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SAR and identification of 2-(quinolin-4-yloxy)acetamides as *Mycobacterium tuberculosis* cytochrome bc_1 inhibitors†‡

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A previous phenotypic screen by GSK identified 2-(quinolin-4-yloxy)acetamides as potent growth inhibitors of *Mycobacterium tuberculosis* (Mtb). We report the results of a preliminary structure–activity relationship (SAR) study of the compound class which has yielded more potent inhibitors. An Mtb cytochrome *bd* oxidase deletion mutant (cydKO) was found to be hypersensitive to most members of the compound library, while strains carrying single-nucleotide polymorphisms of the *qcrB* gene, which encodes a subunit of the menaquinol cytochrome c oxidoreductase (bc_1) complex, were resistant to the library. These results identify that the 2-(quinolin-4-yloxy)acetamide class of Mtb growth inhibitors can be added to the growing number of scaffolds that target the *M. tuberculosis* bc_1 complex.

1. Introduction

The current paradigm for the discovery of antibiotics that can be developed into much-needed new treatments for tuberculosis typically starts with a whole-cell phenotypic screening campaign.[1,2] The majority of these screens use aerobic culture conditions and so by necessity, lead to the discovery of growth inhibitors of replicating *Mycobacterium tuberculosis* (Mtb). Recent efforts have also been directed towards undertaking screening using culture conditions considered more relevant to the *in vivo* disease state,[3,4] or towards Mtb inside host macrophages.[5] Defining the mechanism of action of screening hits obtained in such an agnostic manner can be challenging, however with significant cost reductions, sequencing of resistant mutants has become a productive pathway. Two recent examples of screening campaign hits are the imidazo[1,2-*a*]pyridine amides (IPAs),[2,3,6–8] exemplified by the clinical candidate Q203 (**1**) identified during an Mtb/macrophage assay,[9] and lansoprazole (**2**, Prevacid), identified in a pharmaceutical re-purposing screen designed to identify compounds that protect lung fibroblasts from Mtb-induced cytotoxicity (Fig. 1).[10] Genome sequencing of spontaneous resistant mutants generated for both **1** and **2** revealed a common target of QcrB (Rv2196), which encodes the *b* subunit of the cytochrome bc_1 complex, an essential component of the electron transport chain. In the case of Q203, a mutation of T313 A or T313I in QcrB was observed to cause resistance, while for **2**, a L176P mutation was responsible.

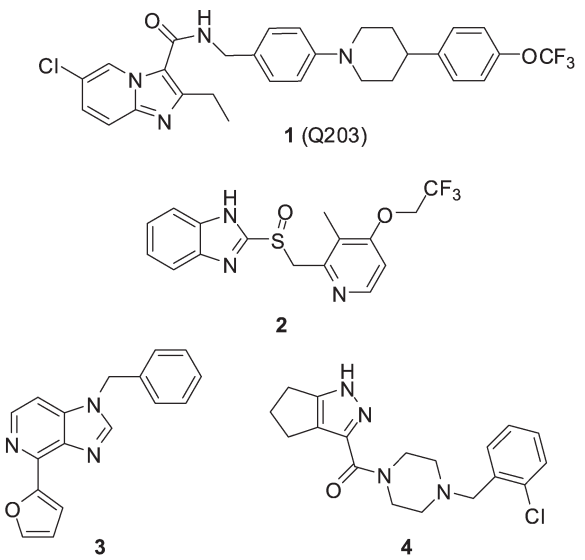

Fig. 1 Structures of cytochrome bc_1 oxidase inhibitors **1**–**4**.

