Synthesis and Evaluation of the Diaminoquinazoline Series as Anti-Tubercular Agents

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Abstract— *The 2,4-diaminoquinazoline class of compounds has previously been identified as an effective inhibitor of Mycobacterium tuberculosis growth. We conducted an extensive evaluation of the series for its potential as a lead candidate for tuberculosis drug discovery. Three segments of the representative molecule N-(4-fluorobenzyl)-2-(piperidin-1-yl) quinazolin-4-amine were examined systematically to explore structure–activity relationships influencing potency. We determined that the benzylic amine at the 4-position, the piperidine at 2-position and the N-1 (but not N-3) are key activity determinants. The 3-deaza analog retained similar activity to the parent molecule. Biological activity was not dependent on iron or carbon source availability. We demonstrated through pharmacokinetic studies in rats that good in vivo compound exposure is achievable. A representative compound demonstrated bactericidal activity against both replicating and non-replicating M. tuberculosis. We isolated and sequenced M. tuberculosis mutants resistant to this compound and observed mutations in Rv3161c, a gene predicted to encode a dioxygenase, suggesting that the compound may act as a pro-drug.*

Keywords— Tuberculosis Mycobacterium tuberculosis Antibacterial activity, 2,4-Diaminoquinazoline Dioxygenase.

I. INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is an infectious disease for which there is still a great need for discovery and development of novel drugs to improve therapy.[1] In 2010 alone, the World Health Organization reported 8.8 million new cases and 1.4 million deaths from the disease.[2] In addition, billions of people harbor latent infections with no clinical symptoms, but with the potential to advance to active form. Current TB treatment requires a combination of four drugs, isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), ethambutol (ETH) for 2 months followed by an additional 4 months of INH and RIF. These drugs have been in use for many decades, contributing to a rise in the emergence of multidrug resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis*. New drugs are needed urgently to shorten the duration of therapy and to treat drug-resistant strains.

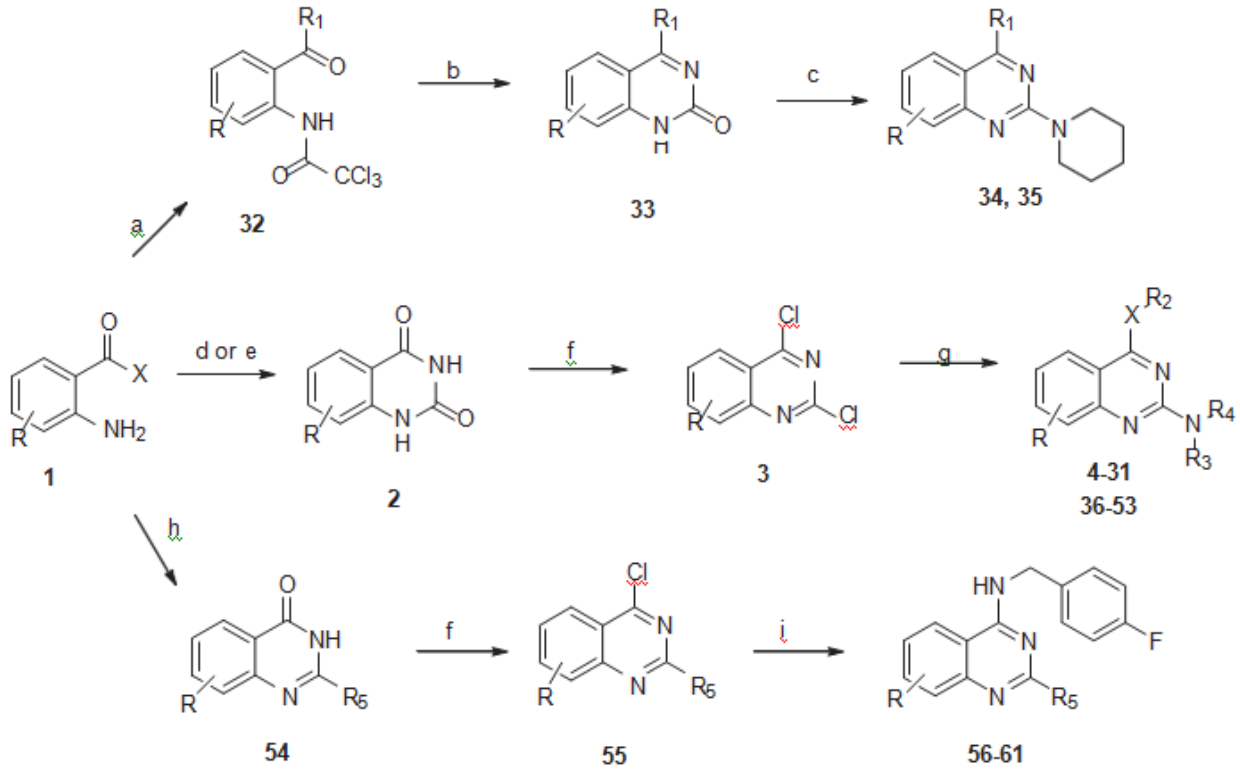
Diaminoquinazolines (DAQ) have been reported with activity against a diverse range of biological diseases including lupus, rheumatoid arthritis, malaria and hypertension.[3] The DAQ series is active against *M. tuberculosis*[4] and effective at preventing the growth of *M. tuberculosis*[5] with

minimum inhibitory concentrations (MICs) reported in the range of 1.3–6.1 **1**g/mL. The DAQ series is less effective against other bacterial species, with weak activity against *Escherichia coli* and *Pseudomonas aeruginosa*, suggesting some element of selectivity.[6]

We were interested in the potential of the DAQ series as a starting point for drug discovery. We conducted an exploratory study to evaluate the potential of the series for progression as a drug lead molecule.

