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Aminopyrazinamides: Novel and Specific GyrB Inhibitors that Kill Replicating and Nonreplicating *Mycobacterium tuberculosis*

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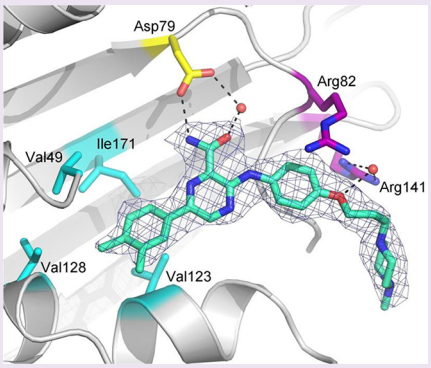
Supporting Information

ABSTRACT: Aminopyrazinamides originated from a high throughput screen targeting the *Mycobacterium smegmatis* (Msm) GyrB ATPase. This series displays chemical tractability, robust structure−activity relationship, and potent antitubercular activity. The crystal structure of Msm GyrB in complex with one of the aminopyrazinamides revealed promising attributes of specificity against other broad spectrum pathogens and selectivity against eukaryotic kinases due to novel interactions at hydrophobic pocket, unlike other known GyrB inhibitors. The aminopyrazinamides display excellent mycobacterial kill under *in vitro*, intracellular, and hypoxic conditions.



Tuberculosis caused by *Mycobacterium tuberculosis* (Mtb) is a leading cause of death due to an infectious agent claiming 1.4 million lives annually.[1] The emergence of multidrug resistant tuberculosis (MDR-TB) and extremely drug resistant tuberculosis (XDR-TB) highlights the need to discover drugs with novel mechanisms of action.[2] The current treatment for drug-susceptible tuberculosis is a four-drug regimen administered for six months. Each of these drugs is more than 40 years old.[3] It is believed that, for a new TB agent to contribute to treatment shortening, it must demonstrate an ability to kill replicating and nonreplicating Mtb.[4,5]

DNA Gyrase is a clinically validated target that catalyzes the essential function of introducing negative supercoils in an ATP-dependent manner.[6,7] The enzyme is a heterotetrameric A_2B_2 protein. Negatively supercoiled DNA is crucial for cellular processes including DNA replication, transcription, and recombination. The druggability of DNA gyrase has been well established through the development of the fluoroquinolone class of antibacterial agents.[8] The enzyme is present in all prokaryotes and is the only type II topoisomerase enzyme in Mtb. This lack of redundancy makes it an attractive target for discovering novel drugs against tuberculosis (TB). While the cleavage-reunion domains of GyrA and GyrB are the target of fluoroquinolones, the GyrB ATPase domain has not been explored extensively for anti-TB drug discovery. Hence, GyrB is an attractive target for developing inhibitors against drug resistant Mtb including clinical isolates resistant to fluoroquinolones.[9,10] This study describes the identification and optimization of a novel class of aminopyrazinamides, which display unique binding interactions at the GyrB ATPase site resulting in selective inhibition of *mycobacterium* GyrB.

Aminopyrazinamides were identified from a high throughput screen (HTS) of the AstraZeneca (AZ) compound collection against *Mycobacterium smegmatis* (Msm) GyrB ATPase (Supporting Information). The initial hit **1** (Table 1) displayed moderate enzyme activity as measured by inhibition of the target enzyme to 50% (IC_{50}) as well as Mtb growth inhibition as measured by minimum inhibitory concentration (MIC). We established the fundamental structure−activity relationship (SAR) by improving the IC_{50} against the target enzyme resulting generally in potent Mtb MICs. A better understanding of SAR would help determine if these GyrB inhibitors bind in a similar manner to the ATPase domain as novobiocin or benzimidazole ureas.[11−13] Three diversification points for SAR were identified for the aminopyrazinamides, namely, site-1, site-2, and the hydrophobic pocket (Figure 1). The primary amide is an essential group at site-1, which is thought to involve a water-mediated hydrogen bonding interaction with aspartic acid (Asp79). The corresponding ester (**2**), acid (**3**), and secondary amide (**4**) failed to display enzymatic inhibition with

