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Discovery of Natural Product-based Antimycobacterial Agents Effective against Non-replicating Bacilli

Abstract

New antimycobacterial molecules that kill non-replicating *Mycobacterium tuberculosis* (Mtb) were identified by screening libraries of synthetic natural products. De novo screening of a 400-membered library of aurachin RE analogs resulted in discovery of UT-317 ((R)-20). UT-317 is a selective vitamin K2 biosynthesis (MenA) inhibitor that killed replicating and non-replicating Mtb at 2.31 $\mu\text{g/mL}$ (MIC) and 0.85 $\mu\text{g/mL}$, respectively. A 50-membered library of capuramycin analogs was evaluated in their enzymatic inhibitory activities against translocase I (MraY/MurX) and prenyl-phosphate-GlcNAc-1-phosphate transferase (WecA). UT-01320 (45) is identified as a selective WecA inhibitor that kills both replicating and non-replicating Mtb at 1.50 $\mu\text{g/mL}$ (MIC) and 2.58 $\mu\text{g/mL}$, respectively. UT-01320 killed the intracellular Mtb much faster than the first-line TB drugs such as isoniazid and rifampicin. A strong antimycobacterial agent, UT-800 (64) was identified by screening of a 50-membered library of pleuromutilin derivatives. UT-800 is a protein biosynthesis (50s ribosome) inhibitor which has activity focused against Mtb. UT-800 killed replicating and non-replicating Mtb at 0.83 $\mu\text{g/mL}$ (MIC) and 1.20 $\mu\text{g/mL}$, respectively. In the course of these works, fluorescent probes, Park's nucleotide-N ϵ -C6-FITC (32, for MraY/MurX) and UDP-glucosamine-C6-FITC (46, for WecA) were developed. These fluorescent probes enable us to screen the polyprenyl-phosphate N-acetylhexosamine 1-phosphate transferase enzyme superfamily (e.g. MraY/MurX, WecA, AlgH, and DPAGT1) in high-throughput manner.

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**Discovery of Natural Product-based Antimycobacterial Agents Effective against
Non-replicating Bacilli**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Shajila Siricilla
May 2017

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DEDICATION

To my husband Manoj Siricilla for his constant support, saneness, and for not letting me lose focus on the big picture of life.

To my parents Ummuselma Thacha Paramban and Abid Poolakkal for their endless love and for teaching me to dream.

To my daughter Zoe Siricilla who kept me rejuvenated with all her liveliness.

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ABSTRACT

New antimycobacterial molecules that kill non-replicating *Mycobacterium tuberculosis* (*Mtb*) were identified by screening libraries of synthetic natural products. De novo screening of a 400-membered library of aurachin RE analogs resulted in discovery of UT-317 ((*R*)-**20**). UT-317 is a selective vitamin K₂ biosynthesis (MenA) inhibitor that killed replicating and non-replicating *Mtb* at 2.31 µg/mL (MIC) and 0.85 µg/mL, respectively. A 50-membered library of capuramycin analogs was evaluated in their enzymatic inhibitory activities against translocase I (MraY/MurX) and prenyl-phosphate-GlcNAc-1-phosphate transferase (WecA). UT-01320 (**45**) is identified as a selective WecA inhibitor that kills both replicating and non-replicating *Mtb* at 1.50 µg/mL (MIC) and 2.58 µg/mL, respectively. UT-01320 killed the intracellular *Mtb* much faster than the first-line TB drugs such as isoniazid and rifampicin. A strong antimycobacterial agent, UT-800 (**64**) was identified by screening of a 50-membered library of pleuromutilin derivatives. UT-800 is a protein biosynthesis (50s ribosome) inhibitor which has activity focused against *Mtb*. UT-800 killed replicating and non-replicating *Mtb* at 0.83 µg/mL (MIC) and 1.20 µg/mL, respectively. In the course of these works, fluorescent probes, Park's nucleotide-*N*^ε-C₆-FITC (**32**, for MraY/MurX) and UDP-glucosamine-C₆-FITC (**46**, for WecA) were developed. These fluorescent probes enable us to screen the polyprenyl-phosphate *N*-acetylhexosamine 1-phosphate transferase enzyme superfamily (e.g. MraY/MurX, WecA, AlgH, and DPAGT1) in high-throughput manner.

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LIST OF ABBREVIATIONS

AA-A	Allylaminomethanone-A
ADC	Albumin, Dextrose, Catalase
ATCC	American Type Culture Collection
ATP	Adenosine Tri Phosphate
BDQ	Bedaquiline
CFU	Colony Forming Units
CFZ	Clofazimine
CoQ	Coenzyme Q (Ubiquinone)
CPS	Capsular Polysaccharides
DAPI	4',6-DiAmidino-2-PhenylIndole
DHNA	Di Hydro Naphthoic Acid
DMF	Di Methyl Formamide
DMMK	De Methyl Menaquinone
DMSO	Di Methyl Sulfoxide
EDTA	Ethylene Diamine Tetra Acetic Acid
EMEM	Eagle`s Minimum Essential Medium
ETS	Electron Transport System
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FIC	Fractional Inhibitory Concentration
FITC	Fluorescein Iso Thio Cyanate
GlcNAc	N- Acetyl Glucosamine

GPT	UDP-GlcNAc:dolichyl-P transferase
HCMI	Hydrazine Carboximidamide
HIV	Human Immunodeficiency Virus
HPLC	High Power Liquid Chromatography
HTS	High- Throughput Screening
INH	Isoniazid
LC-MS	Liquid Chromatography-Mass Spectrometry
LIN	Linezolid
LORA	Low Oxygen Recovery Assay
LTBI	Latent Tuberculosis Infection
MABA	Microplate Alamar Blue Assay
MDR-TB	Multi Drug Resistant Tuberculosis
MIC	Minimum Inhibitory Concentration
MK	Menaquinone
MKH ₂	Menaquinol
MOPS	4-Morpholine Propane Sulfonic Acid
MOX	Moxifloxacin
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>
<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide)
MurNGlyc	N- Glycolyl Muramic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance Spectroscopy

OADC	Oleic Acid, Albumin, Dextrose, Catalase
OBR	Optimum Background Regimen
OD	Optical Density
PEG	Poly Ethylene Glycol
P2HPT	Polyprenyl-Phosphate N-Acetyl Hexosamine 1-Phosphate Transferase Superfamily
PMBN	Polymyxin B Nonapeptide
PTC	Peptidyl Transferase Center
PYZ	Pyrazinamide
RIF	Rifampicin
RNAP	RNA Polymerase
SAR	Structural Activity Relationship
SCV	Small Colony Variant
TB	Tuberculosis
TDR-TB	Totally Drug Resistant Tuberculosis
THF	Tetrahydro Furan
TLC	Thin Layer Chromatography
UDP	Uridine Di Phosphate
UMP	Uridine Mono Phosphate
UV-VIS	Ultra Violet- Visible Spectroscopy
VK	Vitamin K
WHO	World Health Organization
XDR-TB	Extreme Drug Resistant Tuberculosis

CHAPTER 1. INTRODUCTION

Mycobacterium tuberculosis

Mycobacterium tuberculosis (*Mtb*) is an acid-fast bacterium that is responsible for tuberculosis (TB). The bacteria infect the lungs (pulmonary TB), but can infect any part of the body such as the kidney, spine, and brain. The reports of verified cases of TB are increasing worldwide, and TB remains one of the top ten causes of death. The World Health Organization (WHO) reported 1.4 million TB-related deaths and 10.4 million new TB incidents in the year 2015 alone (1). The upsurge in the global occurrence of multidrug-resistant TB (MDR-TB) and extreme drug resistant TB (XDR-TB) complicates the TB chemotherapy (2-4). The emergence of totally drug-resistant TB (TDR-TB) has also been reported in Iran, India and South Africa (5-7).