II. RESULTS AND DISCUSSION

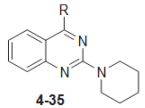
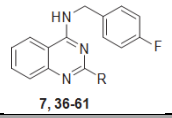
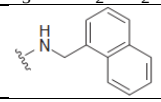
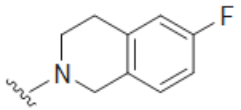
To investigate the biological activity, and the pharmaceutical and pharmacokinetic (PK) properties of the DAQ class of com- pounds, we conducted a systematic structural modification of a lead compound, *N*-(4-fluorobenzyl)-2-(piperidin-1-yl) quinazolin- 4-amine (7). Analogs with key modifications to the piperidine residue at C-2, the 4-fluorobenzylamino residue at C-4 and the quinazoline core structure were synthesized to provide structure activity relationship (SAR) information. For each compound, the biological activity (growth inhibition) was tested against *M. tuber- culosis* in liquid medium; for selected compounds activity was also tested on solid medium.



SCHEME 1. Synthesis of 2,4-substituted quinazolines. Reagents and conditions: (a) X = R_1: Cl_3CCOCl, DMAP, CH_2Cl_2; (b) NH_4OAc, DMSO; (c) (i) $POCl_3$, (ii) Piperidine, *i*-PrOH, reflux; (d) X = OH: KOCN, NaOH; (e) X = NH_2: phosgene; (f) $POCl_3$, *NN*-dimethylaniline, reflux; (g) (i) nucleophile (R_2XH: X = O,S,NH), THF, room temperature, (ii) R_3R_4NH, *i*- PrOH, reflux; (h) X = OH: R_5CONH_2, HCO_2H (i) 4-fluorobenzylamine, THF, room temperature

TABLE 1

EFFECT OF C-2 AND C-4 SUBSTITUTIONS ON BIOLOGICAL POTENCY OF DAQ

 4-35					 7, 36-61				Note
Compd	**R-group**	**MIC**	**I**		**Compd**	**R-group**	**MIC**	***I***	
4	$PhCH_2HN$-	9.2	96		34	H-	469	<30	Compounds were tested for inhibition of *M. tuberculosis* in liquid and on solid medium. The percent inhibition (*I*) of growth at 20 **1**M in liquid medium is reported. Compounds were considered inactive if %*I* <30 at 20 **1**M. Minimum inhibitory concentrations (MIC) were determined using the serial proportion method on solid agar. [a] MIC reported for solid/liquid medium. nd = not determined
5	2-$FPhCH_2HN$-	nd	97		35	4-$FPhCH_2CH_2$-	nd	<30	
6	3-$FPhCH_2HN$-	nd	98		7	Piperidin-1-yl	7.4	97	
7	4-$FPhCH_2HN$-	7.4	97		36	4-(CH_3)piperidin-1-yl	nd	97	
8	3-$MePhCH_2HN$-	nd	99		37	4-(OH)piperidin-1-yl	nd	<30	
9	3-$IPhCH_2HN$-	nd	98		38	4-(NH_2)piperidin-1-yl	nd	<30	
10	4-$MePhCH_2HN$-	nd	98		39	4-(NHMe)piperidin-1-yl	nd	33	
11	4-$MeOPhCH_2HN$-	nd	97		40	4-(NMe2)piperidin-1-yl	nd	<30	
12	4-OCF_3PhCH_2HN-	nd	95		41	4-(CO_2H)piperidin-1-yl	nd	<30	
13	4-$ClPhCH_2HN$-	nd	99		42	3,5-(Me)piperidin-1-yl	nd	<30	
14	4-CF_3PhCH_2HN-	6.6	99		43	4-(CH_2OH)piperidin-1-yl	nd	<30	
15	4-NH_2PhCH_2HN-	nd	<30		44	4-($NHCH_2CO_2H$)piperidin-1-yl	nd	<30	
16	2,4-$FPhCH_2HN$-	nd	97		45	2-(CH_2CO_2H)piperidin-1-yl	nd	<30	
17	3,4-$FPhCH_2HN$-	nd	96		46	H_2N-	93	<30	
18	2,4-$ClPhCH_2HN$-	nd	98		47	MeHN-	35	44	
19	3,4-$ClPhCH_2HN$-	nd	98		48	Me_2N-	34	40	
20	2,5-$ClPhCH_2HN$-	nd	97		49	Pyrrolidin-1-yl	31	97	
21	3-Cl, 4-$MeOPhCH_2HN$-	nd	98		50	Isoindolin-2-yl	nd	78	
22	4-$FPhCH_2O$-	296	<30		51	Piperazinyl	148	<30	
23	4-$FPhCH_2S$-	282	<30		52	4-(4-Anilinyl)piperazin-1-yl	nd	94	
24	MeHN-	206	<30		53	$(HOCH_2CH_2)_2HN$-	nd	<30	
25	*i*PrHN-	25	37		56	(Piperidin-1-yl)CH_2HN-	29	<30	
26	cyclohexylCH_2HN-	nd	99		57	H-	99	<30	
27	4-$CF_3PhCH_2CH_2HN$-	5.7	97		58	Me-	94	<30	
		nd	97		59	F_3C-	nd	<30	
28		39	<30		60	Ph-	76	<30	
29	4-FPhHN-								
30	Piperidin-1-yl	nd	<30		61	Cyclohexyl-	15	47	
31		Nd	94		Rifampicin[a]		0.013/0.004	100	

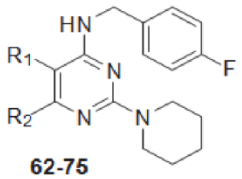
2.1 Exploration of C2 and C-4 substitutions

