Letters

Identification of New Diamine Scaffolds with Activity against *Mycobacterium tuberculosis*

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Abstract: A diverse 5000-compound library was synthesized from commercially available diamines and screened for activity against *Mycobacterium tuberculosis* in vitro, revealing 143 hits with minimum inhibitory concentration (MIC) equal to or less than 12.5 μM. New prospective scaffolds with antitubercular activity derived from homopiperazine, phenyl- and benzyl-substituted piperazines, 4-aminomethylpiperidine, 4-aminophenylethylamine, and 4,4′-methylenebiscyclohexylamine were identified. Compound SQ775 derived from homopiperazine and compound SQ786 derived from benzylpiperazine had potent antimicrobial activity against *M. tuberculosis* in experimental animals in vivo.

Among infectious diseases, tuberculosis (TB) remains the leading single-agent killer in the world: 2 billion people are infected with latent TB and at risk for development of active disease, and annually, more than 8 million people advance to active and contagious TB. More than 2 million people die of pulmonary TB every year.[1−3] Decades of misuse of existing antibiotics and poor compliance with a prolonged TB drug regimen that involves four different drugs for two months and two drugs for an additional four months have created an epidemic of drug-resistant TB. In countries where adequate supplies of the drugs are not readily available, the prevalence of multidrug resistant TB (MDR-TB) is reported to be as high as 50%.[4,5] In the early 1990s, New York City had one of the worst outbreaks of MDR-TB the country has ever experienced, and the city spent nearly $1 billion over 2 years on containment.[6]

Over the past few years, our research efforts have been focused on the discovery of novel scaffolds with antimycobacterial activity and the development of new antitubercular agents that can improve the current therapeutic regimen and be effective in treatment of MDR-TB. We synthesized several combinatorial libraries, totaling over 100 000 compounds, and screened them against *Mycobacterum tuberculosis*. The first library of 63 238 compounds with the 1,2-ethylenediamine pharmacophore of ethambutol (EMB)[7] lead to the identification of a drug candidate, SQ109 (*N*-geranyl-*N*′-(2-adamantyl)ethane-1,2-diamine).[7−9] To further enhance structural diversity, we designed, synthesized, and evaluated for antitubercular activity other diamine libraries, with an emphasis on modification of the linker between two nitrogen atoms, Figure 1. Here we present the synthesis and in vitro screening of a diverse library derived from commercially available "ready-to-use" diamine templates that led to identification of new scaffolds with activity against *M. tuberculosis*. Preliminary in vivo efficacy data of selected hits are also reported.

A targeted diamine library of approximately 5000 compounds was synthesized in one step on 96-well plates from commercially available diamines via solution-phase reductive alkylation in the presence of a polymer-bound trimethylammonium cyanoborohydride. Fifty-nine diamines that included substituted alkylenediamines, piperazines, homopiperazines, biscyclohexylamines, and pyrrolidines (Figure 2) were organized into four groups based on their structure and reactivity and used in the syntheses as mixtures of 20 (group 1), 12 (group 2), 15 (group 3), or 12 (group 4) compounds. We selected 64 aldehydes and 20 ketones as alkylating agents to ensure structural diversity and also taking into consideration previously established structure−activity relationships (SAR) for a 1,2-ethylenediamine series[7] that warranted the use of adamantane-, cyclooctane-, and diphenylethyl-containing molecules. Prior to syntheses, the carbonyl compounds were organized in a 96-well master plate as 1.2 M solutions of individual compounds in 1,2-dichloroethane; they were used in the reactions in 5−10 molar excess. The Supporting Information contains a complete list of the diamines and carbonyl compounds involved in the synthesis of the library.