The current treatment regimen for drug-sensitive TB is a combination of four first-line drugs (rifampicin, ethambutol, isoniazid and pyrazinamide) for six months. The MDR-TB treatment is continued up to 20-28 months with first- and second-line drugs. It takes even longer to treat XDR-TB (8). Therefore, it is crucial to discover new drugs that can greatly shorten and/or simplify treatment regimen for drug-resistant strains of *Mtb*. The development of new TB drugs remains a difficult task. *Mtb* exists in different physiological states in hosts, and its tolerance to general antibacterial agents make TB drug discovery very challenging (9). *Mtb* has a thick cell wall which is composed of peptidoglycans, polysaccharides, glycolipids, and mycolic acid (**Figure 1-1**) (10,11). *Mtb* cell wall forms an impermeable barrier to many organic molecules including biopolymers (11). Such robust cell wall construction is essential for *Mtb* to promote intracellular survival within macrophages (12,13). The high rates of asymptomatic *Mtb* are due to the fact that *Mtb* can exist as the latent TB (LTB) for many years. In LTB infections, *Mtb* is present within the granulomas in alveoli by entering a dormant state (1,14). Therefore, the discovery of small molecules that kill the dormant state of *Mtb* at low concentrations is an important subject in TB drug development.

New TB Drugs

In the last four decades, only two new drugs have been approved for the treatment of *Mtb* infections. U.S. FDA approved bedaquiline in 2012, and delamanid was given the conditional approval by the European agency in 2013 for MDR-*Mtb* infections (15,16). **Table 1-1** summarizes the FDA-approved anti-tuberculosis drugs and their mode of actions. Novel regimens including new and repurposed drugs are being evaluated in different phases of clinical trials for the treatment of drug-susceptible and/or drug-resistant TB (17,18). In the pipeline of TB drugs, only two drug leads belong to new drug classes which exhibit a novel mode of action (**Table 1-2**). Q-203 (Qurient), a cytochrome bc1 complex inhibitor, entered phase I clinical trial. PA-824 (Pathogenesis Corp/Chiron) is a nitroimidazole that kills *Mtb* through intracellular nitric oxide release (19-21).

Figure 1-1. Cell wall of *Mycobacterium tuberculosis* in comparison with Gram-positive and Gram-negative bacteria.

Table 1-1. FDA-approved TB drugs and their molecular targets.

Use	Chemical Class	Name	Molecular Target
First Line	Rifamycin	Rifampicin	Protein Biosynthesis
	Nicotinic acid	Isoniazid	Mycolic acid biosynthesis
	Pyrazine	Pyrazinamide	Increases acidic pH
	Ethylenediamine	Ethambutol	Arabinogalactan biosynthesis
Second Line	Fluoroquinolones	Ofloxacin	DNA Gyrase
		Ciprofloxacin	DNA Gyrase
	Aminoglycosides	Amikacin	Protein Biosynthesis
		Kanamycin	Protein Biosynthesis
		Capreomycin	Protein Biosynthesis
	Isoxazolidinone	Cycloserine	Peptidoglycan Biosynthesis
		Ethionamide	Mycolic acid biosynthesis
	Nicotinamide	Para aminosalicylic acid	Folic acid biosynthesis
Other Drugs	Oxazolidinone	Linezolid	Protein Biosynthesis
	Diarylquinolone	Bedaquiline	ATP Synthase
	Nitroimidazole	Delamanid	Mycolic acid biosynthesis
	Riminophenazine	Clofazimine	

Table 1-2. New TB drug leads in different stages of clinical trial.

Name	Chemical Class	Molecular Target	Phase
PA-824	Nitroimidazole	Intracellular NO release	Phase III
Q-203	Imidazopyridine	Cytochrome bc1 (ETS)	Phase I
SQ-109	Ethylenediamine	Mycolic biosynthesis	Phase II ^a
Meropenem	Carbapenem	Cell wall biosynthesis	Phase II ^a
Faropenem	Carbapenem	Cell wall biosynthesis	Phase II ^a
High dose rifampicin	Rifamycin	RNA polymerase	Phase III
Linezolid	Oxazolidinone	50S ribosome	Phase III
Sutezolid	Oxazolidinone	50S ribosome	Phase II ^a
AZD5847	Oxazolidinone	50S ribosome	Phase II ^a
Moxifloxacin	Fluoroquinolone	DNA replication	Phase III
Gatifloxacin	Fluoroquinolone	DNA replication	Phase II ^a
Levofloxacin	Fluoroquinolone	DNA replication	Phase III
Metronidazole		DNA replication	Phase II ^a
PA824-MOX-PYZ	-	-	Phase II ^a
PA824-BDQ-LIN	-	-	Phase III
Delamanid-OBR	-	-	Phase III

NOTE: PYZ-Pyrazinamide, MOX-Moxifloxacin, BDQ-Bedaquiline, LIN-Linezolid, OBR-Optimized background regimen, ^a Completed.

Although a phase III trial of PA-824 was initiated in combination with moxifloxacin and pyrazinamide, an unexpected suspension of the clinical trial was reported due to toxicity (22,23). In May 2016, the TB Alliance noted that the trial may restart after making necessary addition of safety procedures (22). A different combination of PA-824, bedaquiline and linezolid is currently being evaluated in clinical studies (22,24). SQ-109 (25) reached a phase II trial but the clinical studies were discontinued due to lack of discernable advantage in efficacy over the control group (18,22,25-27). A phase II trial is completed for meropenem/faropenem plus amoxicillin and clavulanic acid. This trial evaluated the rapid bactericidal activity, safety and tolerability in patients with pulmonary tuberculosis (24). A clinical trial will assess the maximum tolerated dose of rifampicin and the rapid killing effect of higher doses of rifampicin against tubercle bacillus in the lungs (24). Novel oxazolidinones and fluoroquinolones have progressed to clinical studies (24). Metronidazole is aimed to develop into a second-line therapy for MDR-TB, and it was evaluated in a phase II study (24). The need for new TB drugs is obvious, and treatment regimens need to be simplified by introduction of new drugs that are effective against MDR-*Mtb* and their dormant state.

Dormant *Mycobacterium tuberculosis*

Mtb is a facultative aerobic bacterium which can also survive under anaerobic conditions. *Mtb* is thought to exist in a non-replicating (dormant) form particularly inside the caseous nodules of host lung granulomas where it has very little access to oxygen and nutrients (28-31). *Mtb* remains in the dormant form in these caseous pulmonary nodules for decades. In patients with compromised immune systems due to HIV/AIDS, the non-replicating bacilli are reactivated and progress the disease (32,33). The dormant form of *Mtb* shows resistance to many clinically used TB drugs. It is believed that the eradication of *Mtb* dormant form requires long-term chemotherapy for tuberculosis (34). If a drug effectively kills the dormant form at low concentrations, such a drug would have a significant effect on the duration of TB therapy.

Potential Targets Effective against Dormant *Mtb*

Dr. Kurosu published a comprehensive review of the potential targets effective against dormant *Mtb* (35). Based on the reported genetic experiments and effects of selective inhibitor molecules on the molecular targets, the genes associated with DNA gyrases, reductases (that produce toxic materials *via* activation of prodrugs), protein kinases, DosS/DosR systems, glyoxylate shunt, methyltransferases, electron transport systems, ATP synthase, menaquinone biosynthesis, protein biosynthesis, and cell wall biosynthesis remain actively expressed in dormant *Mtb* cells.

Table 1-3 summarizes investigational drug leads and FDA-approved drugs that affect the potential molecular targets for dormant *Mtb*. Fluoroquinolones, DNA gyrase inhibitors, were reported to kill dormant *Mtb in vitro*. Moxifloxacin and gatifloxacin

Table 1-3. Potential targets against dormant *Mtb*.

