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Discovery of Benzothiazole Amides as Potent Antimycobacterial Agents

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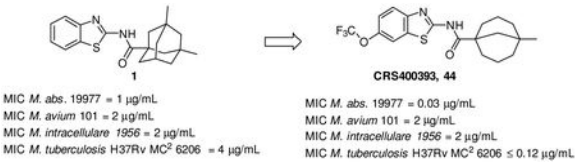
Abstract

From a high throughput screening of commercially available libraries against nontuberculous mycobacteria and *Mycobacterium tuberculosis*, numerous hits were identified with moderate activity. Extensive medicinal chemistry optimization has led to a series of potent benzothiazole amide antimycobacterial agents. Replacement of the adamantyl group with cyclohexyl derivatives and further development of this series resulted in an advanced lead compound, **CRS400393**, which demonstrated excellent potency and a mycobacteria-specific spectrum of activity. MIC values ranged from 0.03-0.12 μg/mL against *Mycobacterium abscessus* and other rapid-grower NTM, and 1-2 μg/mL against *Mycobacterium avium* complex. The preliminary mechanism of action studies suggested these agents may target MmpL3, a mycobacterial mycolic acid transporter. The series has demonstrated *in vivo* efficacy in a proof of concept mouse model of *M. abscessus* infection.

GRAPHIC ABSTRACT:



Keywords

mycobacteria; nontuberculous mycobacteria; MmpL3 inhibitor; tuberculosis; benzothiazole amide; antibiotics; *Mycobacterium abscessus*; *Mycobacterium avium*

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SUPPORTING INFORMATION: Supplementary data (Experimental details and characterization of selected compounds) associated with this article can be found, in the online version, at

Mycobacterial pathogens are intrinsically resistant to most antibiotics, and pose an enormous human health issue (1). While *Mycobacterium tuberculosis* (*Mtb*) has been the subject of extensive drug discovery efforts, the nontuberculous mycobacteria (NTM) pose a unique and under-resourced therapeutic challenge, associated with significant morbidity and mortality (2). There is growing epidemiologic evidence to suggest that NTM cause more infections in the United States than *Mtb* (3), yet there are virtually no antimicrobial drug discovery programs specifically targeting NTM.

Pulmonary disease caused by NTM is especially problematic in patients with underlying host susceptibility such as populations with immunosuppressive medications (4), cystic fibrosis (5, 6), ciliary dyskinesia, smoking-induced lung diseases such as chronic obstructive pulmonary disease COPD (7), HIV infection (8), bronchiectasis and malignancies (9) or underlying defects in IL-12, interferon gamma receptor or Nuclear factor κB essential modulator (NEMO) deficiency (10). *Mycobacterium abscessus* (*Mabs*) complex, in particular, is an emerging NTM infection for which effective therapy is often elusive, burdensome and costly (11, 12, 13, and 14). Chronic lung disease due to NTM (especially *Mabs*) can be particularly difficult to treat, even with multi-drug regimens (15). Recent evidence suggests that drug resistant *Mabs* subspecies *massiliense* can cause human-to-human transmission in cystic fibrosis patients (16, 17).

There have been several hospital acquired outbreaks of multi-drug and disinfectant resistant rapidly growing mycobacteria (RGM). One massive outbreak of skin and soft tissue infections occurred in Brazil by contaminated laparoscope with *M. chelonae* that was resistant to 2% glutaraldehyde (18). Due to the changing epidemiology and transmission dynamics of certain problematic NTM species such as *Mabs*, the threat of worldwide epidemics is escalating (16, 17). Given the emergence of NTM as a public health issue, finding new antimycobacterial agents is of paramount importance. Development of new agents specifically active against NTM is an under-explored area with no approved drug specifically treating NTM infections.

In addition, tuberculosis represents an ongoing public health threat as well, with over two billion people carrying the infection (19). *Mtb* can remain latent for extended periods of time; nonetheless, the large reservoir of latent infection fuels a growing population with active disease, amounting to 10.4 million of new cases per year with 1.7 million deaths in 2016 (20). Treatment regimens involve three to four drugs, administered for six months or longer. Emergence of multidrug resistant (MDR)-*Mtb* has added impetus to drug discovery efforts against this devastating pathogen. Historically, antimycobacterial drug discovery efforts have focused almost exclusively on *Mtb*, with virtually no concerted effort toward extended spectrum agents that cover NTM. In part, this reflects the intrinsic challenge of treating NTM, which are frequently refractive to anti-*Mtb* drugs, thus prompting our research to develop broad spectrum anti-mycobacterial agents. We are particularly excited to have discovered a lead series that exhibits broad antimycobacterial activity against NTM (including both rapid growing and slow growing species) and *Mtb* as well.