Several 2,4-diaminoquinazolines incorporating a single-point variation at C-2-position (4–31) or C-4-position (36–53) of the qui- nazoline template of the reference structure (7) were readily syn- thesized according to Scheme 1, by the nucleophilic aromatic substitution reaction on a key 2,4-dichloroquinazoline intermedi- ate (3). The dichloroquinazolines were either purchased or pre- pared from commercial quinazoline-2,4(1*H*,3*H*)-dione (2) by chlorination with phosphorus oxychloride in *N*,*N*-dimethylaniline under reflux conditions. Where necessary, the quinazolinediones were prepared from corresponding anthranilamides (1) via condensation with phosgene. Regioselective substitution of C-4 chloride in 3 with the appropriate amine was accomplished at room temperature. Nucleophilic substitution at C-2 with the second amine proceeded smoothly at higher temperatures in isopropanol or tetrahydrofuran to give target compounds (4–31, 36–53).[7] The C-4 ether, 4-(4-fluorobenzyloxy)-2-(piperidin-1-yl) quinazoline (22) and the thioether, 4-(4-fluorobenzylthio)-2-(piperidin-1-yl) quinazoline (23) were similarly prepared utilizing the corresponding sodium salt of benzyl alcohol or thiol as nucleophiles. Alternative routes were adapted to incorporate different alkyl and aryl substit- uents at these positions. The preparation of analogs 56–61, bearing aliphatic or aromatic substituents at C-2 began with the condensa- tion of anthranilamide with appropriate aldehydes, followed by chlorination of the resulting 2-substituted quinazolin-4(3*H*)-one(54) with phosphorus oxychloride to give key intermediates[8,9] (55), which were readily converted to the 4-amino analogs 56–61. The synthesis of analogs 34 and 35 bearing alkyl group at C-4 were carried out using 2-aminophenones[10] (1, X = alkyl) as the starting point for similar condensation, chlorination and animation sequence of reactions.

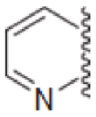
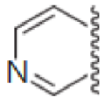
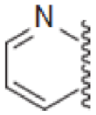
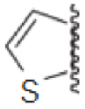
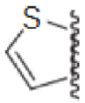
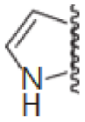
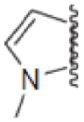
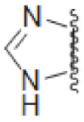
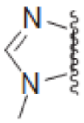
The biological activity for each compound was determined by measuring inhibition of growth against a virulent strain of *M. tuberculosis* in liquid medium (Table 1–3). In liquid medium none of the compounds gave an MIC <20 **1**M, although most com- pounds had activity, that is, >30% inhibition of growth at 20 **1**M and could be ranked loosely based on this value. Several compounds were tested for MIC on solid medium; surprisingly MICs were lower than for liquid medium for several of the compounds (4, 7, 14, 27), but a large variation was seen which was used to inform the SAR analysis.

Aromatic substituents on the benzylamino residue had very little or no influence on activity; analogs incorporating various sub- stituents (F, Cl, Br, Me, OMe, NH_2, CF_3, OCF_3) (5–21) all showed comparable activities. However, the lipophilicity of the C-4 substi- tuent seemed important as replacement of the benzyl with simple groups such as hydrogen (34), methyl (24) or an isopropyl (25) group, resulted in substantial reduction in the inhibitory activity. On the other hand, similar lipophilic groups like the non-aromatic cyclohexylmethyl (26) displayed good activity. The importance of the amino function at C-4 was examined by replacement with an ether (22), thioether (23) and methylene (35) units, all of which resulted in complete loss of activity. The activities of a secondary (tetrahydroisoquinoline, 31) and primary C-4 amine analogs were comparable, precluding significance of any hydrogen-bonding contribution to activity. Homologation of the C-4 benzylamin group to a phenethylamino moiety (27) resulted in a very small improvement in the inhibitory activity. The aniline derivative (29) lacking any methylene spacer between the core and the lipophilic group was relatively less active.

TABLE 2
EFFECT OF CORE REPLACEMENT ON BIOLOGICAL POTENCY



62-75

Compound	R1, R2	MIC	I
Rifampicin[a]		0.013 / 0.004	100
62	H, Me	83	<30
63	Me, H	83	<30
64	Me, Me	nd	<30
65	Benzyl, H	26	68
66	H, Benzyl	27	84
67		74	37
68		30	59
69		37	<30
70		73	nd
71		292	<30
72		nd	<30
73		nd	<30
74		nd	<30
75		nd	<30

Compounds were tested for inhibition of M. tuberculosis in liquid and on solid medium. The percent inhibition (I) of growth at 20 μM in liquid medium is reported. Compounds were considered inactive if %I <30 at 20 μM. Minimum inhibitory concentrations (MIC) were determined using the serial proportion method on solid agar.

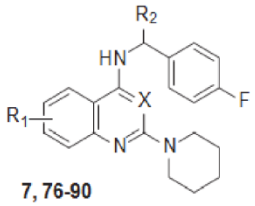
[a] MIC reported for solid/liquid medium. Nd = not determined.

2.2 Exploration of the core

We explored the core quinazoline structure through the synthesis of analogs outlined in Schemes 2–4. All azaquinazoline analogs (67–69) reported were easily prepared from their corresponding 2,4-dichlorides in good yields. We were, however, unable to prepare and isolate the 6-aza analog. Similarly, the thieno[2,3- *d*] pyrimidine (70, 71), pyrrolo[2,3-*d*]pyrimidine (72, 73) and purine (74, 75) analogs were prepared from corresponding dihalides. Other quinazoline core replacement prepared include the quinoline analogs (88, 89, 90) which were similarly prepared from 2,4-dichloro- quinoline. The synthesis of pyrazolo[1,5-*a*]pyrimidine (94) and triazolo[1.5-1]pyrimidine (95) analogs were achieved via a sequential diamination of their respective key dihalide (93)[11] intermediates as illustrated in Scheme 3.