Potential Targets	Drug Leads	MIC _{replicating <i>Mtb</i>}	MIC _{non-replicating <i>Mtb</i>}	References
Protein biosynthesis	Pleuromutilins ^a	0.78-1.56 µg/mL ^a	1.04-1.98 µg/mL ^{a,b}	
	Rifampicin	0.05-0.2 µg/mL	1.5 µg/mL ^b	(47)
	Sutezolid	0.2-0.4 µg/mL	1 µg/mL ^b	(48)
	DA-7157	0.125-0.5 µg/mL	ND	(49)
	Spectinamides	0.8-1.6 µg/mL	0.06% viable cfu/mL at 10µg/mL ^b	(50)
	Linezolid	<0.125-4 µg/mL	65.2 µM ^b	(47,48,51,52)
DNA gyrase	AZD 5847	0.125-4 µg/mL	1 µg/mL ^b	(48,53)
	Moxifloxacin	0.125 µg/mL	15.9 µg/mL ^b	(47,52,54)
	Gatifloxacin	0.06 µg/mL	2 µg/mL ^b	(55)
	Mannich Base derivative	8-16 µg/mL ^c	ND	(56)
	Pyrrolamide derivatives	0.03-0.25 µg/mL	ND	(57)
	AB-1	0.06 µg/mL	1 µg/mL ^b	(58)
	Thiazolopyridone ureas	0.1 µM	ND	(59)
Methyltransferase	-	-	-	
DosS/DosR systems	Phenylcoumarines	-	> 99 % reduction in viability at 131µg/mL	(60)
Protein kinase	Benzo[g]quinoxaline	ND	ND	(61)
	AX20017	6-15 µg/mL	ND	(61-64)
		99% inhibition 10 µM ^d		
	VI-17494	20-50 µM ^d	ND	(61,63)
	VI-17499	20-50 µM ^d	ND	(61,63)
	VI-437	20-50 µM ^d	ND	(61,63)
	MRT67127	8-16 µM	ND	(65)

Table 1-3. (Continued).

Potential Targets	Drug Leads	MIC _{replicating <i>Mtb</i>}	MIC _{non-replicating <i>Mtb</i>}	References
Glyoxylate pathway	Mannich Base derivative	ND	ND	(66)
	3-nitropropionamides	0.16-3.3 μ M	0.04-1.72 μ M ^e	(67,68)
	Phenyl-diketo acids	<1-16 μ M	ND	(69)
	salicylanilide	0.25-8 μ M	ND	(70)
	IMBI-3	0.25-0.5 μ g/mL	ND	(71)
Activation <i>via</i> reductases	Metronidazole	>1000 μ M	8.56 μ M ^b	(72,73)
	PA-824	0.015-0.25 μ g/mL	2-10 μ g/mL ^b	(19,21,74)
	Delamanid	0.012	0.7-4.4 μ g/mL ^b	(75,76)
Electron transport systems	Chlorpromazine	0.23-3.6 μ g/mL ^d	-	(77,78)
	Trifluoperazine	10 μ g/mL	5 μ g/mL ^f	(79)
	Thioridazine	2-4 μ g/mL ^d	7.41-18.53 μ g/mL ^b	(80-82)
			18.53 μ g/mL ^e	
Menaquinone biosynthesis	Clofazimine	0.15-2.5 μ g/mL	0.8-1.5 μ g/mL ^b	(47)
	UT-317 ^a	2.31 μ g/mL ^a	0.85 μ g/mL ^{a,b}	(83)
	7-methoxy-2-naphthol derivatives	3-5 μ g/mL	ND	(84)
	Methyl and phenyl-butanoates	0.64 μ g/mL	1.5 μ g/mL ^b	(85,86)
	Sulfonyl adenosine analogs	ND	ND	(87,88)
F ₁ F ₀ ATP Synthase Cell wall biosynthesis	Bedaquiline	0.002-0.06 μ g/mL	1-2.78 μ g/mL ^b >11.11 μ g/mL ^e	(82,89)
	CPZEN 45	0.2-1.56 μ g/mL	ND	(90)
	UT-01320 ^a	1.56 μ g/mL ^a	2.58 μ g/mL ^{a,b}	(91)

Table 1-3. (Continued).

Potential Targets	Drug Leads	MIC_{replicating <i>Mtb</i>}	MIC_{non-replicating <i>Mtb</i>}	References
Cell wall biosynthesis	Meropenem+Clavulanate	0.32 µg/mL ^g	0.19~12.5 µg/mL ^{b,h}	(92)
	C-2 ester/oxadiazole substituted cephalosporins	>100 µg/mL	0.88 µg/mL ⁱ	(93)

NOTE: ^a Data generated in this study, ^b MIC obtained on *Mtb* cultured in hypoxic conditions, ^c MIC determined against *M. smegmatis*, ^d MIC obtained on intracellular *Mtb*, ^e MIC obtained on nutrient deficient *Mtb*, ^f MIC obtained on nitric oxide induced *Mtb*, and bacteriostatic effect was observed at 5 µg/mL concentration, ^g MIC of meropenem with 5µg/mL clavulanate, ^h MIC of meropenem with 2.5µg/mL clavulanate, ⁱ MIC obtained on multi stress model of non-replicating *Mtb*, ND – Not determined.

progressed to clinical development for use in combination with the other TB drugs (rifampicin, isoniazid and pyrazinamide)(24,36). PA-824 is thought to exhibit activity against dormant *Mtb* by activation *via* reductases (19,20,35). Ser/Thr protein kinases, PknA, B and G play an essential role in signal transduction in dormant *Mtb* (35,37). DosS/DosR, two component regulatory systems in *Mtb* are known to be important for its virulence and is responsible for the regulation of the dormancy state (35,38,39). The glyoxylate shunt is another pathway that was reported to be upregulated during dormancy (40).

Intracellular *Mtb* requires an alternative carbon metabolism because of the scarcity of carbohydrate and amino acid nutrients inside infected cells. *Mtb* in macrophages uptakes simple carbon compounds as carbon sources through the glyoxylate shunt mechanism (41). The key enzymes, malate synthase and isocitrate lyase have been studied in this pathway. Protein biosynthesis pathways have great potential to be effective targets for dormant *Mtb*. Menaquinone biosynthetic pathway is evaluated as a critical component for the survival of *Mtb* due to the fact that it directly links to the ATP production in both replicating and non-replicating *Mtb*. Although the importance of the cell wall for intracellular survival of *Mtb* has been discussed, the cell wall biosynthesis has not yet been established as a target for anti-dormant *Mtb*.

Menaquinone Biosynthetic Pathway

Lipoquinones are involved in the electron transport system (ETS) as electron carriers for the synthesis of ATP. *Mtb* and Gram-positive bacteria utilize menaquinone (MK) in their ETS (42). Gram-negative bacteria use ubiquinone (CoQ) in their ETS in aerobic conditions and menaquinone in anaerobic conditions (43,44) (**Figure 1-2A and B**). Humans utilize ubiquinone and lack a menaquinone biosynthetic pathway. Dhiman et al. have demonstrated that menaquinone synthesis is critical for mycobacterial growth, and its biosynthetic pathway presents a viable drug target (45). Although dormant *Mtb* is known to have less active metabolism and energy reserves, the ATP synthesis should be active in the dormant mycobacteria (46). Murphy et al. demonstrated that eight gene clusters involved in *Mtb* electron transport are upregulated in dormant *Mtb* (94). The ETS couples with F₁F₀ ATPase to produce ATP through oxidative phosphorylation; thus, the menaquinone biosynthetic pathway could be a selective target against *Mtb*. The biosynthetic pathway of menaquinone utilizes seven Men enzymes (MenA-MenG) (**Figure 1-2C**). Menaquinone biosynthesis begins with conversion of chorismate to isochorismate by MenF. MenD catalyzes the addition of α -ketoglutarate to isochorismate to form 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate. Subsequent steps catalyzed by MenC, MenE and MenB form 1,4-dihydroxy-2-naphthoate (DHNA). MenA (1,4-dihydroxy -2-naphthoate prenyl transferase) catalyzes the decarboxylative prenylation of DHNA to form demethylmenaquinone (DMMK). MenG catalyzes methylation of DMMK with S-adenosyl L-methionine (SAM) to complete the synthesis of MK. MenA is a membrane-associated protein that is predicted to have five

