidazo[1,2-*a*]pyridine-3-carboxyamides (**IAP1−6**), this series has the highest SAR potency correlation, contrary to the seven 5,6-heteroaromatic systems we profiled previously.[12] Interestingly, this new series does appear to emulate the SAR observed with the recently disclosed pyrazolo[1,5-*a*]pyridine-3-carboxamides.[39] By screening under the same assay conditions we found that the simple benzyl amide (**5**) had slightly better potency than **IAP1** (which bears the 7-CH_3 group) but was not as potent as the **IAP2** (with the 6-CH_3 group) (Table 1). In our screening campaign we saw a trend that the 2,6-dimethylimidazo[1,2-*a*]pyridine core would often have slightly more potency than its 2,7-dimethylimidazo[1,2-*a*]pyridine isomer[14,15] and that the 6-Cl isomer was better than the 7-Cl isomer.[16] **IAP3** and **IAP4** both had slightly better potency than **6** and **12**, respectively. Of note, **IAP3** displayed much more potency in our assay than the published study (0.03 μM), whereas **IAP4** compared well with its published average value of 0.7 μM (different assay medium and readout). **IAP5** was not as potent as **13** under the same assay conditions, but its lowest reported MIC (0.004 μM, different assay medium and readout) is outstanding as was the MIC of the 6-CH_3 positional isomer (0.005 μM, different assay medium and readout).[14] **IAP6** (**Q203**) demonstrated outstanding potency in the GAS medium (0.1 nM and **16** was 0.9 nM) but it was less potent than **16** in the 7H12 medium supplemented with BSA (26 nM compared to 7 nM, respectively). It is possible that the >250 shift in the activity of **Q203** is due to protein binding, given its higher lipophilicity relative to **16** (ClogP difference of 0.77) or perhaps relative solubility. In general, given the inherent variation of MICs in a whole cell assay, the imidazo[2,1-*b*]thiazole-5-carboxamides (**5**, **6**, **12**, **13**, **16**) were remarkably similar in potency and SAR trends to the benchmark

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Table 2. In Vitro Evaluation of Compounds 6, 15, and 17, Rifampicin (RMP), and Isoniazid (INH) against Clinical Monodrug-Resistant *Mtb* (μM)[a]

	monoresistant MIC					
compound	rRMP	rINH	rSM	rCs	rMox	rKM
6	0.201	0.506	3.97	>10 (46%)	0.571	0.467
15	<0.04	<0.04	0.41	>10 (73%)	<0.04	<0.04
17	<0.04	<0.04	0.46	>10 (69%)	<0.04	<0.04
RMP	>4	0.08	0.06	0.02	0.09	0.06
INH	0.24	>8	0.32	0.53	0.41	0.37

[a]rRMP, *Mtb* resistant to rifampicin (RMP); rINH, *Mtb* resistant to isoniazid (INH); rSM, *Mtb* resistant to streptomycin; rCS, *Mtb* resistant to cycloserin; rMox, *Mtb* resistant to moxifloxacin; rKM, *Mtb* resistant to kanamycin. Percent inhibition at 10 μM denoted in parentheses.

imidazo[1,2-*a*]pyridine-3-carboxyamides (**IAP1−6**) with the largest deviation observed with the biaryl ether analogues. We anticipate more notable differences (particularly in metabolism, metabolites formed, and efficacy) will arise when we complete in vivo assessment of the lead compounds.