TABLE 3

EFFECT OF CORE SUBSTITUTIONS ON BIOLOGICAL POTENCY



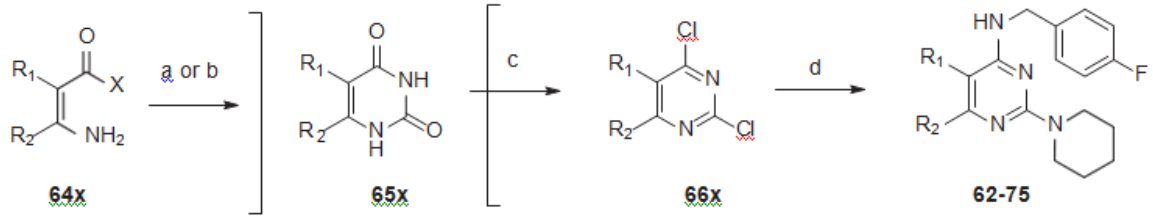
7, 76-90

Compound	R1	R2	X	MIC	*I*
Rifampicin				0.013 / 0.004[a]	100
7	H	H	N	7.4	97
76	5-F	H	N	nd	<30
77	8-NO_2	H	N	nd	<30
78	5-Cl	H	N	nd	<30
79	6-Cl	H	N	nd	49
80	7-Cl	H	N	nd	51
81	8-Cl	H	N	nd	<30
82	5-Me	H	N	nd	98
83	6-Me	H	N	nd	99
84	6-OMe	H	N	nd	97
85	7-OMe	H	N	nd	74
86	8-OMe	H	N	nd	83
87	6,7-OMe	H	N	25	92
88	H	H	C	nd	98
89	H	CH_2OH	C	nd	55
90	H	CO_2H	C	nd	<30
94	–	–	–	–	<30
95	–	–	–	–	<30
103	–	–	–	27.7 / 13.8[a]	98

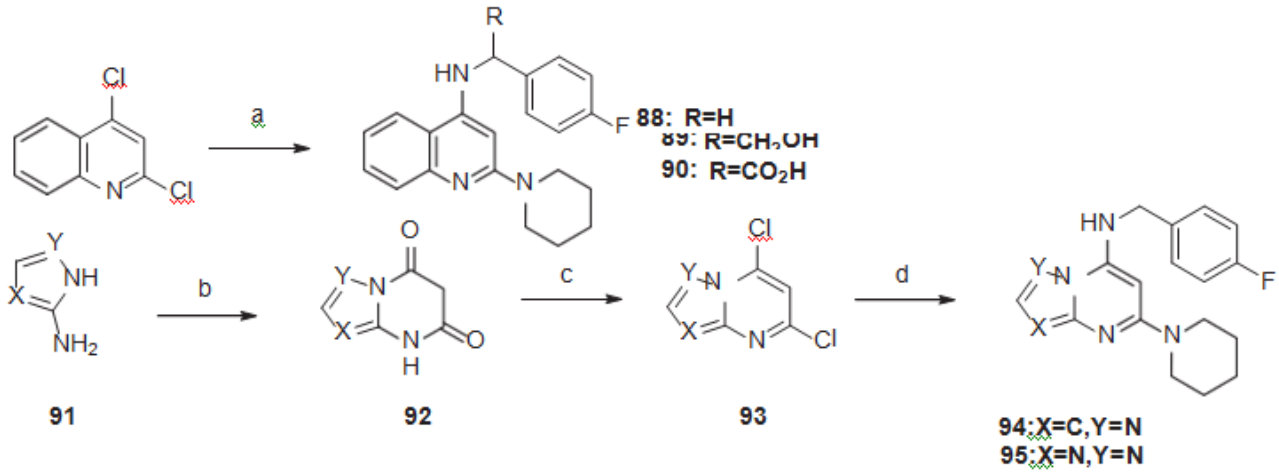
Compounds were tested for inhibition of M. tuberculosis in liquid and on solid medium. The percent inhibition (I) of growth at 20 μM in liquid medium is reported. Compounds were considered inactive if %I <30 at 20 μM. Minimum inhibitory concentrations (MIC) were determined using the serial proportion method on solid agar.

[a] MIC reported for solid/liquid medium. nd = not determined.

The potency of each compound was assessed by growth inhibition against a virulent strain of *M. tuberculosis* in agar and liquid medium (Table 2). Compounds had activity in both liquid and solid medium. Among the various C-2 substituents synthesized, piperidine remained a preferred residue (7) at this position. Pyrrolidine homologs (49, 50) were also accommodated with no loss in activity. Piperazine substitution (51) resulted in complete loss of activity, but all activity was regained with an N-4^{0} -aromatic substituted piperazine (52). Smaller, non-cyclic amines (46–48, 53) showed greater than two-fold reduction in activity. Non-amine substitution such as alkyls or aryls (56–61) resulted in loss of activity with cyclohexyl analog (61) showing the least, a two-fold, reduction. We also investigated variously substituted piperidines (36–45) at the C-2 position for SAR exploration and for physic chemical property modulation. In general, substitution on the C-2 piperidine was detrimental to anti-TB activity.



SCHEME 2. Synthesis of DAQ derivatives with variation in the benzo region. Reagents and conditions: (a) 64x, X = OH: KOCN, NaOH; (b) 64x, X = NH_2: phosgene; (c) $POCl_3$, *N*,*N*-dimethylaniline, reflux; (d) (i) 4-fluorobenzylamine, THF, room temperature, (ii) piperidine, *i*-PrOH, reflux.