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‡ Electronic supplementary information (ESI) available: Materials and methods, and characterisation of compounds **10a–f**, **11a–x** and **5**, **9**, **12a–12aa**. See DOI: 10.1039/c6md00236f

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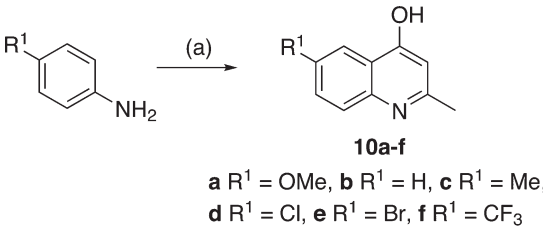
In addition to the chemtypes encompassed by **1** and **2**, further chemically diverse scaffolds have been identified to bind to QcrB,[11] including imidazo[4,5-*c*]pyridine **3** and pyrazole **4**,[12] suggesting the target is somewhat promiscuous (Fig. 1).

GSK recently made publically available the results of a phenotypic screening campaign, whereby a large compound library was assessed for the ability to inhibit the growth of *Mycobacterium bovis* BCG and *M. tuberculosis* H_{37}Rv. The curated results led to the identification of 177 hits covering a number of different structural classes.[13] Amongst this set were five 2-(quinolin-4-yloxy)acetamides (QOAs) (**5–9**, Fig. 2) exhibiting favourable Mtb growth inhibition properties with MIC_{90} Mtb H_{37}Rv 0.3–3.3 μM.

The potency of activity and structural simplicity of this series makes them an attractive target, prompting efforts to explore the structure–activity relationship of the class. During the preparation of this manuscript, two groups have reported the results of their studies of the QOA class, confirming compound potency, selectivity for *M. tuberculosis* and lack of toxicity in a *Danio rerio* (zebrafish) model.[14] Herein we report our efforts in optimizing the *in vitro* anti-tuberculosis activity of the more potent GSK QOA analogue **5** and also identify, *via* use of selective mutant strains, their cellular target as involving the cytochrome bc_1 complex.

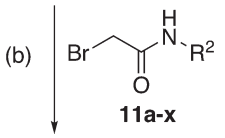
2. Results and discussion

Our preliminary structure–activity relationship study of GSK 358607A (**5**) focused on changes to the (i) 2-methoxyphenylacetamide moiety and (ii) the quinoline 6-position. The target 2-(quinolin-4-yloxy)acetamide library (**12a–12aa**), in addition to GSK compounds **5** and **9**, were prepared in three steps from commercially available materials (Scheme 1). The synthesis began by preparing the required 4-hydroxyquinolines by heating the appropriate aniline with ethyl acetoacetate in polyphosphoric acid to afford, after purification, **10a–f** in yields of 25–77%. Bromoacetamides **11a–x** were prepared in 27–100% yields from the acylation, at −77 °C, of various substituted anilines or alkylamines with bromoacetyl bromide. In the final step, 2-(quinolin-4-yloxy)acetamides **5**, **9** and **12a–12aa** were prepared, in yields of 16–89%, by reaction of 4-hydroxyquinolines with bromoacetamides in either acetone or DMF solvent in the presence of K_2CO_3 base.

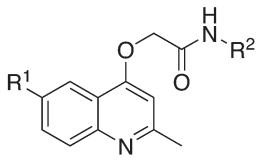


10a-f

a R^1 = OMe, **b** R^1 = H, **c** R^1 = Me,
d R^1 = Cl, **e** R^1 = Br, **f** R^1 = CF_3