Compound **1** in Figure 1 was identified from the high throughput screening of over 350,000 small molecule compounds for *Mabs* whole cell activity (21). Parallel screening of the same

compound collection against *Mtb* allowed us to identify screening hits with broad antimycobacterial spectrum. However, the adamantyl group in compound **1**, although found in some commercial drugs, can be a liability in terms of its high lipophilicity and potential for nonspecific binding. Consequently, our initial strategy for this series involved trimming of the adamantyl group to identify the least amount of lipophilic structure that retains activity.

The general synthetic route of novel mycobacterial inhibitors is outlined in Scheme 1. The synthesis started from substituted 2-amino-benzothiazole intermediates and variably substituted cycloalkyl carboxylic acids under the amide coupling conditions using 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluoro-phosphate (HATU) in the presence of N,N-diisopropylethylamine (DIEA) in dichloroethane (DCE). The products were purified by column chromatography using ethyl acetate and hexanes as eluents and fully characterized by NMR and LC-MS.

The compounds were first screened in a panel of RGM including *M. abscessus* ATCC 19977 (*Mabs*), *M. abscessus* 1, *M. abscessus 21, M. abscessus* 79, *M. abscessus massiliense* 119, *M. fortuitum* 41, *M. chelonae* 93, and *M. peregrinum* ATCC 700686. Since *M. abscessus* is a rapid emerging pathogen, for ease of discussion and limited space only activity for *M. abscessus* ATCC 19977 as a representative of RGM will be discussed, however, all MIC data for the panel of RGM is available in the supplemental materials. Compounds that showed good activity in the panel of RGM were then tested against two slow growing NTM species (SGM), *M. avium* 101 and *M. intracellulare* 1956. Select compounds were then tested against *M. tuberculosis* H37Rv mc^2 6206.

Table 1 summarizes the structure-activity relationship (SAR) of substituted cyclohexanes with 5-trifluomethyl substitution on the benzothiazole, which was found to be one of the early optimal aromatic RHS during previous SAR exploration (19). As one can see, anti-mycobacterial activity was affected by the position and degree of substitution about the cyclohexane ring. Generally, the MICs were elevated as the number of carbons was trimmed resulting in loss of antimycobacterial activity. However, substitution position matters (compound **12** vs **14** and compound **5** vs **6**). The best substitution pattern was 3,3,5-trimethyl cyclohexane (compound **8**). Although the MIC of compound **8** for the rapid grower such as *Mabs* was comparable to the MIC of compound **1** with adamantane as the right hand side (RHS), the activity against slow growers, such as *M. avium* 101 and *M. intracellulare* 1956 activity was diminished.

We kept the 3,3,5-trimethyl-cyclohexane RHS and explored the substitution on the benzothiazole ring to find the optimal substitution pattern (compounds **16 - 37**). Table 2 summarizes the SAR of the left hand side (LHS). It is clear that introducing a single small halogen such as fluorine on the benzene ring at 4, 5, and 6-position resulted in improved spectrum activity (compounds **20**, **27** and **30** vs compound **16**). Di- or trifluoro- substituted analogs **17** and **18** had comparable activity against *Mabs* to the 6-fluoro analog **30**, however the activity against the slow growers was reduced, indicating that position and degree of aromatic substitution has a substantial effect on the spectrum of activity. This effect is further exemplified in comparison of 5,7-difluoro compound **23** with the 5- and 7- mono-