Formation of the final products and composition of the product mixtures were assessed by mass spectrometry by testing two randomly selected columns per plate; 17% of the library was tested. Based on MS data, at least 4788 of the targeted compounds (95% of the library) were formed. As expected, reductive alkylation of secondary amines yielded only monoalkylated compounds. Interpretation of the mass spectra of product mixtures derived from pooled monosubstituted piperazines, for example, was quite straightforward because every starting diamine gave only one product. For the rest of the library, derivatization of the diamines that contained a primary amino group led to the formation of mixtures of mono- and double-alkylated products, and synthesis outcomes were dependent upon the reactivity of both diamine and carbonyl compounds. Diamines with a sterically hindered amino group (3-aminoquinonucledine, for example) formed exclusively monoalkylated products. Reductive alkylation by ketones, regardless of the nature of the starting diamines, proceeded with the sole formation of monoalkylated products. Monoalkylation of the primary amino group was also the only (or a major) process in the reactions that involved aldehydes such as 4-(dimethylamino)-benzaldehyde, 2-nitrobenzaldehyde, indol-3-carboxaldehyde, 1-methyl-2-pyrrolecarboxaldehyde, 4-quinolinecarboxaldehyde, 2,3-dihydroxybenzaldehyde, or 2-hydroxy-4-methoxybenzaldehyde. Sterically hindered 5-norbornene-2-carboxaldehyde and diphenylacetaldehyde always produced a roughly equal mixture of mono- and double-alkylated products. 2-Methoxycinnamaldehyde, hydrocinnamaldehyde, citronellal, and 5-(4-chlorophenyl)furfural favored double alkylation, and in some cases, the reactions with these compounds proceeded even further.

The prepared library was advanced into in vitro screening without purification, since identified hits would be later resynthesized and purified to confirm activity. The in vitro

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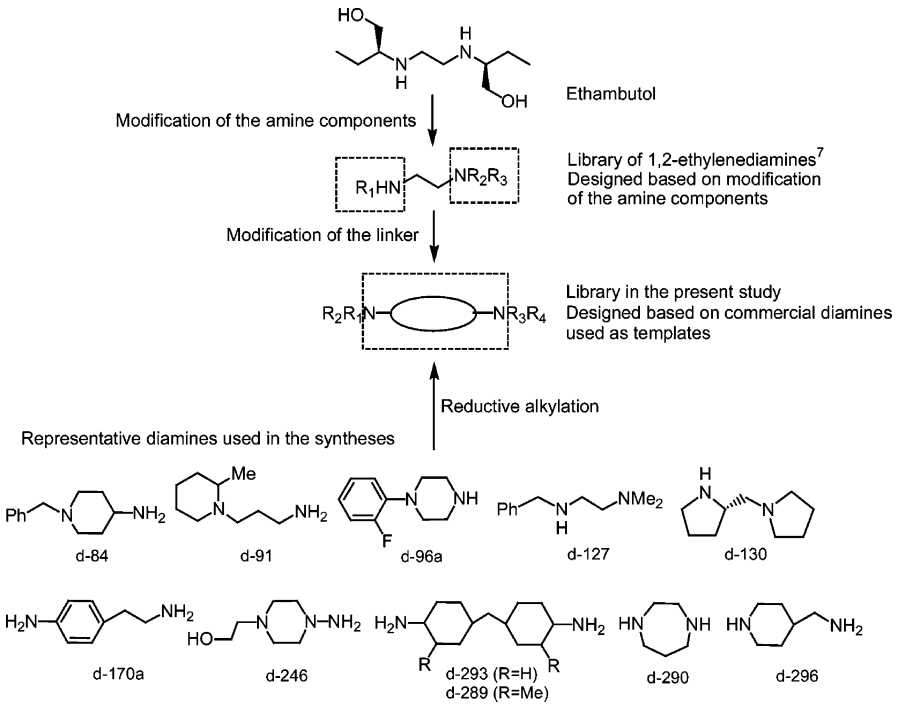


Figure 1. Evolution of synthesized diamine-based libraries.