Figure 1-2. Electron transport system and menaquinone biosynthetic pathway.
 A) Electron transport system couples with F₁F₀ ATPase to produce ATP in *Mtb*. Phenothiazines inhibit NADH dehydrogenase II and bedaquiline inhibit F₁F₀ ATPase. Menaquinone is the only lipoquinone used as an electron carrier in *Mtb* ETS. B) MK transfers two electrons in ETS for the ATP synthesis and reduces to menaquinol (MKH₂). C) Menaquinone (MK) biosynthetic pathway: MenA is inhibited by our aurachin RE analogs.

transmembrane segment by SOSUI (95). Our group has studied the function of the MenA enzyme towards the development of new drug leads against MDR-*Mtb* and dormant *Mtb* (45,96-98).

To date, several groups have identified menaquinone biosynthesis inhibitors and validated MenB, MenE, and MenA as drug targets for development of new antibacterial agents (**Figure 1-3**). Lu et al. reported a series of adenosine-phosphate mimics which showed MenE enzyme inhibitory activity (87,88). Matarlo et al. demonstrated that the 4-oxo-4-phenylbutanoate derivatives are effective inhibitors of MenB and exhibit antibacterial activity against MRSA and *Mtb* (86). Xu et al. identified that an oxythiamine derivative is an anti-Staphylococcal MenD inhibitor (99).

MenA is the sixth enzyme of the MK pathway. Our group has pioneered design and synthesis of a series of selective MenA inhibitors (83,97). The identified MenA inhibitors showed significant growth inhibitory activities against drug-resistant Gram-positive bacteria including *Mtb*. Significantly, the selective MenA inhibitors killed non-replicating *Mtb* at equal or lower to/than the MIC concentrations for replicating *Mtb* (93,96). Choi et al. reported 2-naphthol-based MenA inhibitors with bactericidal activity against *Mtb* and methicillin-resistant *S. aureus* (MRSA) (84).

Cell Wall Biosynthesis

The thick cell wall of *Mtb* is known to play an important role in its intracellular survival (100,101). Peptidoglycan (PG) biosynthesis has been extensively studied, but only a few enzymes associated with PG biosynthesis (e.g. transpeptidase of penicillin binding proteins (PBPs) and MurA) were of interest to the drug industry for antibacterial drug discovery. Comparisons with gene expression studies of *Mtb* at the exponential phase and the non-replicating state indicated that the genes associated with extracellular materials (arabinoxyl transferases, lipoarabinomannan biosynthesis, mycolic acid biosynthesis, and other enzymes associated with reconstructions of cell walls) are up-regulated in dormant *Mtb* (102). Therefore, inhibition of the committed steps of the mycolyl-arabino galactan biosynthesis will affect the intracellular growth and dormancy of *Mtb* cells. We have studied polyprenyl-phosphate N-acetylhexosamine-1-phosphate transferase enzyme superfamily (P2HPT) (103). In *Mtb*, WecA catalyzes the first membrane-associated step that anchors the phospho-GlcNAc moiety of UDP-GlcNAc to decaprenyl phosphate (C₅₀-P), leading to the formation of mycolyl-arabino galactan (104) (**Figure 1-4**). We expect that inhibition of WecA could impair the structure of the mycolyl-arabino galactan in *Mtb*, significantly increasing intracellular killing effect in macrophages or epithelial cells.

WecA homologs include MraY/MurX, TagO, WbcO, WbpL, RpgG, WbaP and eukaryotic UDP-GlcNAc:dolichyl-phosphate transferase (GPT) (105-107). MraY/MurX catalyzes the first membrane-anchored step in PG biosynthesis (108). MurX is specific to UDP-N-acetyl muramyl-pentapeptide (Park's nucleotide), forming decaprenyl-

Figure 1-3. Menaquinone biosynthesis inhibitors.

1 - The MenE inhibitor reported by Lu et al, **2** - The MenB inhibitor reported by Matarlo et al, **3** - The MenD inhibitor reported by Xu et al, **(R)-20** - A menA inhibitor, This study (Chapter 2), **5** - The MenA inhibitor reported by Choi et al.

Figure 1-4. Biosynthesis of peptidoglycan and mycolyl-arabinogalactan in *Mtb*. MurX and WecA utilize decaprenyl phosphate. MurX catalyzes transformation from Parks nucleotide to lipid I, while WecA transfers from UDP-GlcNAc to C₅₀-P-P-GlcNAc. Tunicamycin, CPZEN-45 and muraymycin D1 are inhibitors of both WecA and MurX. We identified a selective WecA inhibitor, UT-01320 (**45**).

diphosphoryl-N-acetylmuramyl-pentapeptide (lipid I) (**Figure 1-4**). We demonstrated that MurX inhibitors are effective in killing only replicating *Mtb* under aerobic conditions. On the contrary, WecA inhibitors are effective in killing non-replicating *Mtb* under oxygen-depleted conditions. To date, only tunicamycin, CPZEN 45, and muraymycin D1 have been demonstrated to have enzyme inhibitory activity against both MurX and WecA (90,109,110). We identified, for the first time, that a capuramycin derivative, UT-01320 kills non-replicating *Mtb* by targeting WecA selectively.

Protein Biosynthesis

Bacterial protein biosynthesis is a clinically validated drug target. However, there is very little information related to the translation steps and dormant form of *Mtb*. Rifamycin, DNA-dependent RNA polymerase inhibitor is active against non-replicating *Mtb* at higher concentrations. Aminoglycoside antibiotics bind to the 30S bacterial ribosomal subunit that effectively kill non-replicating *Mtb in vitro*. The oxazolidinone class of protein biosynthesis inhibitors (e.g. linezolid and sutezolid) are also effective in killing non-replicating *Mtb* (48,50,111,112). We confirmed effectiveness of antimycobacterial protein biosynthesis inhibitors for non-replicating *via* the Wayne dormancy model and low oxygen recovery (LORA) assays. Although several protein biosynthesis inhibitors are listed in the second-line TB drugs (e.g. amikacin and capreomycin), the narrow safety margin of FDA-approved protein biosynthesis inhibitor drugs discourage physicians from applying them to treat MDR- and XDR-*Mtb* infections (113-116).

Pleuromutilin and its derivatives have been studied for development of new anti-Staphylococcal infections. To date, very few pleuromutilin derivatives have been developed for MDR-*Mtb* infections, and their efficacy against non-replicating *Mtb* is not known. **Figure 1-5** illustrates the mode of actions of protein biosynthesis inhibitors and the binding site of pleuromutilin in 50s ribosomes. Pleuromutilin interferes with both the A-site and P-site of the 50s ribosomal subunit (117,118). It was evident that the pleuromutilin binding site overlaps only partially with the linezolid (an A-site binder) binding site in the 50s ribosome (118,119). In a cell-based screening of pleuromutilin derivatives against a drug sensitive *Mtb* (H37Rv), several new molecules exhibited bactericidal activity with an MIC of below 1.0 µg/mL. The identified derivatives displayed significant effect in killing non-replicating *Mtb in vitro*.