fluoro compounds (compounds **27** and **36**) and with 4,6-difluoro compound **18**. Larger halogens such as chloride and bromide provided less activity than the fluoride at 4 or 5-position of the benzothiazole (compounds **19**, **25**). However, at the 6-position, all halogen substitutions (compounds **28**, **29** and **30**) resulted in much improved activity against *Mabs* in comparison to unsubstituted compound **16**. At the 6-position, trifluoromethyl (compound **8**) and trifluoromethoxy (compound **32**) analogs were also very active against *Mabs* while dimethylamine (compound **31**), methoxy (compound **34**), and trifluoromethylsulfanyl (compound **35**) analogs lost activity against *Mabs*. Interestingly, the 5,7-disubstituted analogs **22**, **23** and **24** were highly potent against *Mabs* and *Mtb* but with activity ranging from MIC 8 - >64 μg/ml against *M. avium* and *M. intracellulare*, respectively. In general, although most of the compounds had good activities against the RGM such as *Mabs*, the loss of activities against SGM is evident. We don't have a good explanation yet for this observation, but we speculate that the elevated MIC for SGM may be due to the bovine albumin in the MIC testing media Middlebrook 7H9 broth since this series of compounds is highly protein bound (> 99%). The best one in this subset of compounds was **26** with MIC = 0.25 μg/mL against *Mabs* and ≤ 0.12 μg/mL against *Mtb*, although it lacked activity against SGM as discussed above. For the sake of better understanding the SAR, when the adamantane was installed with the best left hand side (Figure 2, **CRS400226**, **15**), the broad spectrum activity was recovered (22).

We hypothesized that loss of activity against SGM in Table 1 may be due in part to the lack of fourth substitution on the 1-position of the cyclohexane ring. To examine this hypothesis, we synthesized two pairs of analogs with the cycloalkyl ring RHS methylated at 1-position as seen in Table 3. Importantly, the methylation at 1-position on both the cyclohexane and cycloheptane (compounds **39** and **41)** recovered the SGM activity and also resulted in improvement of anti-*Mtb* activity while the non-methylated counterparts (compounds **38** and **40**) were ≥ 16-fold less active than the methylated compounds against SGM.

Encouraged by these results, we identified additional non-adamantane, tetra-substituted cycloalkyl carboxylic acids at the 1-position as building blocks to synthesize the final compounds. One analog stood out as a major breakthrough in improving the antimycobacterial activity, a compound derived from 5-methylbicyclo[3.3.1]nonane-1-carboxylic acid (compound **52**, **CRS400359**) with MIC as low as 0.06 μg/mL against *Mabs* and MIC = 2 μg/mL against the slow growers *M. avium 101* and *M. intracellulare* 1956. **CRS400359** was also potent against *Mtb* with MIC = 1 μg/mL. Follow-up analogs (compounds **42 - 51**) with the best substitution patterns from earlier SAR were synthesized based on the new, exciting RHS as shown in Table 4 to find the optimal LHS substitution. Most of the compounds demonstrated great antimycobacterial activity, not only against the RGM, but also the SGM and *Mtb*. One of the best compounds was compound **43** (**CRS400393)**, demonstrating MIC = 0.03, 2, and ≤ 0.12 μg/mL against *Mabs, Mycobacterium avium* complex (MAC), and *Mtb* respectively. This was a significant development in activity against mycobacteria and also indicated that the adamantane moiety was not necessary to maintain the broad spectrum antimycobacterial activity. Although our initial plan was to reduce the lipophilicity of the series, the loss of activity against mycobacteria by trimming the adamantane group indicated that lipophilicity may be needed

for binding to the target mmpL3. The level of improvement in potency of **CRS400359** and **CRS400393** from the initial hit compound **1** warranted further characterization of these compounds in ADME, PK, efficacy and toxicity.

Although the primary focus of the project was to improve the antimycobacterial activity of the benzothiazole amide hit series to identify potent antimycobacteral agents, understanding the mechanism of action of the compound series was also part of this effort. Due to the structural similarity of the initial hit compound **1** to the known inhibitors of mmpL3 (the trehalose monomycolate transporter protein large), a mycolic acid transporter that plays important role in mycobacterial outer membrane formation, we believed the benzothiazole amide series also targeted mmpL3. We selected compounds **18** and **32** to confirm the mechanism of action. The data suggested that these compounds affect the transfer of mycolic acids, the mycobacterial outer membrane building blocks, to their cell envelope acceptors in both *Mabs* and *Mtb*, most likely through the inhibition of mmpL3 (23).

Compounds with good antimycobacterial activities were further profiled in a secondary panel of Gram-positive and Gram-negative bacteria to test for selectivity of spectrum. All compounds tested showed no activity below 32 μg/mL. **CRS400393** was also characterized in time kill kinetic studies (23) and it was bacteriostatic against *Mabs*, for 3 days, followed by a drop of 2 Log10 CFU after 5 days. Importantly, no regrowth was observed following a single addition of compound due to emergence of resistance (23).