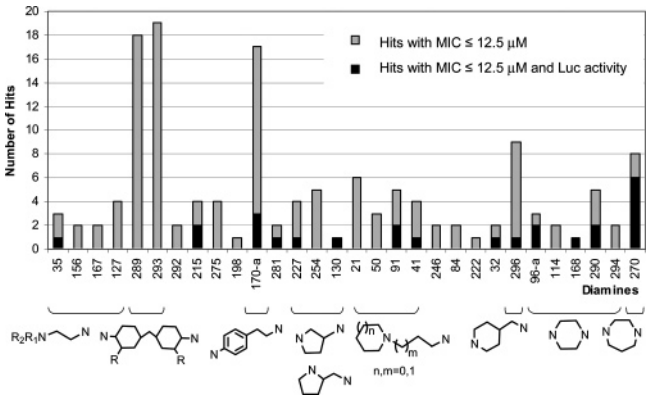


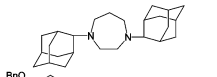
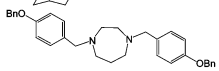
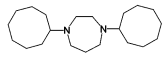
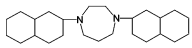
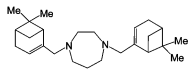
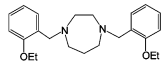
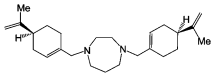
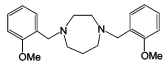
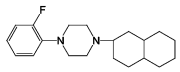
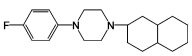
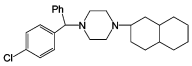
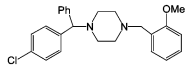
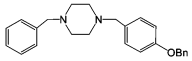
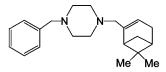
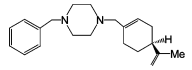
Figure 2. Occurrence of the starting diamines in the hit molecules and scaffolds with demonstrated activity against *M. tuberculosis*.

testing against *M. tuberculosis* was carried out by (1) direct determination of minimum inhibitory concentration (MIC) in a broth microdilution assay and (2) a high-throughput screening bioluminescence assay (luciferase (Luc) assay) with a recombinant *M. tuberculosis* strain containing promoter Rv0341 fused to firefly luciferase. This promoter is activated when the bacteria encounter certain antibiotics such as EMB, isoniazid (INH), and ethionamide that act on targets involved in mycobacterial cell wall biosynthesis.[7,8] The recombinant *M. tuberculosis* harboring the P_{0341}−luciferase fusion produces light when the bacteria are treated with these antitubercular agents. While MIC is a direct measure of bacteria growth inhibition, the Luc assay allowed us to determine whether a compound interferes with the cell wall biosynthesis. Thus, by combining these two assays, we were able to identify compounds capable of inhibiting *M. tuberculosis* growth in vitro presumably through damaging the bacterial cell wall components. Compounds were screened (as prepared) in pools within a range of 50 to 0.39 μM concentrations per each individual compound based on an assumption that every synthesized compound was produced with the theoretical yield of 100%; commercially available EMB (of 99% purity) was used as a positive control in each assay. The MIC of EMB ranged from 6.25 (1.73) to 3.13 μM (0.86 μg/mL). Wells with MIC equal to or less than 12.5 μM regardless of the status in the Luc assay and wells with MIC equal to or less than 50 μM and Luc activity that exceeded background luminescence of untreated control by 1.5 times were considered active and were selected for deconvolution. Out of 336 synthesized compound mixtures, 176 showed antimycobacterial activity and were deconvoluted. Resynthesized individual compounds in each active well were tested for inhibitory activity against *M. tuberculosis*, as well as for the activation of the P_{0341}−luciferase fusion using the same in vitro screening assays. As a result, 143 hits with MIC equal to or less than 12.5 μM were identified in the broth microdilution assay; among those, 25 compounds were also active in the Luc assay.

We found that the vast majority of identified hits were derived from five diamines: 4,4′-methylenebis(cyclohexylamine) (d-289) and its analogue 4,4′-methylenebis(2-methylcyclohexylamine) (d-293), 2-(4-aminophenyl)ethylamine (d-170.a), 4-(aminomethyl)piperidine (d-296), and homopiperazine (d-270), see Figures 1 and 2. Both biscyclohexylamines yielded at least 37 hits with MIC equal to or less than 12.5 μM, but none of them were active in the Luc assay, suggesting that this compound class either targeted a different biological pathway than cell wall biosynthesis or represented compounds with nonspecific toxicity toward the target pathogen in the screens. Similarly, hit compounds derived from 2-(4-aminophenyl)ethylamine (d-170a) and from 4-(aminomethyl)piperidine (d-296) showed little activity in the Luc screening assay (Figure 2). Homopiperazine hits were found to be the most interesting, since they demonstrated significant activity in both bacterial growth inhibition and the Luc assay: out of eight hits derived from homopiperazine, six were active in both screens. These compounds likely target mycobacterial cell wall components.