11a-x

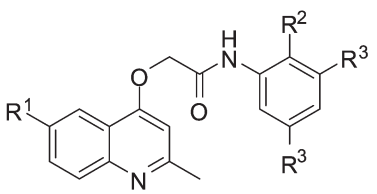


5, 9, 12a-aa

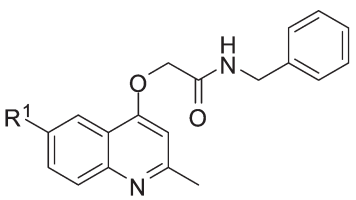
12a-v R^1 = OMe
a R^2 = Ph, **b** R^2 = 3-OMe-Ph,
c R^2 = 4-OMe-Ph, **d** R^2 = 2,4-diOMe-Ph,
e R^2 = 3,4-diOMe-Ph, **f** R^2 = 3,5-diOMe-Ph,
g R^2 = 3,4-dioxomethylene-Ph, **h** R^2 = 3-Br-Ph,
i R^2 = 4-Br-Ph, **j** R^2 = 2-Cl-Ph,
k R^2 = 4-Cl-Ph, **l** R^2 = 4-Me-Ph,
m R^2 = 4-Ph-Ph, **n** R^2 = 3,4-dioxomethylene-Ph-CH_2,
o R^2 = 2-phenethyl, **p** R^2 = 2-(2,5-diOMe-Ph)-ethyl,
q R^2 = 2-(3,4-diOMe-Ph)-ethyl,
r R^2 = 2-(3,4-dioxomethylene-Ph)-ethyl,
s R^2 = 3-phenylpropyl, **t** R^2 = 2-methoxyethyl,
u R^2 = prop-2-yn, **v** R^2 = *n*-pentyl

12w-aa R^2 = 2-OMe-Ph
w R^1 = H, **x** R^1 = Me, **y** R^1 = Cl,
z R^1 = Br, **aa** R^1 = CF_3

Scheme 1 Synthesis of target compounds **5**, **9**, **12a–12aa**. Reagents and conditions: (a) ethylacetoacetate, PPA, 130 °C, 2 h, yield: 25–77%; (b) bromide **11a–x**, K_2CO_3, DMF or acetone, 5–21 h, yield: 16–89%.



5 (GSK 358607A) R^1 = R^2 = OMe, R^3 = H
6 (GSK 749336A) R^1 = OMe, R^2 = H, R^3 = Me
7 (TCMDC-142552) R^1 = Br, R^2 = R^3 = H



8 (GSK 124576A) R^1 = Br
9 (GSK 463114A) R^1 = OMe

Fig. 2 Structures of GSK hit compounds **5–9**.

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The library of analogues were tested for whole-cell growth inhibition of *Mycobacterium tuberculosis* (Mtb) $H_{37}Rv$ under a variety of growth conditions and different readouts of growth and minimum inhibitory concentration (MIC) values were determined at either 7 or 14 days (Table 1). Using laboratory-adapted strain Mtb $H_{37}Rv$ grown in Middlebrook 7H9 media with microplate Alamar Blue assay (MABA) readout after 14 days (Table 1, data column 1) revealed that the GSK hits **5** and **9** exhibited MICs of 1.1 and 2.3 μM respectively (entries 1 and 2). Of the phenylacetamide sidechain analogues prepared, examples containing either no substituent (**12a**, entry 3), or 2-chloro (**12j**, entry 12) or 4-methyl (**12l**, entry 14) substitution were slightly more potent than GSK compounds **5** and **9**. The remaining phenylacetamide sidechain analogues (**12b–12i**, **12k**, **12m**, entries 4–11, 13, 15) were either equipotent or less active than the original hits. In most cases introduction of conformational flexibility in the form of 2-phenethyl (**12o**, **12p**, **12q**, **12r**) or 3-phenylpropyl (**12s**) acetamide sidechains yielded compounds that were poor growth inhibitors. The exception to this trend was **12n**, a 3,4-dioxomethylenebenzyl derivative, which exhibited an MIC of 0.53 μM, being slightly more potent than the unsubstituted GSK benzyl analogue **9**. Acetamide sidechains that incorporated no aryl moiety (*i.e.* **12t** 2-methoxyethyl, **12u** prop-2-yn and **12v** *n*-pentyl, entries 22–24) were also significantly less active than the original hit compounds. Overall, four compounds **12a**, **12j**, **12l**, **12n** were identified as being more potent growth inhibitors than the original GSK hit compound. In general, bulky, lipophilic substituents of limited conformational flexibility at R_2 improved the antimycobacterial activity of the compounds, while R_1 being methoxyl was critical for activity. Similar trends in overall structure–activity relationship were observed by Pissinate *et al.*, where they arrived at conclusions regarding the requirement for a bulky lipophilic group at R_2, finding a 2-naphthyl derivative to be potently antimycobacterial.[14a]