The long term goal of the project is to identify compounds that have the drug like properties. Thus, one question that we needed to answer was whether these compounds would affect metabolic enzymes such as cytochrome P450 (CYP) since the patients infected with NTM will likely take more than one drug due to the potential for bacterial resistance. Select mycobacterial inhibitors **9, 28**, **29** and **32** were screened in an *in vitro* panel of six common isoforms of CYP enzymes CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 (Table 5). Compound **32** appeared to show the least propensity for CYP inhibition, with IC_{50} for all isoforms > 20 μM. Compound **9** had IC_{50} below 20 μM only for CYP2D6 isoform (1.4 μM). Compound **28** and **29** hit three of the six isoforms with IC_{50} less than 20 μM. Compounds **CRS400359** and **CRS400393** were also tested against CYP2B6 and CYP3A4, the two more relevant CYP enzymes for pulmonary delivery, at 10 μM concentration, and neither of the compounds inhibited the enzymes more than 50% at such concentration (data not shown).

The compounds were also screened in a hemolysis assay for potential toxicity. None of the compounds tested showed hemolysis at the highest concertation of 128 μg/ml. The compounds generally had low solubility (data not shown) and high protein binding in human serum (> 99%). Select compounds were also screened for cytotoxicity towards the HepG2 cell line and compound **32** had IC_{50} > 100 μM.

Early in the program, representative compounds **9** and **28** were selected for pharmacokinetic studies in mice. Oral bioavailability of these compounds was 53% and 75%, respectively, when dosed at 100 mg/kg. The plasma clearance (CL) after intravenous (IV, 10 mg/kg) administration was 1.7 L/hr/kg and 1.5 L/hr/kg for compounds **9** and **28**, respectively.

Compound **18** was further tested orally in a single dose toxicity study at 100 mg/kg and 300 mg/kg. All mice appeared normal with no abnormal behavior observed through the course of the study.

Although the compounds were well tolerated with reasonable bioavailability, the poor solubility and lipophilicity present challenges for oral pharmaceutic development. Instead, intrapulmonary delivery methods for treatment of lung infections such as NTM and *Mtb* were explored. An earlier lead compound, **CRS400226** (Figure 2), was further developed for this delivery pathway and demonstrated *in vivo* efficacy as proof of concept for the approach (23). With the significant advances made in broad spectrum anti-mycobacterial activity, along with the supporting data demonstrating tolerability, we are looking forward to advancing lead compounds such as **CRS400393** (compound **43**) into *in vivo* efficacy and tolerability studies using the intra-pulmonary delivery approach previously validated for this project.

In summary, starting from a high throughput screening hit, we applied an iterative process of lead optimization and made substantial progress toward the development of compounds as antimycobacterial agents. NTM are a diverse group of pathogens, and most drugs show a wide range of potencies, even between different strains within the same species. By testing for both *Mtb* and NTM activity in the medicinal chemistry optimization process, we sought to maintain broad antimycobacterial spectrum of activity. Over the course of several hundred compounds, this process resulted in progressive improvement of MIC potency from the initial lead compound **1** to much more advanced compounds such as **CRS400393**, generating increasingly potent compounds against RGM while maintaining activity against SGM and *Mtb*. Further compound optimization to improve the antimycobacterial activities and physicochemical properties and evaluation of pulmonary delivery methods is needed. Development and optimization of inhaled formulation vehicles and *in vivo* screening of efficacy, tolerability and pharmacokinetics will be important advances toward the development of a drug candidate such as compound **CRS400393** for the treatment of mycobacterial lung infections. The benzothiazole amide series holds promise for development of a novel therapeutic agent with broad antimycobacterial activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS:

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ABBREVIATIONS:

NTM	nontuberculous mycobacteria
Mtb	Mycobacterium tuberculosis

SAR	structure activity relationship
Mbs	*Mycobacterium abscessus* ATCC 19977
MIC	minimum inhibitory concentration
RHS	right hand side
LHS	left hand side
ADME	absorption, distribution, metabolism, and excretion
PK	pharmacokinetics
CYP	cytochrome P450
RGM	rapid growing mycobacteria
SGM	slowly growing mycobacteria

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- A series of potent benzothiazole amide antimycobacterial agents identified.
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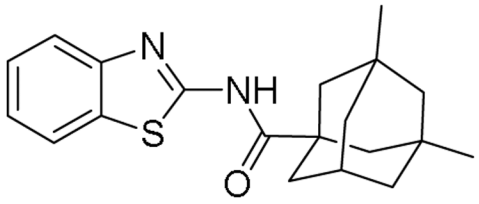
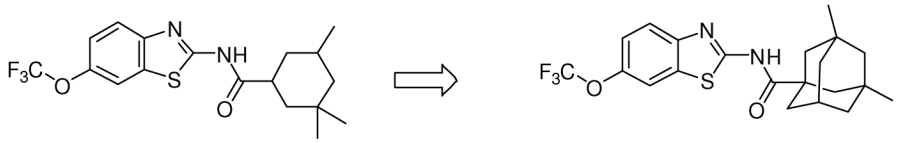
- The series may target MmpL3, a mycobacterial mycolic acid transporter.
- The series demonstrated in vivo efficacy in a mouse model of M. abscessus infection.