Among the new scaffolds with antitubercular activity, we also identified a series of phenyl- and benzyl-substituted piperazines:[10] when combined, 1-(2-fluorophenyl)piperazine (d-96.a), 1-piperonylpiperazine (d-114), 1-(4-fluorophenyl)piperazine (d-168),

Table 1. Structures and in Vitro Data of Homopiperazine and Piperazine Hit Compounds

Compd.	Structure	MIC in screen, μM[a]	MIC μM[b]	IC_{50}, μM[c]	SI[d]	Activity in the Luc assay;[e] %	LogP[f]
	Homopiperazines						
SQ775		6.25	1.56	132	84.6	126	5.98
SQ776		12.5	3.13	56	18	not active	6.29
SQ777		12.5	1.56	74	47	81	6.57
SQ778		1.56	1.56	54	35	127	7.4
SQ779		3.13	3.13	110	35	113	6.99
SQ780		1.56	1.56	71	46	70	4.04
SQ781		12.5	6.25	81	13	not active	7.14
SQ782		6.25	6.25	105	17	138	2.98
	Piperazines						
SQ765		6.25	12.5	70	6	145	ND[g]
SQ774		12.5	12.5	86	7	157	5.49
SQ783		12.5	6.25	47	8	not active	6.01
SQ784		12.5	6.25	65	10	not active	3.85
SQ786		3.13	6.25	54	9	not active	4.02
SQ788		6.25	6.25	64	10	not active	4.36
SQ789		3.13	3.13	60	19	not active	4.44

[a] Results obtained during screening of deconvoluted plates; the MICs determined by broth microdilution method. The MIC of EMB (control) ranged from 1.73 (6.25) to 0.86 μg/mL (3.13 μM). [b] MIC of purified compounds determined by broth microdilution method; 10 mM stock solutions in methanol were used. MIC of the controls: INH 0.0625 μg/mL (0.46 μM); RIF 0.03 μg/mL (0.03 μM). [c] Determined with HepG2 cell line in the MTS assay. [d] Selectivity index, SI = IC_{50}/MIC. [e] Presented as the percent of activity of a tested compound compared to the activity of EMB (100%). [f] Calculated using the algorithm and database statistics of the ACD. [g] Not determined.

1-benzylpiperazine (d-290), and 1-(4-chlorobenzhydryl)piperazine (d-294) gave 13 hits with 5 active in both screening assays.

Other identified scaffolds (Figure 2) were 1-(2-aminoethyl)-piperidine (d-21), 1-(2-aminoethyl)pyrrolidine (d-50), 1-(2-aminoethyl)piperazine (d-41), and pipecoline d-91 (total 18 hits), tryptamines d-215 and d-275 (8 hits), and 3-aminopyrrolidine (d-227), pyrrolidine d-130 (Figure 1), and (*S*)-(+)-2-(aminomethyl)pyrrolidine (d-254) (10 hits).

Within the carbonyl compounds, the most frequently occurring structural fragments in the hit molecules were contributed by 4-benzyloxybenzaldehyde (14 hits), 5-(4-chlorophenyl)-furfural (11 hits), decalone (12 hits), 2-methoxy-1-naphthaldehyde (9 hits), and (*S*)-perillaldehyde (8 hits). We also found similarities with the 1,2-ethylenediamine series: some of the hits in this library contained earlier described[7] myrtenyl, geranyl, and adamantane fragments derived from (−)-myrtenal (11 hits), (*S*)-citronellal (10 hits), geranylacetone (13 hits), and 2-adamantanone (8 hits).

After identification of pharmacophores with antimycobacterial activity, we focused mostly on the homopiperazine series, since these compounds were active against *M. tuberculosis* in both broth microdilution and Luc assays and were easily synthesized. Eight symmetrically substituted homopiperazines SQ775−SQ782 were resynthesized on a larger scale, purified to ensure at least 95% purity, and retested against *M. tuberculosis*. Determined by the same microdilution method, the MIC of purified hits fell within a 1.56−6.25 μM range and in most cases confirmed the previous MIC results obtained during the screening of deconvoluted plates (Table 1). INH and rifampicin (Rif), both commercially available from Sigma, were used as controls, with MICs of 0.46 (0.0625) and 0.03 μM (0.03 μg/mL), respectively.