Dissertation Hypothesis and Specific Aims

A fundamental problem in the treatment of tuberculosis (TB) is the requirement of long-term treatment with multiple drugs. Because the treatment for latent TB infection is a reason for long duration of therapy, the discovery of a drug that effectively kills non-replicating (dormant) *Mtb* will have the potential to significantly reduce TB treatment time. During the Ph.D. period (2011-2016), I have studied three drug targets that are

Figure 1-5. Mode of action of protein biosynthesis inhibitors against *Mtb*.

A) Pleuromutilins and linezolid inhibit peptidyl transfer in elongation step. Rifampicin inhibits RNA polymerases (RNAP) and aminoglycosides inhibit ribosomal translocation step. B) Pleuromutilins and linezolid bind to different sites at 50S ribosomal subunit.

essential for maintaining the viability of dormant form of *Mtb*: menaquinone (vitamin K₂), mycolylarabinogalactan and protein biosynthetic pathways. The hypothesis was that, validation of selective inhibitors of these molecular targets in our *in vitro* assays will provide valuable insight into the development of new TB drugs that are effective in killing dormant *Mtb*.

Aim 1. Discovery of Selective MenA Inhibitors

Mtb uses menaquinone in both aerobic and anaerobic conditions as an electron carrier for ATP synthesis, while humans solely use ubiquinone. We identified that a quinolone natural product, aurachin RE exhibited MenA enzyme (the sixth enzyme in the menaquinone biosynthetic pathway) inhibitory activity and a broad-spectrum of activity against Gram-positive bacteria including *Mtb* (83,120). A 400-membered library of aurachin RE analogs available in our group were screened by a de novo assay strategy that can identify selective MenA inhibitors with a narrow spectrum of activity focused against *Mtb*.

Aim 2. Discovery of WecA-type Phosphotransferase Inhibitors

Undecaprenyl-phosphate α -N-acetylglucosaminyl 1-phosphate transferase (WecA) enzyme is an essential enzyme for the biosynthesis of mycolyl-arabinogalactan conjugate and growth of *Mtb*. Maintenance of integrity of the cell wall is important for *Mtb* to survive in macrophages, and thus inhibition of WecA-type phosphotransferase is believed to be effective in killing non-replicating *Mtb*.

Capuramycin is a natural product that inhibits peptidoglycan biosynthesis by selectively targeting MurX-type phosphotransferase. We discovered that a chemical modification of capuramycin alters its molecular target and one of the analogs UT-01320 (**45**) kills non-replicating *Mtb* at low concentrations. In order to identify selective WecA inhibitors, we have developed new WecA- and MurX-type assay methods using the fluorescent probes **32** and **46**. Robustness of our fluorescent-based assays is demonstrated by screening a 50 membered-library of capuramycin analogs. Through *in vitro* profiling of the selective WecA inhibitor **45** is performed.

Aim 3. Discovery of New 50S Ribosome Inhibitors: Antimycobacterial Pleuromutilin Analogs

Pleuromutilin analogs exhibit strong 50S ribosome inhibition and have been developed for the infections caused by drug resistant strains of Gram-positive bacteria. In our program of extended spectrum of activity of pleuromutilin, our group has generated a library of new analogs and screens via the MABA and LORA assays. We identify novel pleuromutilin analogs with a narrow spectrum of activity towards *Mtb* that effectively kills replicating and non-replicating *Mtb* at very low concentrations. *In vitro* and *in vivo*

toxicity studies are performed to advance an antimycobacterial pleuromutilin for *in vivo* efficacy studies using an infected animal model.

CHAPTER 2. MENAQUINONE BIOSYNTHETIC PATHWAY*

Function of ubiquinone (coenzyme Q₁₀) as a component of the mitochondrial respiratory chain in human is well established (“the chemiosmotic theory”, Mitchell, 1978) (121-123). In prokaryotes, especially in Gram-positive bacteria, menaquinone transfers two electrons in a process of either aerobic or anaerobic respiration. On the other hand, a majority of Gram-negative organisms utilize ubiquinone under aerobic conditions and menaquinone under anaerobic conditions in their electron transport systems (44,124,125). Therefore, inhibitors of menaquinone biosynthesis or specific inhibitors of enzymes associated with electron transport systems have great potential for the development of novel and selective drugs against MDR Gram-positive bacteria (96). The electron transport system couples with ATP synthase to produce ATP through oxidative phosphorylation. Bacterial ATP synthase, F₁F₀-ATPase, is a viable target for treatment of MDR *Mtb* infections. A diarylquinoline, bedaquiline, is an inhibitor of *Mtb* ATP synthase that exhibited a remarkable activity against *Mtb* (126). However, only few studies have investigated the electron transport system for development of new antibacterial drugs (96). Weinstein and co-workers reported the inhibitors of type II NADH: menaquinone oxidoreductase that effectively killed *Mtb in vitro* and they concluded that type II NADH dehydrogenase could be a unique and interesting antimicrobial target (77). We have reported that the inhibition of MenA (1,4-dihydroxy-2-naphthoate prenyltransferase), which catalyzes a formal decarboxylative prenylation of 1,4-dihydroxy-2-naphthoate (DHNA) to form demethylmenaquinone (DMMK) in menaquinone biosynthesis (**Figure 2-1**), showed significant growth inhibitory activities against drug resistant Gram-positive bacteria including *Mtb* (97,98). In menaquinone biosynthesis, MenD (2-succinyl-5-enoylpyruvyl-6-hydroxy-3-cyclohexane-1-carboxylic acid synthase), MenE (an acyl-CoA synthase), and MenB (1,4-dihydroxynaphthoyl-CoA synthase) have recently been studied for the development of novel drug lead for Gram-positive pathogens including *Mtb* (88,127-129).

In an attempt at finding a new pharmacophore for MenA inhibitors, we identified a new quinolone natural product, aurachin RE, **6 (Figure 2-2A)** which exhibited MenA enzyme inhibitory activity as well as a wide antimicrobial spectrum against Gram-positive bacteria (120). As there is an overall structural resemblance between aurachin RE and our 1st generation MenA inhibitors (**7 and 8 (Figure 2-2B and C)**) or menaquinone, aurachin RE’s antibacterial activity could be attributed to a synergistic effect of respiratory chain and menaquinone biosynthesis inhibitory activities. The identification of aurachin RE’s MenA enzyme inhibitory activity encouraged us to redesign and synthesize chiral MenA inhibitor molecules, in which *primary* or *secondary* alcohol was introduced in the side chain of the 1st generation MenA inhibitor molecules

* Adapted with permission. Debnath, J., Siricilla, S., Wan, B., Crick, D. C., Lenaerts, A. J., Franzblau, S. G., and Kurosu, M. (2012). Discovery of selective menaquinone biosynthesis inhibitors against *Mycobacterium tuberculosis*. *J. Med. Chem.* 55 3739-3755.

Figure 2-1. Biosynthesis of menaquinone in *Mtb*.

MenA catalyzes the prenylation of DHNA to form DMMK, this is one step prior to the formation of menaquinone.

Figure 2-2. MenA inhibitors.

A) Aurachin RE is a natural product with MenA inhibitory activity. B and C) Our first generation MenA inhibitors (7 and 8).

(97,98). Our lab has generated over 400 molecules with >95% purity either in solution or on polymer-support, and these molecules were evaluated *in vitro* (IC₅₀) against menA enzyme and bacterial growth inhibitory assays (38). **Figure 2-3** illustrates our assay scheme to identify selective MenA inhibitors against *Mtb*. Out of these molecules, 26 compounds were identified to exhibit the *in vitro* biological activities which met the assay criteria summarized in **Figure 2-3**. Based on obtained SAR from a 400-membered library, it became evident that the topology of the N atom in the inhibitor molecules plays an important role in selectivity of the MenA enzymatic and bactericidal activities (*Mtb* vs. *S. aureus*). As summarized in **Figure 2-4**, selective mycobactericidal molecules (**9–13**) possess the secondary or tertiary amine in the near center of the molecules (highlighted moieties in **9–13** in **Figure 2-4**), whereas the topology of the N atom of the molecules possessing antibacterial activities against both *Mtb* and *S. aureus* (**14–17**) locates the right half of the molecules (highlighted moieties in **14–17** in **Figure 2-4**). We have identified selective antimycobacterial MenA inhibitors in their racemic forms. In order to obtain insight into the effect of chirality of new MenA inhibitors (**9–13** in **Figure 2-4**), we commenced syntheses of the optically active forms of the identified inhibitors (**Figure 2-5**).