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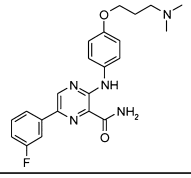
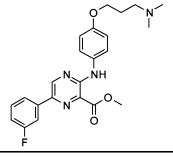
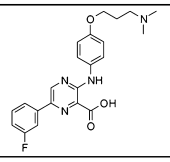
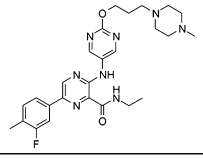
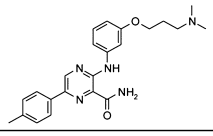
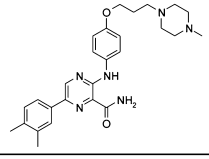
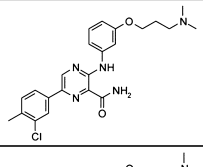
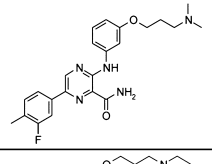
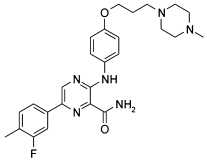
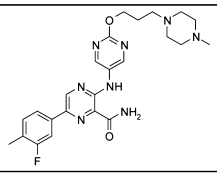
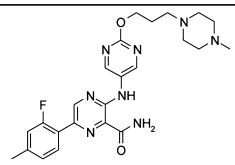
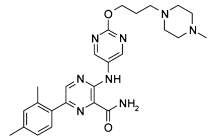
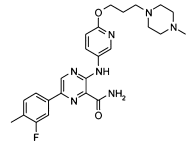
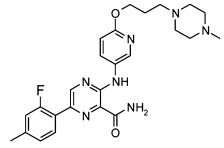
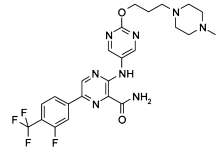
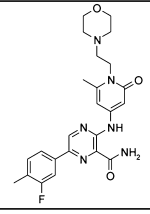
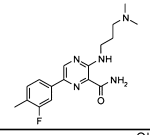
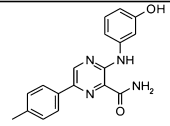
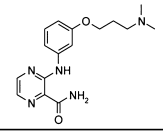
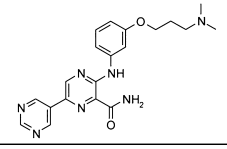
Table 1. Aminopyrazinamides are Potent Inhibitors of *M. smegmatis* GyrB and Inhibit the Growth of Replicating *M. tuberculosis* in Broth

No.	Structure	GyrB IC_{50} (μM)	Mtb MIC (μg/ml)
1		1.7	16
2		>50	32
3		>50	>32
4		>50	16
5		0.229	4
6		0.69	1
7		0.28	2
8		0.029	0.70
9		0.042	0.5
10		0.009	0.5

No.	Structure	GyrB IC_{50} (μM)	Mtb MIC (μg/ml)
11		<0.002	0.5
12		0.010	1
13		0.006	0.5
14		0.02	<0.5
15		<0.002	<0.5
16		0.022	2
17		>50	>32
18		0.362	>32
19		>50	>32
20		>50	>32

weak or no Mtb MICs. On the basis of the known benzimidazole ureas,[11−13] we believe that the aryl or heteroaryl groups at site-2 (**5**−**16**) may be involved in cation-pi interactions with a conserved arginine residue, while a second

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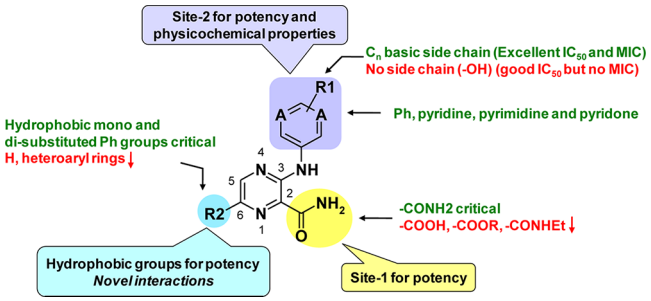

Figure 1. Structure–activity relationship of aminopyrazinamides.

arginine residue may be involved in hydrogen bonding interactions with either the ether O of the basic side chain or an aryl ring N (**1**, **10**–**15**). This was supported by the loss of enzymatic and cellular potency for compound **17**, which lacks an aryl group. The basic side chain attached to the aryl or heteroaryl ring at site-2 was found to be essential for cellular potency, as its removal resulted in a dramatic loss of Mtb MIC despite a sub micromolar IC_{50} (**18**). These predicted interactions at site-1 and site-2 are conserved across known GyrB inhibitors.[11−13] The substituted phenyl ring at C-6 of the pyrazine ring is required for enzymatic activity. The disubstituted phenyl ring shows improved potency compared to the monosubstituted phenyl ring (**5** vs **8**).