To select candidates for in vivo efficacy testing in mice, synthesized compounds were evaluated[8] for (a) predicted membrane permeability based on log *P* values (www.acdlabs.com), (b) in vitro cytoxicity in HepG2 cells to determine IC_{50},[11] and (c) selectivity index (SI), the ratio of IC_{50} to MIC, as an estimate of a therapeutic window, see Table 1. Compounds with the best data, SQ775 (MIC 6.25 μM, SI 17, log *P* 5.98) and SQ780 (MIC 1.56 μM, SI 18, log *P* 4.04) were selected for in vivo efficacy evaluation in murine models of tuberculosis. Compound SQ782 (MIC 6.25 μM, SI 13, log *P* 2.98) was postponed due to structural similarities with SQ780.

We added compound SQ786 as a representative of the piperazine series to the studies with the two homopiperazines in animal models of TB. SQ786 demonstrated a MIC of 6.25 μM on *M. tuberculosis*, relatively low cytotoxicty (IC_{50} 54 μM), SI of 9, and an acceptable-to-good oral bioavailability[12] log *P* value of 4.02, Table 1. But unlike its higher homologues, SQ786 was not active in the Luc assay, implying that its target is not presumably involved in bacterial cell wall biosynthesis. Overall, out of 13 piperazine hits, the MICs of 7 compounds were reconfirmed (Table 1), and 6 compounds demonstrated poorer than expected activity against *M. tuberculosis* with MIC values of 25−50 μM.

In vivo testing of SQ775, SQ780, and SQ786 was conducted using a rapid (3 week) screening model for TB infection based on active drug prevention of infection-induced weight loss in mice.[13] This screen can predict quickly and with reasonable accuracy the efficacy of both known and experimental drugs, Figure 3. Briefly, C3H mice were inoculated intravenously (iv) with 10^6 CFU of virulent *M. tuberculosis* H37Rv to develop rapid and progressive TB disease. Chemotherapy was initiated 7 days after inoculation and continued for 10 days. Experimental compounds were administrated daily by gavage at 10 mg/kg (dose has not been optimized). Uninfected mice, infected untreated mice, and infected mice treated with INH at 25 mg/kg[13] were used as the controls. Body weight of mice in all groups (6 per group) was monitored from day 0. By day 10, infected placebo control mice began to lose weight, and by day 20, the mice in this group lost more than 25% of their body weight (the sign of terminal illness). Treatment with SQ775 and SQ786 prevented weight loss and delayed mortality: by day 21 following challenge, the mice treated with SQ775 or SQ786 lost only 12% of their initial body weight. Compound SQ780 was ineffective in this model (21% weight loss). Preliminary results from in vivo testing of SQ775 and SQ786 in a low-dose

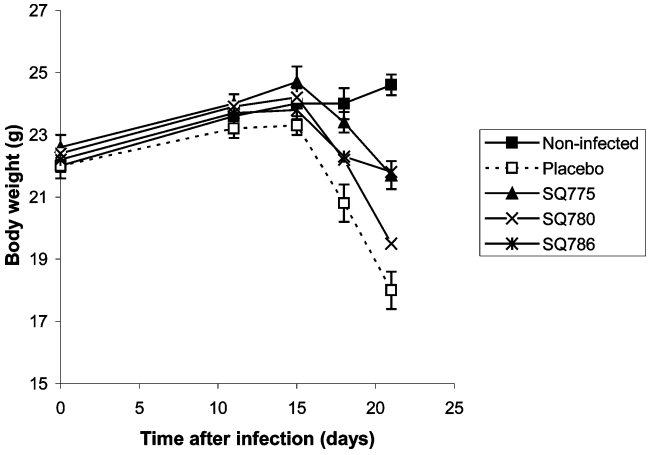


Figure 3. In vivo efficacy testing of SQ775, SQ780, and SQ786 in the rapid mouse model of TB—dynamics of body weight of mice infected with *M. tuberculosis* and treated with SQ775, SQ780, and SQ786 at 10 mg/kg by oral administration. In this model, isoniazid at a dose of 25 mg/kg (used as a positive control) fully prevented body weight loss (data are not shown; the dynamics of body weight were identical to those of uninfected control mice).

mouse model of chronic TB infection[14] suggest that these compounds might serve as leads to develop new potent antibacterial compounds.[15]

In conclusion, we identified new prospective scaffolds with activity against *M. tuberculosis* in vitro and in vivo. Synthesis of new compounds based on these potentially valuable scaffolds is in progress.

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Supporting Information Available: Complete list of diamines and carbonyl compounds used in the library preparation, detailed experimental procedures, ^{1}H NMR and MS spectra for individual compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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