Results and Discussion

Selective *Mtb* MenA Inhibitors: Assay Strategy

Antimicrobial spectrum focused against *Mtb* (selective antimycobacterial agent) is preferable for TB chemotherapy (130). We realized that the peptide sequences of the *Mtb menA* and *S. aureus menA* gene products are only 32% identity and 50% similarity in the BLAST experiment (131). Indeed, we have identified several molecules that exhibit selective MenA enzyme and bacterial growth inhibitory activities against *Mtb*; more than a 10-fold higher inhibitory activity against *Mtb* than *S. aureus*. In order to develop MenA inhibitors which are selective against *Mtb*, molecules generated in this program were first evaluated in MenA enzyme inhibitory assays. Only molecules exhibited *Mtb* MenA activity over *S. aureus* MenA (IC₅₀ < 20 μ M, >60 μ M against *Mtb* MenA and *S. aureus* MenA, respectively) were evaluated in bacterial growth inhibitory assays (MICs) using *Mtb*, *S. aureus*, and *E. coli* (**Figure 2-3**). The molecules exhibited good activity only against *Mtb* (MICs for *Mtb*, *S. aureus*, and *E. coli* are <12.5, >60, and >125 μ g/mL, respectively) were evaluated in *E. coli* growth inhibitory assays under anaerobic conditions followed by menaquinone supplementation assays (*E. coli* utilize only menaquinone in their electron transport system under anaerobic conditions) (43). This assay confirmed that the molecules killed bacteria by targeting MenA enzyme or electron transport systems (*vide infra*). Oxygen consumption assay using *Mtb* identifies electron transport system inhibitors; in this assay, selective MenA inhibitors should not show activity at concentrations below the MIC against *Mtb* (132,133). Selective antimycobacterial MenA inhibitors confirmed *via E. coli* growth inhibitory assays (under anaerobic conditions) and oxygen consumption assays were evaluated for their activity

Figure 2-3. Screening strategy for selective MenA inhibitor drug leads against *Mtb*.

Structures of selected MenA inhibitor molecules		Bactericidal activity ^a	
		<i>Mtb</i>	<i>S.aureus</i>
	<p>Aurachin RE (6)</p>	+	+++
9		++	-
10		++	-
11		++	-
12		+++	-
13		++	-
14		+	+++
15		++	+
16		++	+++
17		+++	+
	<div>Mtb Selective inhibitor</div> <div>Mtb + Gram-Positive</div>		

Figure 2-4. Topology of N atom in *Mtb* selective compounds.

+++ (0.5-8.0 µg/mL), ++ (8.0-15.5 µg/mL), + (15.5-30.0 µg/mL), - (>50 µg/mL),

^a Minimum inhibitory concentration in µg/mL.

Figure 2-5. Optically active compounds and their derivatives.

against non-replicating *Mtb* via the low oxygen recovery assays (LORA) and nutrient deficient model assay (Loebel). LORA assay is an *In vitro* modification of Wayne's model and was developed by Dr. Scott Franzblau and colleagues of Illinois TB Research Institute, USA (47). LORA assay evaluates potency against non-replicating *Mtb* cells under low oxygen conditions and Loebel model assay measures the potency against non-replicating *Mtb* cells under nutrient depleted conditions.

MenA Enzyme Inhibitory Assays

MenA enzymatic assays of generated molecules were originally performed with [³H] farnesyl diphosphate and MenA containing membrane fraction (45). This assay requires a significant effort to separate demethylmenaquinone (DMMK) from the reaction mixtures and includes the significant cost for [³H]farnesyl diphosphate. In order to simplify the procedure and to reduce the cost of MenA enzyme inhibitory assays, we have developed a MenA assay using HPLC. In new MenA assay developed by our group, the MenA product, demethylmenaquinone can readily be quantitated *via* UV absorbance (DMMK; λ 2325 nm).

We performed a preliminary screening of the activity of compounds synthesized (**Figure 2-5**) at the single concentrations of 100 μ M. In these MenA enzyme inhibitory assays, the optically active molecules were assayed against *Mtb* and *S. aureus* MenA. IC₅₀ values of the molecules which exhibited activity against *Mtb* MenA and inactivity against *S. aureus* MenA were calculated. Of 86 optically active molecules synthesized, 26 molecules exhibited *Mtb* MenA inhibitory activity and were inactive against *S. aureus* MenA at 100 μ M concentrations. Dose-response plots (DMMK formation vs. concentrations of inhibitor) were obtained for 26 molecules to determine IC₅₀ values. Significant effect of chirality in the MenA enzyme inhibitory activity was observed in the molecules in classes A and D. On the contrary, an obvious effect of chirality in MenA enzyme inhibitory activity of the molecules in classes B, C, and E was not observed; the racemic forms and each enantiomer did not show noticeable difference in MenA enzyme inhibitory activity. It is noteworthy that the carbamate analog (*R*)-**19** group showed 6-fold better MenA enzyme inhibitory activity compared to the alcohol form (*R*)-**18**. Similar improvement of enzymatic inhibition by modification of the alcohol with the carbamate group is observed in the molecules in class D.

Bacterial Growth Inhibitory Assays

All molecules that showed activity against MenA in a preliminary assay at 100 μ M concentrations were evaluated for their mycobacterial growth inhibitory activity *via* the microplate alamar blue (MABA) and low oxygen recovery (LORA) assays (47,74,134). Briefly, the MABA assay is a colorimetric assay that uses the color change of resazurin to evaluate *Mtb* growth inhibitory activity under aerobic conditions. On the other hand, the LORA assay evaluates potency against non-replicating *Mtb* cells under low oxygen conditions using a luminescent reporter strain. Significantly, in all cases the

MIC values obtained from the LORA assays are lower than those from the MABA assays. To the best of our knowledge, it is the first observations that the molecules killed non-replicating *Mtb* at concentration below the MIC against *Mtb* grown under aerobic conditions; in all cases the values of MIC_{LORA}/MIC_{MABA} were <1 (**Table 2-1**). In the class A molecules (*R*)-**9** was two-fold more potent than *rac*-**9** and (*S*)-**9** in the MABA assays. Similar to an observed trend in MenA enzyme inhibitory assays, the carbamate (*R*)-**19** could improve over 5-fold increase in mycobactericidal activity compared to *rac*-**9**. (*R*)-**19** exhibited a significant activity in the LORA assay; the MIC value of (*R*)-**19** is 1.7-fold more effective in killing non-replicating bacteria than rifampicin (MIC_{LORA} 1.47 µg/mL). It is worth mentioning that (*R*)-**19** was the most active in killing non-replicating *Mtb in vitro* among antimycobacterial drugs (approved by FDA) and preclinical drugs tested in our laboratory. Regardless of the stereochemistry of the chiral centers, the molecules in class B, C, and E did not show noticeable difference in antimycobacterial activity in the both MABA and LORA assays. The molecules in class D are regioisomers at the benzophenone moiety of the class A molecules. Similar to the molecules in class A, pronounced effect of the stereochemistry of secondary alcohol on antimycobacterial activity was observed in the molecules in class D. Contrary to the chirality effect observed in the molecules in class A, the molecules possessing *S*-configuration (in class D) exhibited better antimycobacterial activity than the corresponding *R*-configuration molecules. The effect of carbamate group on enzyme and bacterial growth inhibitory activities was not observed in the molecules in class D; the MABA MIC value of the carbamate (*S*)-**23** was equal to the corresponding alcohol (*S*)-**24**. Nonetheless, the MABA MIC for the *rac*-alcohol **12** could be improved two-fold by the formation of carbamate; the MABA MICs of *rac*-**12** and *rac*-**23** were 6.25 and 3.25 µg/mL, respectively. Antimycobacterial activity was improved by increasing the hydrophobicity of the side chain (C8 vs. C6) in the molecules of class A, whereas noticeable bactericidal effect by increasing hydrophobicity of the side chain was not observed in the molecules of class D. All molecules summarized in **Figure 2-5** did not exhibit bactericidal activity against *S. aureus* at 60 µg/mL concentrations; lack of anti-staphylococcal activity of these molecules was well-correlated with their *S. aureus* MenA enzyme inhibitory activity (IC₅₀ >60 µM against *S. aureus* MenA).