1

MIC *M. abs*. 19977 = 1 μg/mL
MIC *M. avium* 101 = 2 μg/mL
MIC *M. intracellulare 1956* = 2 μg/mL
MIC *M. tuberculosis* H37Rv MC^2 6206 = 4 μg/mL

Figure 1.
Structure and activity of Compound 1



32

MIC *M. abs*. 19977 = 0.5 μg/mL
MIC *M. avium* 101 > 64 μg/mL
MIC *M. intracellulare 1956* > 64 μg/mL
MIC *M. tuberculosis* H37Rv MC^2 6206 = 1 μg/mL

CRS400226, 15

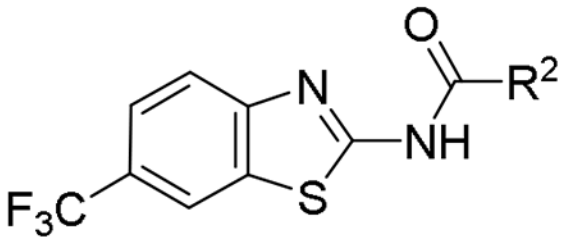
MIC *M. abs*. 19977 = 0.25 μg/mL
MIC *M. avium* 101 = 2 μg/mL
MIC *M. intracellulare 1956* = 1 μg/mL
MIC *M. tuberculosis* H37Rv MC^2 6206 = 1 μg/mL

Figure 2.
Structure of Compound 32 and CRS400226

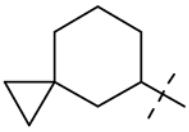
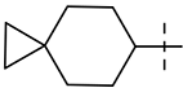
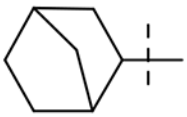
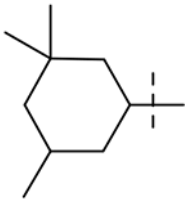
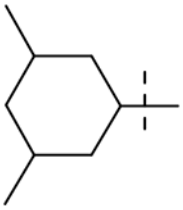
Scheme 1.
Synthetic scheme of benzothiazole amide antimycobacterial agents

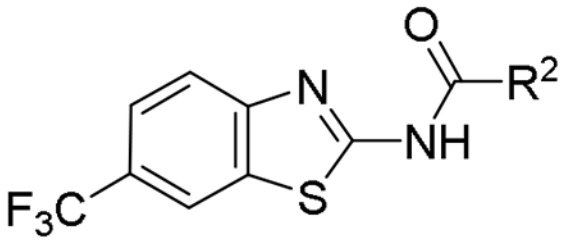
Table 1.

Structure Activity Relationship of Cyclohexane Analogs – RHS

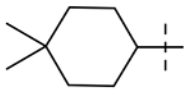
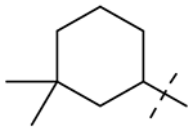
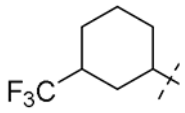
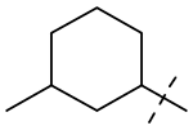


5 - 14

Cmpd#	R^2	MIC (μg/mL) *M. abs.* 19977	*M. avium* 101	*M. intracellulare* 1956	*M. tuberculosis* H37Rv
5		4	> 64	> 64	NT *
6		32	64	> 64	32
7		> 64	32	32	NT
8		0.5	> 64	> 64	2
9		1	> 64	> 64	4



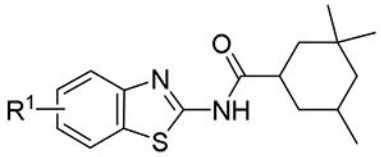
5 - 14

Cmpd#	R^2	MIC (μg/mL)			
		M. abs. **19977**	***M. avium*** **101**	***M. intracellulare*** **1956**	***M. tuberculosis*** **H37Rv**
10		> 64	64	64	NT
11		> 64	> 64	> 64	NT
12		64	> 64	> 64	NT
13		2	16	16	NT
14		2	16	16	16

*NT = not tested

Table 2.