SCHEME 3. Synthesis of DAQ derivatives with variation in the core. Reagents and conditions: (a) (i) piperidine, 75 °C, (ii) benzylamine, $Pd_2(dba)_3$, BINAP, *t*-BuONa, toluene, reflux (b) $CH_2(CO_2Et)_2$, EtONa, EtOH, heat; (c) $POCl_3$, *NN*-dimethylaniline, reflux; (d) (i) a benzylamine, *n*-BuOH, Et_3N, 110 °C, (ii) piperidine, 180 °C.

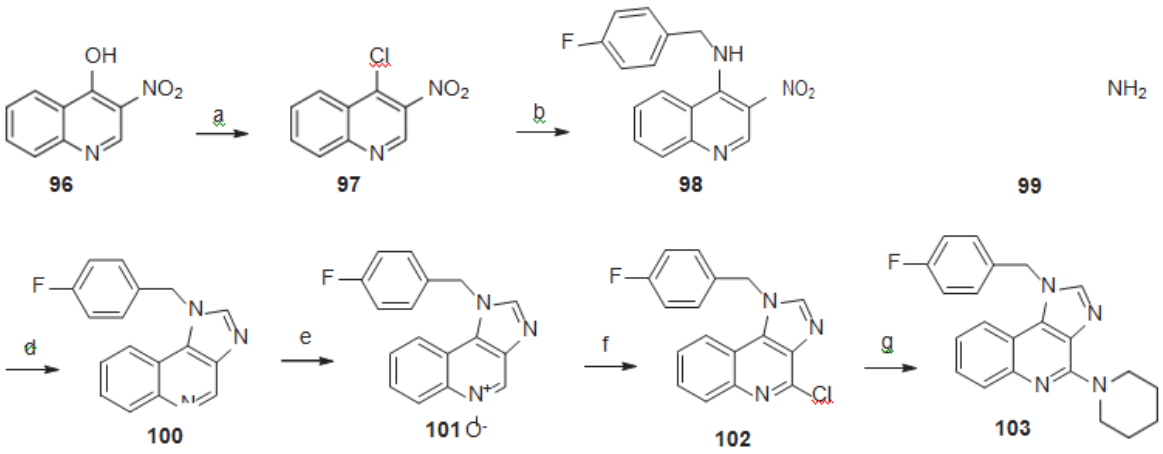
The results also indicated that the benzo portion of this core is important to anti-TB activity. This was inferred from the very weak biological activity of core replacement analogs such as the substituted pyrimidine derivatives (62–66), thiophenopyrimidines (70–71), the diazaindoles (72–73) and purine analogs (74, 75). A change to an azaquinazoline core (67–69) was tolerated but with relatively weaker activity compared to the parent quinazoline. To explore the role of the quinazoline ring nitrogens, we prepared and tested the analogous quinoline analogs (88–90). The retention of activity for this core indicated a non-essentiality of N-atom at N-3 position which

prompted us to investigate other analogs based on this core. The pyrazolo[1,5-*a*]pyrimidine (94) and triazolo[1.5-1]pyrimidine (95) analogs, synthesized as representative extension of this core were essentially inactive but an imidazoquinoline derivative (103) displayed moderate activity.

Substitutions at the C-5, C-6, C-7, and C-8 positions of the quinazoline core followed a general pattern in which electron-donating substituents (82–87) retained activity while electron-withdrawing groups had a negative impact (76–81).

2.3 Other analogs

We prepared a number of scaffold analogs that incorporated changes at N-atom at position-3. The imidazoquinoline derivative (103) was prepared from commercial 3-nitroquinolin-4-ol (96) according to Scheme 4.[12] Chlorination of 96 with phosphorus oxychloride in *N*,*N*-dimethylformamide gave 3-nitro-4-chloroquinoline (97) which was subsequently subjected to nucleophilic substitution of the chloride by 4-fluorobenzylamine to furnish *N*-(4-fluorobenzyl)-3-nitroquinolin-4-amine (98). Catalytic reduction of 98 led to the diamine derivative 99, a precursor to the imidazoquinoline (100). The imidazoquinoline, prepared via condensation of 99 with refluxing triethyl orthoformate, was oxidized with peracetic acid to its N-oxide (101), and then chlorinated to provide the chloroimi- dazoquinoline (102). Finally, 102 was refluxed with piperidine in *N*,*N*-dimethylformamide to provide the target compound, 1-(4-fluorobenzyl)-4-(piperidin-1-yl)-1*H*-imidazo[4,5-*c*] quinoline (103).



SCHEME 4. Synthesis of an imidazoquinoline derivative. Reagents and conditions: (a) $POCl_3$, DMF, heat; (b) 4-fluorobenzylamine, EtOH, Et_3N; (c) 5% Pt/C, H_2, ethyl acetate; (d) $HC(OEt)_3$, toluene, reflux; (e) CH_3CO_3H, CH_2Cl_2, heat; (f) $POCl_3$, CH_2Cl_2, reflux; (g) piperidine, DMF, Et_3N, reflux.

2.4 The DAQ series show bactericidal activity

In order to probe the biological profile of the DAQ series, we conducted a number of additional microbiological assays. We selected compound 14 since it had relatively good activity in solid assays with an MIC of 6.6 **1**M. The most active compound (14) was tested for bactericidal activity in aerobic, replicating culture and under non-replicating conditions generated by nutrient starvation (Fig. 1). Interestingly, compound 14 was bactericidal against M. tuberculosis under both conditions. In aerobic culture a loss of 4 log viability was seen over 7d and similarly 3 logs of kill was seen over 7 days under starvation conditions.