***E. coli* Growth Inhibitory Assays under Anaerobic Conditions**

In aerobic conditions, *E. coli* uses ubiquinone in its ETS. However, menaquinone is the lipoquinone used in anaerobic growth conditions. *E. coli* grown in anaerobic conditions may be used as a convenient surrogate for *Mtb* for initial screening of menaquinone inhibitors. We evaluated several known inhibitors of different targets for their effects on *E. coli* under anaerobic conditions, including electron transport system and menaquinone biosynthesis (**Table 2-2**). Only menA and electron transport inhibitors killed *E. coli* under anaerobic conditions. **Figure 2-6A** summarizes the effectiveness of inhibitors of different targets against *E. coli* under anaerobic conditions. Evaluation of gram negative bacterial inhibitors is a challenge due to efflux pumps. Bacterial efflux pumps are known to be active in anaerobic conditions, therefore an efflux pump inhibitor,

Table 2-1. Antibacterial activities against *Mtb*/*S. aureus*, and MenA enzyme inhibitory activities of all selected molecules.

Compound	MIC(μ g/mL)			MenA Inhibition (μ M)		
	<i>S.aureus</i> ^a	<i>Mtb</i> (MABA)	<i>Mtb</i> (LORA)	<i>S.aureus</i> ^b	<i>Mtb</i> ^b	IC ₅₀
Class A						
<i>rac</i> -9	>60	12.5	5.20	-	+	15.0
(<i>S</i>)-9	>60	12.5	5.59	-	+	7.5
(<i>R</i>)-9	>60	6.25	4.91	-	+	17.0
(<i>S</i>)-18	>60	12.5	4.91	-	+	16.0
(<i>R</i>)-18	>60	6.25	5.59	-	+	9.0
(<i>S</i>)-19	>60	12.5	2.94	-	+	9.0
(<i>R</i>)-19	>60	2.31	0.85	-	+	1.5
(<i>S</i>)-20	>60	12.5	12.5	-	+	15.0
(<i>R</i>)-20	>60	2.31	0.85	-	+	1.2
Class B						
<i>rac</i> -10	>60	12.5	1.88	-	+	15.0
(<i>S</i>)-10	>60	12.5	1.88	-	+	25.0
(<i>R</i>)-10	>60	12.5	1.46	-	+	20.0
(<i>S</i>)-21	>60	12.5	4.93	-	+	20.0
(<i>R</i>)-21	>60	12.5	4.93	-	+	15.0
(<i>R</i>)-22	>60	6.25	1.43	-	+	15.0
Class C						
<i>rac</i> -11	>60	12.5	3.00	-	+	15.0
(<i>S</i>)-11	>60	12.5	3.00	-	+	17.0
(<i>R</i>)-11	>60	12.5	3.00	-	+	14.0
Class D						
<i>rac</i> -12	>60	6.25	2.82	-	+	15.0
<i>rac</i> -23	>60	3.25	2.83	-	+	7.5
(<i>S</i>)-12	>60	1.50	1.43	-	+	4.5
(<i>R</i>)-12	>60	6.25	2.85	-	+	7.5
(<i>S</i>)-23	>60	1.50	1.43	-	+	3.5
(<i>R</i>)-23	>60	6.25	5.20	-	+	9.5
(<i>S</i>)-24	>60	1.50	1.45	-	+	1.5
(<i>S</i>)-25	>60	1.50	1.40	-	+	1.5
Class E						
<i>rac</i> -13	>60	12.5	1.88	-	+	20.0
(<i>S</i>)-13	>60	12.5	1.88	-	+	15.0
(<i>R</i>)-13	>60	12.5	1.46	-	+	15.0
(<i>S</i>)-26	>60	12.5	2.93	-	+	20.0
(<i>R</i>)-26	>60	12.5	4.93	-	+	20.0
(<i>S</i>)-27	>60	12.5	5.54	-	+	20.0
(<i>R</i>)-27	>60	12.5	5.54	-	+	15.0
RIF		0.2	1.47			
INH		0.1	>128			

Table 2-1. (Continued).

NOTE: ^a MIC determined by broth dilution method, ^b At 100μM concentration of the compounds, + (MenA inhibition), - (No MenA inhibition), MABA -Microplate alamar blue assay, LORA -Low oxygen recovery assay, RIF- Rifampicin, INH – Isoniazid.

Table 2-2. Activity of compounds against *E. coli* under anaerobic conditions.

Target	Compounds	Inhibition under Anaerobic Conditions	Inhibition under Aerobic Conditions (µg/mL)
MenA	AA-A, 7	++	>200
	UT – 317 ((<i>R</i>)- 20)	++	>200
	UT – 281R	++	>200
	UT – 292R	++	>200
ETS	Chlorpromazine	++	60 ^a
	Pentachlorophenol	++	20 ^a
	Methylene blue	++	>200
	Hydrazinecarboximidamide	++	>200
	Atovaquone	-	>200
	Trifluoperazine	-	100
	Clofazimine	-	>200
	Resazurin	-	>200
	Doxycycline	+	1.5 ^a
Protein synthesis	Pleuromutiline	+	25
	Capreomycin	+	>25
	Chloramphenicol	+	4 ^a
	Spectinomycin	+	25
	Tobramycin	+	4
	Amikacin	+	4
	Erythromycin	-	30 ^a
	Clindamycin	-	12.5-50 ^a
	Linezolid	-	>200
	Kanamycin	-	19
	Rifampicin	+	16
RNA polymerase	Ciprofloxacin	+	5
DNA Gyrase	Trimethoprim	+	>200
Folic acid biosynthesis	Sulfamethoxazole	+	>200
Efflux pumps	Verapamil	+	>200
Cell permeability	Gramicidine	-	>200
PG biosynthesis	Penicillin G	-	64 ^a
	Cephalosporin C	-	64 ^a
	Vancomycin	-	>200
Vitamin K analog	Warfarin	-	>200
Membrane perturbing	Polymyxin B nonapeptide	-	64 ^a

NOTE: +++ Bactericidal (<25 µg/mL), + Bacteriostatic, - No inhibition at 200 µg/mL,
^aReported MIC values.

Figure 2-6. *E. coli* growth inhibitory assays under anaerobic conditions.