When Mtb $H_{37}Rv$ was grown in the same media (7H9/ADC/Tween) but using optical density as the growth readout,

Table 1 *In vitro* activity of **5**, **9**, **12a–12aa** against *Mycobacterium tuberculosis* $H_{37}Rv$ in various assays and media and an Mtb cytochrome *bd* oxidase mutant cydKO

		MIC (μM)			
Entry	Compound	$H_{37}Rv$ MABA 2 week[a]	$H_{37}Rv$ 1 week[b]	$H_{37}Rv$ 2 week[c]	cydKO 2 week[d]
1	**5**	1.11	2.21	53.92	0.14
2	**9**	2.32	3.57	>148	0.59
3	**12a**	0.62	1.21	>155	0.12
4	**12b**	6.53	141.88	>142	0.57
5	**12c**	1.11	2.21	>142	0.14
6	**12d**	2.04	24.58	32.69	0.18
7	**12e**	24.58	>131	>131	3.14
8	**12f**	24.58	>131	>131	2.04
9	**12g**	2.13	4.26	>136	0.19
10	**12h**	>125	92.20	>125	0.37
11	**12i**	1.94	>125	>125	0.17
12	**12j**	0.56	26.35	53.25	<0.067
13	**12k**	3.36	103.70	>140	0.20
14	**12l**	0.45	0.45	>149	<0.071
15	**12m**	1.51	>125	>125	0.12
16	**12n**	0.53	0.53	24.71	<0.063
17	**12o**	54.22	142.69	>143	4.45
18	**12p**	60.90	121.80	>122	7.62
19	**12q**	122.00	>122	>122	90.13
20	**12r**	31.69	≧127	>127	3.04
21	**12s**	25.79	68.59	68.59	2.14
22	**12t**	121.55	>164	>164	41.06
23	**12u**	>176	>176	>176	21.98
24	**12v**	19.75	158.03	158.03	0.95
25	**12w**	>78	38.77	>155	7.13
26	**12x**	9.30	56.48	>74	0.45
27	**12y**	103.70	26.35	>140	1.09
28	**12z**	>125	23.42	>125	0.50
29	**12aa**	>128	>128	>128	>128
	PAS[e]	0.3	0.3	0.6	0.6
	Linezolid[e]	2.3	2.3	2.3	1.56
	3[e]	1.56	12.5	>25	0.31

MIC values are the average of two independent assays. Assay protocols are described in ref. 8. [a] MIC against $H_{37}Rv$ grown in 7H9/ADC/Tween media. MIC determination using microplate Alamar Blue assay (MABA) after 2 weeks post compound addition. [b] MIC against $H_{37}Rv$ grown in 7H9/ADC/Tween media. MIC determination after 1 week post compound addition. [c] MIC against $H_{37}Rv$ grown in 7H9/ADC/Tween media. MIC determination after 2 weeks post compound addition. [d] MIC against *cyd* knock-out strain of $H_{37}Rv$ grown in 7H9/ADC/Tween media. MIC determination after 2 weeks post-compound addition. [e] Linezolid, PAS (*p*-aminosalicylic acid) and **3** were used as positive controls.

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Table 2 *qcrB* Mutants are resistant to compounds that target the bc_1 complex

Entry	Compound	Fold resistance to qcrB mutant						
		A317V	M342T	W312G	A396T	M342I	A317T	S182P
1	**5**	46.9	1.4	46.9	4.1	6.1	24.5	15.9
2	**9**	>250	3.0	47.0	3.9	3.9	23.5	15.7
3	**12a**	>1250	3.8	235.0	5.0	1.8	30.0	19.5
4	**12c**	63.9	4.1	24.5	3.1	3.1	24.5	15.9
5	**12g**	134.3	2.1	17.1	5.6	1.4	32.9	32.9
6	**12l**	>32.5	>2	>16.3	>2	1.0	>12.5	>16.3
7	**12n**	>130	>12.5	>25	>6.3	>12.5	>32.5	>95.8
8	**3**[a]	>81	20.2	>81	20.2	20.2	10.1	61.3
	PAS[a]	0.5	0.3	0.5	0.3	0.5	0.2	0.5