FIGURE 1. The representative DAQ exhibits bactericidal activity. Kill kinetics of *M. tuberculosis* by 14 in aerobically growing cells (squares) and under starvation conditions (triangles). For the aerobic kill curve, cells were inoculated to a starting OD_{590} of 0.01 into 7H9/OADC containing 124 ΙM of 14 and CFU/mL were enumerated at indicated time points. For the starvation-kill curve, cells were harvested at mid-log phase, re-suspended in phosphate buffered saline to an OD_{590} of 0.01 and maintained for 2 weeks at 37 °C. Compound 14 was added (124 ΙM) and CFU/mL determined at indicated time points following compound addition. The limit of detection was 10, as indicated by the dotted line

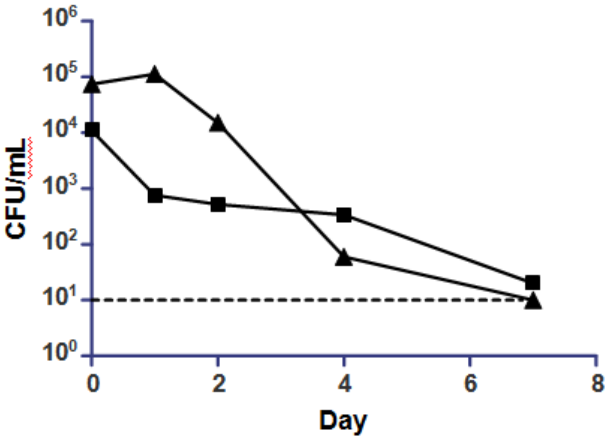
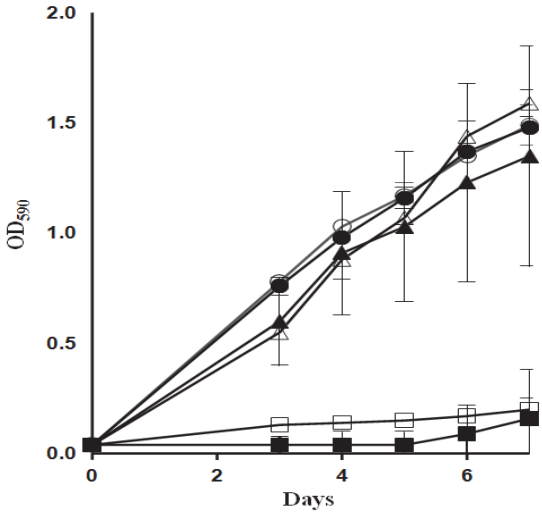


FIGURE 2. DAQ inhibit growth of *M. tuberculosis* under iron-excess conditions. Cells were inoculated to a starting OD_{590} of 0.04 into growth medium containing no compound 14 (circles), 28 mM compound 14 (triangles) and 56 mM compound 14 (squares). Growth was monitored in the presence (solid symbols) and absence (open symbols) of 40 mg/mL hemin supplementation.



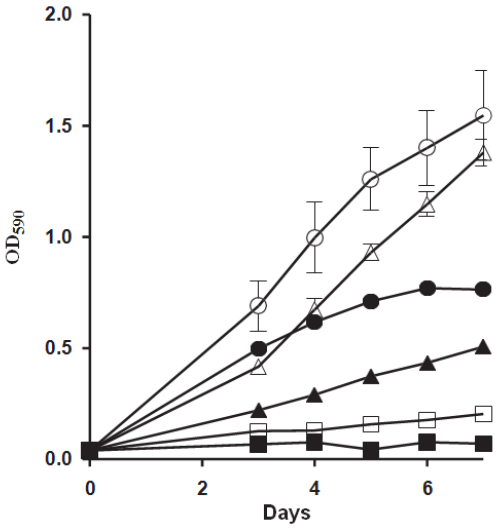


FIGURE 3. DAQ inhibition of *M. tuberculosis* is not dependent on carbon source. Cells were inoculated to a starting OD_{590} of 0.04 into growth medium containing no compound 14 (circles), 14 mM compound 14 (triangles) and 28 mM compound 14 (squares). Growth in medium containing palmitic acid (solid symbols) was compared to that containing OADC (open symbols) supplement

TABLE 4
PHARMACOKINETIC PARAMETERS FOR DAQS FOLLOWING A SINGLE 10 MG/KG ORAL GAVAGE DOSE IN RATS

Compd	MW	*C* Log *P* (day)	NV PSA	VDss (L/kg)	CL (mL/min/kg)	PO AUC (ng*hr/mL)	Bioavail (%F)
7	336.41	5.05	41.1	12.2 ± 2.7	115 ± 21	245 ± 66	16.7 ± 3.3
21	382.89	5.45	50.3	9.3 ± 1.0	133 ± 16	132 ± 20	10.3 ± 1.3
24	242.32	3.46	41.1	5.7 ± 0.6	179 ± 13	209 ± 134	21.8 ± 13.0
34	213.28	2.65	29	4.5 ± 0.2	153 ± 9	196 ± 82	17.6 ± 6.3
36	350.44	5.57	41.1	25.7 ± 18.3	179 ± 79	171 ± 32	21.7 ± 14.0
44	409.46	0.606	90.4	5.0 ± 1.2	99.2 ± 18.7	0.84 ± 0.89	0.05 ± 0.05
46	268.29	3.46	63.8	13.3 ± 0.2	154 ± 25	376 ± 48	34.0 ± 0.7
47	282.32	4.29	49.8	9.2 ± 1.0	95.3 ± 7.7	1610 ± 247	92.2 ± 18.9
48	296.35	4.38	41.1	8.1 ± 1.0	85.5 ± 11.6	1310 ± 339	65.9 ± 12.4
62	300.38	4.17	41.1	12.8 ± 0.9	131 ± 21	82 ± 16	6.4 ± 0.3
103	360.43	5.05	34	14.2 ± 6.2	201 ± 26	27.4 ± 6.5	3.4 ± 1.0

Compounds were administered to rats with a single oral dose of 10 mg/kg. Concentrations in plasma were determined at various time points over 24 h and pharmacokinetic parameters calculated using Watson (version 7.4; Thermo Fisher Scientific). Clearance (CL), volume of distribution (VDss), area-under-the-curve (PO AUC) and bioavailability (%F) are given. Molecular weight (MW); Calculated partition coefficient (C log P, daylight software); Topological polar surface area (TPSA, novartis software) are also given.