Conversely, the removal of the phenyl ring or introduction of heteroaryl rings at C-6 resulted in complete loss of IC_{50} and Mtb MIC (**19**–**20**) at the highest concentration tested. We believe this novel hydrophobic interaction is unique to this scaffold and has not been reported for other GyrB inhibitors. Overall, the aminopyrazinamide series demonstrates a good correlation between GyrB IC_{50} and Mtb MIC (Figure S1, Supporting Information).

Furthermore, to confirm the above hypothesized interactions, a parallel strategy was devised to understand key interactions at the enzyme active site with the aid of X-ray crystallography. The crystal structure of a 22 kDa fragment of the N-terminal domain of Msm GyrB in complex with compound **6** confirms the two key interactions at site-1 and site-2 (Figure 2). The primary carboxamide at site-1 makes two hydrogen bonds with Asp79: one directly and a second *via* water. The phenyl group at site-2 forms pi stacking interactions with Arg82, while the ether O of the basic side chain makes a water-mediated hydrogen bond with the side chain of Arg141. The *N*-methyl piperazine-propoxy group at C-4 of the phenyl ring orients toward the solvent. The crystal structure also provided evidence of additional novel interactions involving a hydrophobic pocket. The 3,4-dimethyl phenyl group is seen to be oriented into a hydrophobic pocket beneath the active site loop (Figure 2), thus explaining the SAR at this position. Overlay of GyrB structures from other isozymes onto the structure of Msm GyrB in complex with compound **6** suggests this unique hydrophobic pocket may account for the specificity observed at the enzyme level (Figure S2, Supporting Information). This is likely due to steric interference of the hydrophobic substituent with the amino acid residues in this part of the active site of other GyrB isozymes. We hypothesize the hydrophobic substituent also hinders binding to the ATP pocket of eukaryotic kinases, thus minimizing potential off target activity (Figure S3 and Table S1, Supporting Information). The unique specificity of our aminopyrazinamide series is in contrast to other known DNA gyrase inhibitors

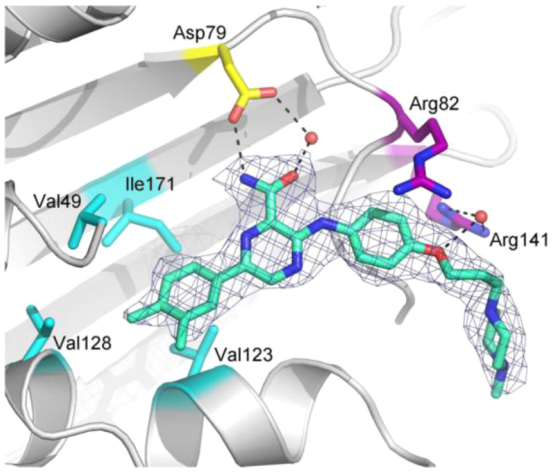

Figure 2. *M. smegmatis* GyrB ATPase domain double loop-deletion mutant (MsmGyrB-EP8) in complex with compound **6**. Seen here are site-1 interactions with Asp79 (yellow); cation-pi stacking with Arg82 and water-mediated interactions with Arg141 at site-2 (purple); and hydrophobic pocket interactions (cyan). Figures were prepared using PyMol (Schrödinger LLC). Final 2Fo-Fc electron density for bound compound **6** is shown contoured at 1σ. MsmGyrB-EP8 is shown as a gray cartoon with selected side-chains in stick representation. Residues 50 to 61 have been omitted for clarity.

(such as novobiocin), which are much broader spectrum agents. This enzyme level specificity was further confirmed by poor inhibition of *E. coli* DNA gyrase and lack of MICs against Gram-positive and Gram-negative pathogens (Table 2).

Table 2. Aminopyrazinamides Specifically Inhibit Mycobacterial GyrB

compound	**10**	**11**	**16**	novobiocin
Msm[a] GyrB IC_{50} (μM)	0.009	<0.0025	0.022	0.008
Eco[b] GyrAB IC_{50} (μM)	1.45	18.65	8.37	0.006
Mtb MIC (μg/mL)	0.5	0.5	2	2
Eco MIC (μg/mL)	>32[e]	>32	>32	>4
Spn[c] MIC (μg/mL)	>32	>32	>32	0.5
Sau[d] MIC (μg/mL)	>32	>32	>32	0.062

[a]*Msm* = *Mycobacterium smegmatis*. [b]*Eco* = *Escherichia coli*. [c]*Spn* = *Streptococcus pneumoniae*. [d]*Sau* = *Staphylococcus aureus*. [e]>: end points not determined.

The aminopyrazinamide series, while being potent ATPase inhibitors of the Msm gyrase, are either very weakly active or inactive against DNA gyrase from other Gram-positive and Gram-negative enzymes, thus achieving excellent target pathogen specificity.