[a] Compound **3** (positive control), PAS (*p*-aminosalicylic acid, negative control).

there was apparent bacterial outgrowth as evidenced by time-dependent outgrowth of the cells in the presence of the compound resulting in a dramatic shift in apparent MIC (Table 1, data columns 2 and 3). Similar outgrowth was also observed for *M. tuberculosis* BSG001 (*M. tuberculosis* H_{37}Rv transformed with the bacterial luciferase-encoding vector pMV306hsp + LuxAB + G13 + CDE)[15] grown in Middlebrook 7H9 media (data not shown). This discordance between growth MIC and the Alamar Blue MIC has been previously reported to be a characteristic of compounds that inhibit the respiratory bc_1 complex of *Mycobacterium tuberculosis*.[12] The ability of cells to overcome the growth inhibitory effect of these compounds could at least in part be driven by compensatory upregulation of the alternate oxygen-dependent cytochrome *bd* oxidase pathway.[16] Evidence for this upregulation-based protection model is based upon the findings of a number of groups, whereby cytochrome *bd* oxidase knock-out strains (ΔcydKO) of mycobacteria are hyper-susceptible to electron transport chain inhibitors[17] including those compounds that target bc_1.[8,11,12] As summarized in Table 1 (data column 4), most of the test set of compounds did indeed exhibit enhanced MIC potency towards the Mtb ΔcydKO strain *versus* laboratory-adapted Mtb H_{37}Rv. Based upon the magnitude of MIC enhancement evident in Table 1, it was concluded that the majority of the analogues, and GSK compounds **5** and **9**, are inhibitors of cytochrome bc_1 oxidase. Further direct evidence for the bc_1 oxidase inhibiting properties of **5** and **9** and analogues **12a**, **12c**, **12g**, **12l** and **12n** was obtained when the compounds were found to exhibit reduced potency against a series of QcrB subunit mutants of the cydKO strain of Mtb (Table 2). The seven defined Mtb cydKO *qcrB* amino acid mutants, A317V, M342T, W312G, A396T, M342I, A317T and S182P, were originally generated in response to imidazo[4,5-*c*]pyridine **3** with homology modeling of QcrB identifying all mutations to be located proximal to the binding site of stigmatellin.[12] In

Table 3 *In vitro* cytotoxicity of **5**, **9**, **12a**–**12aa** against HepG2 cells during growth on galactose and glucose

Entry	Compound	GalactoseCC_{50} (μM)	GlucoseCC_{50} (μM)
1	**5**	19.6	>142
2	**9**	>149	>149
3	**12a**	118.1	>155
4	**12b**	24.7	43.0
5	**12c**	>142	>142
6	**12d**	>131	>131
7	**12e**	>131	>131
8	**12f**	>131	>131
9	**12g**	39.6	80.0
10	**12h**	>125	>125
11	**12i**	>125	>125
12	**12j**	>140	>140
13	**12k**	71.1	89.9
14	**12l**	41.5	>149
15	**12m**	>126	>126
16	**12n**	86.9	>131
17	**12o**	83.9	61.4
18	**12p**	>122	>122
19	**12q**	>122	>122
20	**12r**	>127	>127
21	**12s**	50.7	>137
22	**12t**	>164	>164
23	**12u**	>176	>176
24	**12v**	106.3	>158
25	**12w**	8.64	43.9
26	**12x**	>74	>74
27	**12y**	99.2	140.1
28	**12z**	>125	>125
29	**12aa**	>128	>128
	3[a]	>25	>25
	Antimycin A[a]	0.017	>50

IC_{50} values are the average of two independent assays. Assay protocols are described in ref. 18. [a] Compound **3** and antimycin A were used as positive controls.