Only one compound, **16**, was moderately active against nonreplicating (latent) *Mtb* with an MIC of 1 μM, whereas the LORA MIC of rifampicin was 0.24 and 4 μM for **IAP6** (**Q203**). This suggests that the imidazo[2,1-*b*]thiazole-5-carboxamides are more selectively active against replicating *Mtb*, consistent with targeting oxygen-dependent respiration through the QcrB−bc_1 complex. Additionally, only one compound, **13**, showed unacceptable toxicity to VERO cells with an IC_{50} of 14 μM (whereas analogous biaryl ether **IAP5** was more toxic at 9 μM). Noting that compound **6** had quite a large MIC shift between the two assays, we evaluated the potency of imidazo[2,1-*b*]thiazole-5-carboxamides **6**, **13**, **15**, **16**, and **17** and imidazo[1,2-*a*]pyridine-3-carboxamides **IAP3**, **IAP5**, and **IAP6** in six different media, by two different readouts (optical and MABA), and at two different times (weeks 1 and 2). This screening showed that these compounds are uniformly potent but that the absolute MIC will vary depending on the medium, readout, and time of reading (Table S5). Compounds with the lowest ClogP (**6** and **15**) had the largest MIC range, whereas the most lipophilic compounds (**IAP6** and **16**) had smaller MIC ranges in all of these additional *Mtb* screens.

We evaluated a subset of compounds (**6**, **15**, and **17**) against a panel of monodrug-resistant strains of *Mtb* (Table 2).[29] The compounds were exceptionally potent against these strains with the exception of one strain. The cycloserine-resistant phenotype of this strain is unlikely associated with its resistance to this series of QcrB inhibitors but is more likely associated with other adaptations that may have occurred during in vitro growth.

Next, these same three representative compounds (**6**, **15**, **17**) were screened in a Kirby−Bauer agar diffusion assay[35] against a panel of bacteria that included four Gram-positive strains (*Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Mycobacterium vaccae*) and four Gram-negative strains (*Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Burkholderia dolosa*). At 2 mM, these compounds were inactive against all of the control organisms except for potent activity observed against *M. vaccae*, as expected (Table S2 in the Supporting Information). When screened against other nontuberculosis mycobacteria (NTM), the same compounds showed good activity (0.4−3 μM) against *Mycobacterium kansasii* and *Mycobacterium bovis* BCG, moderate activity (<4 μM) against *Mycobacterium chelonae* and *Mycobacterium avium*, and no activity against *Mycobacterium abscessus* and *Mycobacterium marinum* (>10 μM) (Table S3 in the Supporting Information). The poor potency against *M. marinum* may be due to the presence of a second *ctaD* homologue[40] (which encodes the terminal aa3-type supercomplex in *Mtb*), which is also found in *Mycobacterium smegmatis* to which these compounds were also inactive (data not show). Lack of potency against *M. abscessus* is not surprising as only a limited number of drugs are active against *M. abscessus* due to a number of mechanisms including slow growth, the presence of a waxy impermeable cell wall, which acts as a physical and chemical barrier, export systems, and neutralizing enzymes.[41]

As mentioned previously, we had suspected that the structure of the imidazo[2,1-*b*]thiazole-5-carboxamide analogues would mimic that of the analogous imidazo[1,2-*a*]pyridine-3-carboxamides, and this structural overlap may be a key to explaining their similar potency to *Mtb*. As such, two imidazo[2,1-*b*]thiazole-5-carboxamides, **13** and **17** (Figure 3B and Table S1 in the Supporting Information), and two imidazo[1,2-*a*]-pyridine-3-carboxamides, **IAP1** and **IAP5** (Figure 3B and Table S1 in the Supporting Information), were crystallized and X-ray structures determined. The resulting structures were subsequently compared with each other (Figure 3A and Figures S1 and S2 in the Supporting Information).