Aminopyrazinamides are highly bactericidal against both replicating and nonreplicating Mtb (Figure 3) and have potent intracellular activity against Mtb residing within macrophages. These compounds exhibited exposure and time-dependent killing kinetics against Mtb grown in Middlebrook 7H9 broth under aerobic conditions. The extent of kill reached >6 log units by day 14 at a drug concentration of 32 μg/mL. Similarly, a 4-log unit of kill was observed following 7 days of drug exposure on hypoxia induced nonreplicating Mtb. The excellent kill observed with this chemical series as compared to novobiocin could be attributed to additional unique hydrophobic interactions with GyrB exploited by aminopyrazinamides.[9,10]

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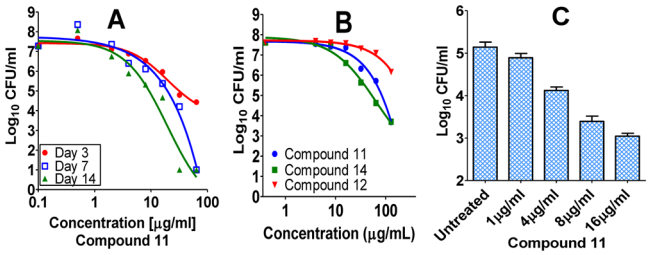


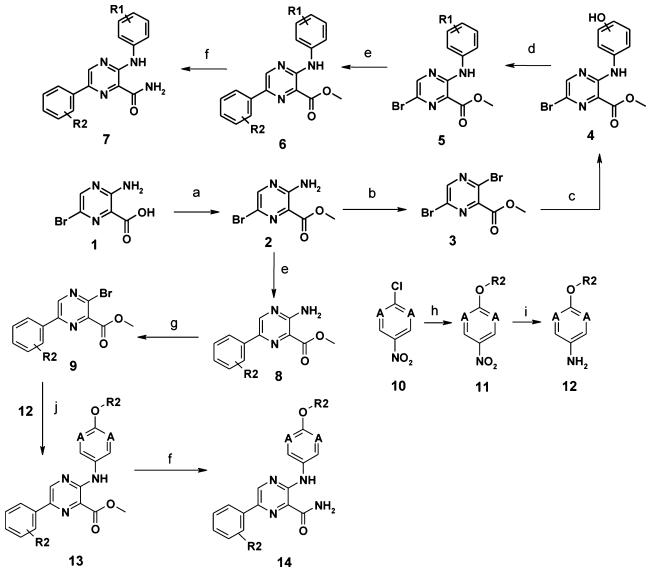
Figure 3. Aminopyrazinamides are highly bactericidal against *M. tuberculosis*. (A) Killing kinetics of compound **11** against replicating Mtb in 7H9 broth. (B) Cidality of aminopyrazinamides against hypoxia induced nonreplicating Mtb. (C) Compound **11** is bactericidal against intracellular Mtb in THP-1 macrophages.

Single-step mutants of Mtb with reduced susceptibility to pyrazinamides arose at a frequency of 2.1×10^{-11} when Mtb cells were treated with 4 μg/mL of compound **11** (Table S2, Supporting Information). Sequencing of the *gyrB* gene from this resistant clone revealed a single nucleotide change in the Mtb *gyrB* gene resulting in an amino acid substitution at position 157 (Gly157 → Ser). The Gly157 → Ser mutation on GyrB is in close vicinity to Arg180 → His or Ala92 → Ser mutation known to confer resistance to novobiocin and benzimidazoles, respectively, in Mtb (Figure S4, Supporting Information).[9,10]

The representative potent compounds in the series were profiled for preliminary DMPK (drug metabolism and pharmacokinetics) properties (Table 3). The equilibrium solubility for this series seems to be poor, but the physiological relevant FassiF (fast state simulated intestinal fluid, pH 6.5) solubility is in the range of 80 μM and 1 mM. Further, the HCl salts of these compounds have very good equilibrium solubility. The significant improvement of solubility (FassiF or HCl salts) can be attributed to the basic nature of the scaffold. The protein binding of the series was generally on the higher side with percentage free fraction between 1 and 4. The predicted % liver blood flow (LBF) was normally in the lower range for mouse, human microsomes, and rat hepatocytes, respectively, and the corresponding half-life were on higher side for microsomal stability as compared to hepatocytes. The MIC data against *Candida albicans* and *Saccharomyces cerevisiae* suggests that compounds in the series are selective for bacteria and inactive on eukaryotes. Furthermore, none of these compounds showed any membrane disruption activity in a red blood cell hemolysis assay (expressed as minimum lytic concentration (MLC)). We have also observed >95% THP-1 macrophage viability following 7 days of compound exposure at 32 μg/mL using a sensitive tetrazolium dye assay. This data clearly implies that the aminopyrazinamides are selective to mycobacteria and have a high selectivity index against eukaryotic targets as measured by the lack of cytotoxicity (MIC > 32 μg/mL) against *C. albicans*, *S. cerevisiae*, and THP-1 macrophages (Table 3).