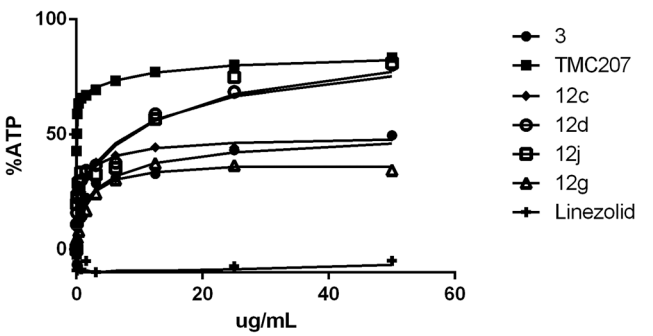

Fig. 3 ATP depletion (%) under anaerobic conditions for test compounds **12c**, **12d**, **12g**, and **12j**.

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the present study, the magnitudes of the fold-resistance observed for the test compounds against these seven cydKO mutants confirms cytochrome bc_1 oxidase as a cellular target of 2-(quinolin-4-yloxy)acetamides. Compounds that inhibit cytochrome bc_1 oxidase have previously been shown to deplete intracellular ATP levels in anaerobic cells.[9] We found that our 2-(quinolin-4-yloxy)acetamides were able to reduce ATP levels within 24 hours of exposure under anaerobic conditions in a similar manner to TMC207[3,9] (Bedaquiline, a F_0F_1 ATP synthase inhibitor) and **3**, a known cytochrome bc_1 oxidase inhibitor[12] (Fig. 3) (and ESI‡ Fig. S1–S3). ATP levels were not reduced by the cell-wall synthesis inhibitor linezolid.

Previous groups have determined the selectivity of antitubercular cytochrome bc_1 oxidase inhibitors towards *Mycobacterium tuberculosis*, with little or no detectable antibacterial activity being observed towards panels of Gram-positive or Gram-negative bacteria.[8,10] We can confirm the recent report by Pissinate *et al.*,[14a] where they noted the absence of antibacterial activity of the 2-(quinolin-4-yloxy)acetamide compound class towards *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Acinetobacter baumannii*. In the present study, no activity was observed towards *Escherichia coli* 25922, *Staphylococcus aureus* XEN36, *Mycobacterium smegmatis* and *M. abscessus* (data not shown).

The cytotoxicity of the 2-(quinolin-4-yloxy)acetamide library was determined using HepG2 cells, with the assay media containing either glucose or galactose. The use of galactose forces the cells to rely on mitochondrial oxidative phosphorylation rather than glycolysis for growth.[18] While five of the test compounds (**12b**, **12g**, **12k**, **12o**, **12w**; Table 3, entries 4, 9, 13, 17, 25) were considered to be mildly cytotoxic independent of glucose/galactose-based media, two analogues (**5**, **12l**) exhibited mild to moderate levels of cytotoxicity ($CC_{50} < 50$ μM) only towards cells grown in galactose-containing media. This latter result identifies these two compounds, which includes GSK hit **5**, as being potential inhibitors of mitochondrial respiration.

3. Conclusions

In conclusion, our preliminary structure–activity relationship investigation of the anti-tuberculosis activity of 2-(quinolin-4-yloxy)acetamides has identified four analogues (**12a**, **12j**, **12l**, **12n**) as being more potent growth inhibitors than the original GSK 358607A (**5**) hit compound, exhibiting submicromolar MIC values. We have established that this compound class targets QcrB, an essential component of the electron transport chain. Knowledge of the cellular target of 2-(quinolin-4-yloxy)acetamides will now facilitate structure-based drug design as we seek to optimize the anti-tuberculosis potency of this series.

Author contributions

N. P. and B. R. C. synthesized the compound library. H. I. B., C. E. B., J. D. and S. W. performed antibacterial testing. P. S. T. performed cytotoxicity testing. B. R. C. wrote the manuscript with contributions from all co-authors.

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