Structure Activity Relationship of Cyclohexane Analogs – Left Hand Side (LHS)



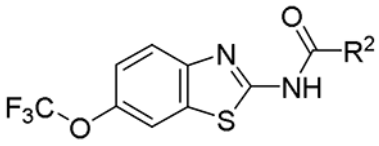
16 - 38

Cmpd#	R^1	MIC (μg/mL)			
		M. abs. 19977	*M. avium* 101	*M. intracellulare* 1956	*M. tuberculosis* H37Rv
16	H	2	8	8	8
17	4,5,6-triF	0.5	> 64	> 64	1
18	4,6-diF	0.5	64	4	1
19	4-Cl	16	> 64	16	NT *
20	4-F	2	8	8	4
21	4-F-6-Br	1	> 64	16	1
22	5,7-diCl	0.25	8	16	1
23	5,7-diF	0.25	8	8	0.25
24	5,7-diMe	0.5	> 64	> 64	0.25
25	5-Br	32	> 64	> 64	NT
26	5-CF_3	0.25	> 64	> 64	≤ 0.12
27	5-F	1	16	16	4
28	6-Br	0.5	> 64	> 64	2
8	6-CF_3	0.5	> 64	> 64	2
29	6-Cl	0.5	16	4	4
30	6-F	0.5	8	8	4
31	6-NMe_2	32	> 64	> 64	NT
32	6-OCF_3	0.5	> 64	> 64	1
33	6-$OCHF_2$	1	> 64	8	2
34	6-OMe	1	16	8	8
35	6-SCF_3	16	> 64	> 64	2
36	7-F	0.5	8	16	1
37	7-OH	16	16	4	NT

* NT = not tested

Table 3.

Structure Activity Relationship of Cyclohexane Analogs – Alpha-methylation



39 - 42

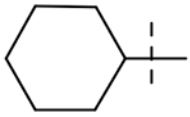
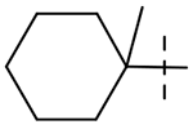
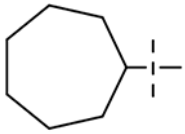
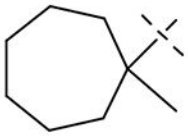
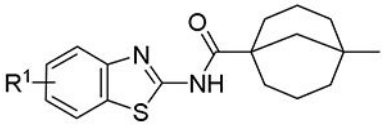
Cmpd#	R^2	MIC (μg/mL) *M. abs.* 19977	*M. avium* 101	*M. intracellulare* 1956	*M. tuberculosis* H37Rv
38		32	> 64	> 64	64
39		0.25	8	4	4
40		> 64	> 64	> 64	64
41		0.12	8	4	0.5

Table 4.

Structure Activity Relationship of Cyclohexane Analogs – With the Best RHS



43 - 53

Cmpd#	R^1	MIC (μg/mL)			
		M. abs. 19977	*M. avium* 101	*M. intracellulare* 1956	*M. tuberculosis* H37Rv
42	5-Br	0.06	4	1	≤ 0.12
43	5,7-diCl	0.03	2	2	≤ 0.12
44	5,7-diMe	0.12	8	8	0.25
45	5,7-diF	0.06	4	4	0.25
46	4,5,6-triF	0.12	> 8	8	≤ 0.12
47	4,6-diF	0.12	4	4	0.25
48	6-CF_3	0.25	> 8	4	0.25
49	6-Br	0.12	8	4	0.5
50	6-Cl	0.12	8	4	0.5
51	6-$OCHF_2$	0.12	8	4	1
52	6-OCF_3	0.06	2	2	1

Table 5.

CYP Inhibition of Benzothiazole Amides

Cmpd#	CYP IC_{50}(μM)						
	CYP 2B6	**CYP 2C8**	**CYP 2C9**	**CYP 2C19**	**CYP 2D6**	**CYP3A4-Midazolam**	**CYP3A4-Testosterone**
9	> 20.0	> 20.0	> 20.0	> 20.0	1.40	> 20.0	> 20.0
28	12.8	> 20.0	> 20.0	12.7	1.60	> 20.0	> 20.0
29	14.9	> 20.0	> 20.0	15.5	0.20	> 20.0	> 20.0
32	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0