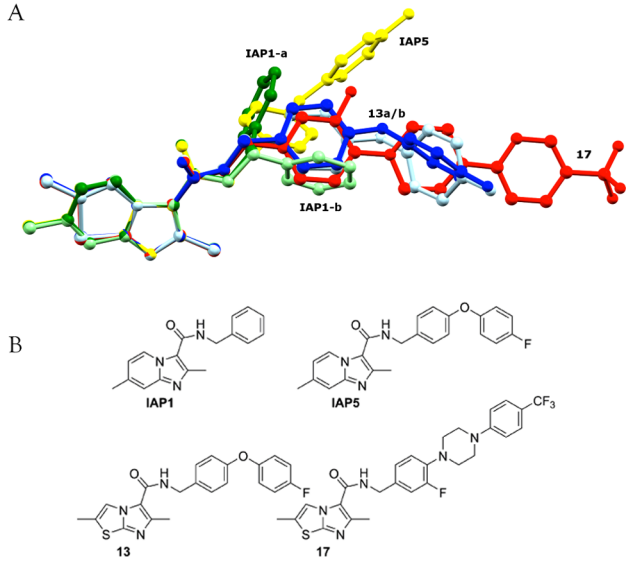

Figure 3. (A) Overlay of compounds **13** and **17** with **IAP1** and **IAP5**. Legend: **13a**, blue; **13b**, light blue; **17**, red; **IAP1a**, green; **IAP1b**, light green; **IAP5**, yellow. (B) Structures of compounds **13**, **17**, **IAP1**, and **IAP5** crystallized and used in overlay.

Structural features that stand out include an indication that the biaryl ether moiety of **IAP5** is more out of the plane

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compared to that of biaryl ether **13**, simple benzyl amine **IAP1**, and **17** (Figure 3). All of these compounds display great potency (Table S1 in the Supporting Information), suggesting that, as expected, in solution these pendant moieties are dynamic and fluxional. However, in the solid state there is a much higher correlation between the orientations of the pendant groups as can be seen in Figure 3, particularly with the two scaffold analogues (**13** and **17**) bearing the same biaryl ether benzyl amide (Figure S1 in the Supporting Information). Similarly, both imidazo[2,1-*b*]thiazole-5-carboxamide compounds (**13** and **17**) had high pendant group correlation (Figure S2 in the Supporting Information). It should be noted that in the solid state, compounds **IAP1** and **13** have two crystallographically independent molecules present in the asymmetric unit (designated **IAP1a**, **IAP1b**, **13a**, and **13b**). Although they are crystallographically independent, they are chemically identical. This observation supports the claim above that these compounds are likely to be fluxional in solution. This information will hopefully be useful for docking studies in the QcrB homology model.[20,22]

Because the target of this class is of great interest, compounds **6**, **13**, **15**, **16**, and **17** as well as **IAP3** and **IAP6** were screened against a panel of six QcrB mutants[20] known to be resistant to other scaffolds targeting the bc_1 complex.[17,18] Specific mutations in QcrB conferred resistance to these compounds, although some of the mutants previously shown to be resistant to other inhibitors of this subunit remained sensitive (Table S4). In addition, a strain with a deletion of genes encoding the cytochrome *bd* oxidase,[20] as predicted, was hypersusceptible to these compounds due to the inability of this mutant to redirect electrons to this alternative oxygen-dependent terminal oxidase.

Finally, two compounds (**16** and **17**) were evaluated ex vivo in J774A.1 macrophages[36,37] infected with the Erdman *Mtb* strain (Figure S6 and Table S6 in the Supporting Information). Each compound was administered at three concentrations (0.05, 0.5, and 5 μM) following infection, and cultures were then incubated for 6 days. Both compounds demonstrated a modest inhibition of growth relative to the untreated control with relatively flat dose responses (compared to a dose-dependent drug such as rifampin). At the concentrations tested, only static activity was observed. We intend to probe whether this static activity is concentration or time dependent by determination of the time kill kinetics in both replicating and nonreplicating conditions. Results will be reported in due course.

In conclusion, we present the imidazo[2,1-*b*]thiazole-5-carboxamides as a promising new scaffold for *Mtb* inhibitors that target QcrB with excellent activity against replicating and drug-resistant strains, low toxicity to VERO cells, and the ability to reduce bacterial burden in macrophages. Our future assessment will include profiling this series in vivo.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00154.

Experimental procedures, additional SAR, synthesises and ^{1}H and ^{13}C NMR of all new compounds, and details of all microbiological and antibacterial (*Mtb*, NTM, broad spectrum, QcrB mutant panel) studies (PDF)

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Notes

The authors declare no competing financial interest.

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