2.5 Lack of iron chelating activity

The amine at the C4 position has the potential to form a chelating center with the N3 of the core, although it would be weak. Therefore, we considered the possibility that the compound might act via chelation of iron in cultures leading to an intracellular iron shortage. To test this possibility, we monitored growth in liquid in the presence of hemin to supply additional iron to the cells, since we already knew that hemin was transported into *M. tuberculosis*.[13]

Under these conditions 56 **1**M of compound 14 was sufficient to prevent growth completely, regardless of hemin supplementation (Fig. 2). These data suggest that it is unlikely that the compound acts via iron chelation, in agreement with the structural features of 14 which are devoid of any signature chelation motifs.

2.6 Carbon source dependency

Previously published data suggested that the DAQ series as a class of compounds had significantly lower MICs in liquid medium than those we found.[5] One of the major differences in the method-ology for determining MIC was the carbon source in the growth medium, in our case the simple sugar glucose, as opposed to the fatty acid palmitate (hexadecanoate). Since the catabolic pathways for utilizing these carbon sources differ, it is possible this altered metabolism might be reflected in different MICs. We determined whether compound potency was carbon source-dependent using the same strain of *M. tuberculosis*. Growth was monitored in liquid medium and the MIC was determined on solid medium. No difference was seen, with the liquid MIC of 28 **1**M in both growth media, and the solid MIC of 16 **1**M (Fig. 3). Thus we discounted the possi- bility that the potency of the compound series is dependent on the carbon source.

2.7 Evaluation of pharmacokinetic properties in rats

To determine the in vivo exposure a set of twelve compounds was selected and evaluated for pharmacokinetic (PK) properties in rats (Table 4). The selection was guided by a multiparameter analysis of all compounds. Calculated compound properties taken into consideration included lipophilicity (*C* log *P*), distribution coefficient values at pH = 7.4 (*C* log *D*), molecular weight

(MW), topological polar surface area (TPSA), number of hydrogen bond donors (HBD) and acidity/basicity parameter (pK_a).

Sprague-Dawley rats were administered a single dose of the test compound either iv at 1 mg/kg or orally at 10 mg/kg (Table 4). The volume of distribution was generally high for all compounds. Plasma clearance was rapid for all compounds and the absolute oral bioavailability was moderate (10–35%) for most compounds tested. However, two structurally similar analogs, 47 and 48, were well-absorbed with bioavailability of 92% and 66% (%*F*), respectively. The carboxylate analog, 44, registered the poorest oral expo- sure, presumably due to slow, passive permeability as a result of the high polarity conferred by the amino carboxylate and a drastically reduced lipophilicity ($C \log P = 0.6$).

2.8 Resistant mutant isolation for target identification studies

Drug resistance in *M. tuberculosis* is largely mediated by chromosomal mutations. For example, mutations may modify the drug target, inactivate bacterial enzymes that activate pro-drugs, lead to decreased membrane permeability, or up-regulation of efflux pumps. To ascertain the mechanism(s) responsible for resistance to 14 we isolated spontaneous resistant mutants to this compound and analyzed 3 resistant mutants by whole genome sequencing. Resistant mutants were isolated which showed a four-fold shift towards resistance to compound 14. We sequenced the entire gen- ome in order to identify single nucleotide polymorphisms. In all three cases, mutations were observed in Rv3161c, a non-essential and poorly characterized gene predicted to encode a dioxygenase (Table 5). We observed an insertion of the transposable IS*6110* element and a 2 base pair deletion which should both result in gene inactivation. The third mutation results in the substitution of a cysteine residue at position 115 with tryptophan. This non-conservative amino acid substitution is also likely to inactivate the resultant protein product. Our findings are consistent with compound 14 functioning as a pro-drug in wild-type *M. tuberculosis*. Since Rv3161c is not essential, it seems unlikely this is the target. We propose that Rv3161c is able to modify the DAQ compounds and that the resulting metabolite is the active species. Inactivation of Rv3161c would thus lead to resistance.

TABLE 5
DAQ RESISTANCE RESULTS FROM MUTATIONS IN THE PUTATIVE DIOXYGENASE, RV3161C

Strain	Compound 14 solid MIC (1M)	Rv3161c mutation
H37Rv	6.6	N/A
RM1	25	IS6110 at nt 801
RM2	25	GT deletion at 175-176
RM7	25	C115 W

Comparison of wild-type (H37RvLP) and 3 spontaneous resistant mutants to compound 14. Solid MIC (mM) values to Compound 14 are indicated. Whole genome sequencing indicated that all three resistant strains contain mutations in Rv3161c as shown. RM: resistant mutant. N/A: not applicable. nt: nucleotide.

III. CONCLUSION

Based on previous reports of sub-micromolar MIC activity of the DAQ series in TB assays, the main goal of our work was to deter- mine whether the series is a viable starting point for drug lead development. Surprisingly, we found the activity of the series in liquid medium was limited and anti-tubercular activity was weaker than previously reported. We hypothesized that this dis- parity was a result of variation in assay conditions, the most noticeable difference being a change in carbon source. However, we saw no improvement in anti-tubercular activity of these compounds when tested using palmitate as a carbon source. In previous work, cell viability was determined by metabolic activity (using Alamar blue), whereas we measured bacterial growth (by OD and fluorescence). This may be indicative of mode of action, since compounds might be able to reduce metabolic activity more efficiently than prevent growth.