The synthesis of the aminopyrazine carboxamide (Scheme 1) commenced with the esterification followed by diazotization of the amino group from commercially available 3-amino, 6-bromopyrazine-2-carboxylic acid **21**. The key intermediate phenol **24** was prepared in excellent yield by SNAr using a slight excess of aminophenol. The subsequent *Mitsunobu* alkylation was done using polymer-supported triphenylphosphine, in order to ease purification, followed by a *Suzuki* cross-coupling reaction under microwave conditions. The subsequent compound **26** was treated with methanolic ammonia under microwave conditions to afford the final compound **27**. In the

Scheme 1[a]



[a]Synthesis of aminopyrazinamides: (a) H_2SO_4 (cat), MeOH, 80−90%; (b) isoamyl nitrite, $CuBr_2$, DMF, 80−90%; (c) aminophenol, 50−70%, (d) DIAD, polymer supported PPh_3, 30 min, 60−80%; (e) aryl boronic acid, Pd(dppf)Cl_2·DCM, CsF, MeOH, MW, 120 °C, 20 min, 60−98%; (f) 7 N ammonia in MeOH, 30 min, 60−80%; (g) *t*-butyl nitrite, $CuBr_2$, DCM, 25−30%; (h) R2-OH, NaH, or K_2CO_3, DMF, 50 °C, 30−50%; (i) H_2, Pd/C, 90−100%; (j) $Pd_2(dba)_3$·$CHCl_3$, xanthene, Cs_2CO_3, 1,4-dioxane, 40−50%. DIAD = diisopropyl azodicarboxylate; DCM = dichloromethane; MW = microwave; DMF = *N*,*N*-dimethylformamide.

Table 3. Aminopyrazinamides Exhibit Cellular Selectivity against *M. tuberculosis* and Preliminary DMPK Propoerties

compound	11	12	14
equillibrium solubility (μM)	10 (86)[a] (3700)[b]	11 (117)[a]	6 (832)[a]
mouse % LBF $_{pred}$ (microsomes)/$t_{1/2}$ (min)	15.5/>180	0.63/>180	1.93/>180
human % LBF $_{pred}$ (microsomes)/$t_{1/2}$ (min)	7.6/77	6.1/85	4.3/101
rat % LBF $_{pred}$ (hepatocytes)/$t_{1/2}$ (min)	19/25	12.2/26	22/26
PPB (% free)	1.0	3.1	4.2
Cal[c] MIC (μg/mL)	>32	>32[f]	>32
RBC[d] MLC (μg/mL)	>32	>32	>32
Sce[e] MIC (μg/mL)	>32	>32	>32
THP-1 Macrophages at 32 μg/mL	>95% viability	>95% viability	>95% viability

[a]FassiF. [b]HCl salt. [c]*Cal* = *Candida albicans*. [d]RBC = Red blood cells. [e]*Sce* = *Saccharomyces cerevisiae*. [f]>: end points not determined.

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case of aminopyrimidinyl or aminopyridinyl analogues, the basic side chains were introduced by nucleophilic substitution reaction on substrate **30** followed by reduction to **32**. Compound **22** was subjected to a *Suzuki* cross-coupling reaction under microwave conditions followed by a *Buchwald* reaction using compound **32**. The subsequent compound **33** was treated with methanolic ammonia to afford the final compound **34**.

In conclusion, we report a novel class of inhibitors, aminopyrazinamides, which target the *mycobacterial* GyrB ATPase with chemical tractability, robust SAR, and potent antitubercular activity. In addition, these compounds have promising attributes of specificity due to unique interactions at a hydrophobic pocket, unlike other known GyrB inhibitors. Compounds in the series have demonstrated excellent mycobacterial kill under *in vitro*, intracellular, and hypoxic conditions. Given the desperate need for new anti-TB agents, we believe the aminopyrazinamide class has the potential for further optimization to build in DMPK properties through systematic medicinal chemistry exploration.

METHODS

The experimental details can be found in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Details of the synthesis of all compounds, details of structure determination, and details of biological assays is available. The atomic coordinates and structure factors for enzyme−ligand complex have been deposited in the protein data bank (pdb accession code 4b6c). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

‖These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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