However, good activity was seen on solid medium for these compounds where the MICs were lower than in liquid medium (even using the same carbon source, glucose). This was surprising, since in our experience MICs on solid medium are normally higher by several-fold. This may reflect a true difference in bacterial physiology or metabolism between the two states. Culture on solid medium may reflect better the physiological state of bacteria dur- ing infection, particularly in granulomas or in biofilms.

We conducted a systematic exploration of a diaminoquinazoline compound series for inhibitory activity against *M. tuberculosis*. SAR efforts around N-3 replacements led to a fused ring in the form of an imidazoquinoline (103) with an improvement in biological activity and an opportunity for SAR development on an alternate scaffold. PK evaluation indicated there is no obvious barrier to property optimization. Our data demonstrated that the DAQ series had an encouraging microbiological profile with bactericidal activity against replicating and non-replicating *M. tuberculosis*, suggesting that the target of the series is important for bacterial viability. Future work to identify the target could allow screening for alter- native scaffolds with improved properties and biological activity. These properties may have implications in the application of these compounds as anti-TB agents. In addition, the DAQ series could be useful tools for probing cell death mechanisms and for target identification studies.

IV. EXPERIMENTAL SECTION

4.1 Determination of minimum inhibitory concentration (MIC)

M. tuberculosis H37Rv was grown in Middlebrook 7H9 medium containing 10% OADC (oleic acid, albumin, dextrose, catalase) supplement (Becton Dickinson) and 0.05% w/v Tween 80 (7H9-Tw-OADC). Liquid MICs were performed in 96-well plates as described.[14] Briefly, compounds were solubilized in DMSO and assayed using a 10-point two-fold serial dilution with the highest concentration of 20 **1**M. Bacterial growth was measured by OD after 5 days of incubation at 37 °C; growth curves were fitted to the Gompertz model. The MIC was defined as the minimum con- centration required for complete growth inhibition. In order to generate an MIC, two points of complete inhibition were required; where MICs could not be calculated the % inhibition of growth at 20 **1**M was recorded. Inhibition of >30% was recorded as active, <30% was recorded as inactive. Data are from one run. The assay was validated according to NCGC guidelines with reproducibility between runs of <2-fold for MIC values.[14] MIC99 was determined on solid medium

(Middlebrook 7H10 plus 10% v/v OADC) using the serial proportion method[15]: MIC was defined as the minimum concentration required to prevent 99% of growth.

4.2 Kill kinetics

For aerobic kill curves, cells were inoculated to a theoretical OD_{590} of 0.01 (theoretical) in 10 mL medium containing Compound 14. For the starvation-kill determinations, 10 mL of culture was grown to mid-log phase, harvested by centrifugation for 10 min at 4000 rpm, and re-suspended in phosphate buffered saline. Cells were maintained as standing cultures at 37 °C for 2 weeks prior to the addition of compound to ensure that cells had adapted to the non-replicating state. Colony forming units were enumerated by plating dilutions of cells onto compound free plates at the indicated time points.

4.3 Growth curves

Growth curves were carried out in 16 mm diameter glass tubes with 2 mm stirrer bars containing 5 mL of 7H9-Tw-OADC medium plus 40 lg/mL hemin where required; 2 tubes were inoculated to a theoretical OD_{590} of 0.04. Cultures were incubated at 37 °C with stirring. For studies using palmitate as a carbon source, OADC and Tween 80 were omitted and medium was supplemented with 5 g/L BSA fraction V, 0.8 g/L NaCl, 0.05% v/v Tyloxapol and 0.25 mM palmitic acid.

4.4 Pharmacokinetics

Male Sprague-Dawley rats (250–350 g) were purchased from Harlan (Indianapolis, IN). The Institutional Animal Care and Use Committees at Covance and Harlan approved all animal procedures. Three rats were dosed per treatment group. Plasma samples were collected over 24 h in an intravenous/oral crossover study with a 1 day washout period between drug administrations. On day 0, compound was administered by intravenous bolus injection (1 mg/kg; 2 ml/kg, Captisol 20% w/v/$NaPO_4$ buffer 25 mM, pH2, q.s) and on day 1 by oral gavage (10 mg/kg; 10 mL/kg HEC 1% w/v/P80 0.25% v/v/AF 1510-US 0.05% v/v/DIW, q.s.). Arterial blood samples were collected at the following times after dose administration: 0 (oral arm only), 0.08 (intravenous arm only), 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h.

Compound concentrations in plasma were quantified by LC-MS/ MS. All samples were mixed with an organic internal standard solution to precipitate protein and centrifuged. The resulting supernatants were analyzed. Analytes were separated using a Betasil C18 (2.1 20 mm, 5 lm; Thermo Fisher Scientific, Waltham, MA) with gradient elution. All analytes were detected in positive ion mode reaction monitoring (Sciex API 4000 triple quadrupole mass spectrometer equipped with a TurboIonSpray interface; Applied Biosystems/MDS, Foster City, CA): The dynamic range of the assays was 1 to either 1250 or 5000 ng/mL for all analytes. Samples with analyte concentrations above the upper limit of quantification were diluted with matrix to within the assay range; concentrations below the lower limit of quantification (BQL) were reported as BQL. All bioanalytical assays met acceptance criteria for accuracy (<±30% relative error) and precision (<30% relative SD). Noncompartmental pharmacokinetic parameters were calculated using Watson (version 7.4; Thermo Fisher Scientific).

4.5 Resistant mutant isolation and characterization

Resistant mutants were isolated by plating 10^7, 10^8, or 10^9 bacteria onto agar plates containing 5X and 10X the solid MIC of compound. Resistant colonies were streaked onto plates contain- ing compound and MICs were determined on solid medium to con- firm resistance. Genomic DNA was prepared[16] and whole genome sequencing performed17; mutations were confirmed by PCR and sequencing.

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