A) Summary of bactericidal and bacteriostatic effect of compounds against *E. coli* under anaerobic conditions by target. B) Effect of polymyxin B nonapeptide (PMBN) on MIC of allylaminomethanone-A (AA-A, **7**) in inhibition of *E. coli* under anaerobic conditions. Amount of PMBN added was 25 μg/mL. C) Rescue study - Exogeneous menaquinone was added in varying concentrations to evaluate if the *E. coli* cells under anaerobic conditions can be rescued from the effect of AA-A and HCMI.

verapamil was supplied in the assay along with inhibitor molecules to reduce the efflux of inhibitor molecules. Additionally, we supplemented membrane perturbing agent, polymyxin B nonapeptide (PMBN) to improve permeation of compounds into cells. In anaerobic conditions, verapamil did not show a major effect on the MICs of inhibitor compounds while supplementation of PMBN (25 µg/mL) was able to reduce the MIC of AA-A, **7** from 50 µg/mL to 25 µg/mL (**Figure 2-6B**). AA-A and other MenA inhibitors were ineffective against *E. coli* under aerobic conditions but displayed different effectiveness profile by being bactericidal under anaerobic conditions.

Our group has previously demonstrated that *Mtb* or *S. aureus* treated with the MenA inhibitors could not be rescued completely even at higher concentrations of exogenous menaquinone (MK/VK₂). In contrast, growth inhibition of *E. coli* by the MenA inhibitor could be rescued by supplementation of VK₂ (6 µg/mL) under anaerobic conditions. *E. coli* growth inhibition rescued by addition of VK₂ may be attributed to the degree of permeability of VK₂ through cell envelope. Although lack of activity of MenA inhibitors against Gram-negative bacteria grown under aerobic conditions have been demonstrated, all MenA inhibitors identified in this program showed growth inhibition of *E. coli* at 5~10 µg/mL concentrations under anaerobic conditions, and the inhibitory effect of MenA inhibitor was rescued by supplementation of VK₂ (**Figure 2-6C**). Therefore, these convenient cell-based assays using *E. coli* under anaerobic conditions can be utilized to confirm that the inhibitor molecules kill Gram-positive bacteria including *Mtb* by targeting MenA biosynthesis or bacterial electron transport systems.

Oxygen Consumption Assays

The basic concept underlying bacterial oxygen consumption assay is that changes in the rate of oxygen uptake result in a change in the oxygen concentrations. The oxygen consumption by bacterial concentrations greater than 10⁸ cfu/mL bacteria is proportional to the concentration of bacteria. Effect of the inhibitor molecule on electron transport by the quantitation of decolorization of methylene blue, which is a well-known redox dye, has been unambiguously demonstrated. Oxygen-consumption assays of (*R*)-**19** and (*S*)-**24** showed decolorization of methylene blue at concentrations (12.5 µg/mL) above the MIC of each molecule (MICs 2.31 and 1.50 µg/mL, respectively) against *Mtb*. Thus, we have concluded that (*R*)-**19** and (*R*)-**24** are very weak (or are not) electron transport system inhibitors, and thus, antimycobacterial activity of these molecules are attributed by targeting menaquinone biosynthesis.

Cytotoxicity of MenA Inhibitors

In order to obtain insight into potential toxicity of identified inhibitor molecules, all antimycobacterial MenA inhibitors were evaluated in *in vitro* cytotoxicity assays using Vero monkey kidney cells and HepG₂ human hepatoblastoma cells. Most MenA inhibitors possessing the benzophenone group showed IC₅₀ of 1–6.5 µg/mL against two mammalian cell lines; selectivity indexes (IC₅₀ in mammalian cells/MIC against *Mtb*) of

a series of benzophenone MenA inhibitors identified in this program were less than 2.49. On the other hand, O-methyl oxime derivatives in class A showed approximately 10-fold less cytotoxic than the corresponding benzophenone derivatives in *in vitro* cytotoxicity assays (**Figure 2-7**); (*R*)-**20** showed an encouraging *in vitro* activity/toxicity ratio (a SI value >10). It is believed that electrophilicity of the benzophenone moiety of (*R*)-**19** can be diminished by O-methyl oxime formation of the benzophenone. Thus, the benzophenone O-methyl oxime may not be a good electron acceptor that interferes with redox systems of mammalian cells.

Antibacterial Activities of (*R*)-**19** and (*R*)-**20** against Drug-resistant *Mtb* and Other *Mycobacterium* Species

Two MenA inhibitors molecules (*R*)-**19** and (*R*)-**20** showed MIC values of 2.31 µg/mL against *Mtb* (H₃₇Rv, a common laboratory strain). We resynthesized (*R*)-**19** and (*R*)-**20** and determined MICs against several other *Mycobacterium* species and drug-resistant *Mtb*. As summarized in **Table 2-3**, (*R*)-**19** and (*R*)-**20** showed mycobactericidal activity against rifampicin- and isoniazid-resistant strains at slightly lower concentrations (entries 2 and 3). (*R*)-**19** and (*R*)-**20** also killed other *Mycobacterium* species such as *M. bovis*, *M. intracellulare*, and *M. smegmatis* at relatively low concentrations (3.0~6.5 µg/mL). Thus, we concluded that new MenA inhibitors identified in this program kills *Mycobacterium* species selectively and are especially effective in killing *Mtb* at low concentrations. MenA inhibitors (*R*)-**19** and (*R*)-**20** effectively inhibited growth of drug-resistant *Mycobacterium* species, indicating that MenA is a valid drug target to develop new drugs for MDR-*Mycobacterium tuberculosis*.

Conclusions

We have identified novel MenA (1,4-dihydroxy-2-naphthoate prenyltransferase) biosynthesis inhibitors by assay guided inhibitor design. We screened aurachin RE analogs through a detailed assay strategy. The identified MenA inhibitors demonstrated either very weak (or no) inhibitory activities of bacterial electron transport systems. A series of MenA inhibitors identified in this program exhibited antimicrobial spectrum focused against *Mtb*. Selective activity against *Mtb* is ideal in TB drug discovery due to the fact that TB chemotherapy requires long regimen, and broad-spectrum anti-TB agents may cause resistance to other bacteria during TB chemotherapy. The carbamates (*R*)-**19** and (*R*)-**20** in class A showed significant growth inhibitory activities against non-replicating *Mtb* (MIC_{LORA}, 0.85 µg/mL) with the MIC_{LORA}/MIC_{MABA} value of 0.37 (MIC_{LORA}/MIC_{MABA} = 7.35 for rifampicin). The effectiveness against non-replicating *Mtb* was also confirmed *via* assays using a modified Wayne model and nutrient deficient Loebel model assays (72,135,136) (137). Among antimycobacterial agents tested in our laboratories, the inhibitor (*R*)-**19** and (*R*)-**20** were the most active in killing non-replicating *Mtb in vitro*. A convenient assay with an *E. coli* model under hypoxic conditions was established and was used to screen compounds against MenA. This model may be used as a convenient surrogate for *Mtb* hypoxia.

Figure 2-7. Optically active compounds with selective anti *Mtb* activity.

Table 2-3. MICs of selected compounds against *Mycobacterium* spp.

Compounds	MIC ^a			
	(R)-19	(R)-20	RIF	INH
<i>Mtb</i> H ₃₇ Rv	2.31	2.31	0.07	0.24
<i>Mtb</i> H ₃₇ Rv INHr ^b	2.91	1.52	0.03	>8
<i>Mtb</i> H ₃₇ Rv RIFr ^c	2.91	1.51	>4	0.05
<i>M. bovis</i> BCG	3.00	2.95	-	-
<i>M. intracellulare</i> ATCC 15984	6.50	6.50	-	-
<i>M. smegmatis</i> ATCC 607	6.50	6.50	-	-

NOTE: *Mtb* - *Mycobacterium tuberculosis*, MIC - Minimum inhibitory concentration, RIF – Rifampicin, INH – Isoniazid, ^a MIC determined by broth dilution method (Microplate alamar blue method-MABA), ^b INH resistant *Mtb*, ^c RIF resistant *Mtb*.

MenA inhibitors identified in this program strongly suggested that menaquinone biosynthesis is important in maintaining mycobacterial viability under conditions of restricted oxygen and nutrient stress (138). MenA inhibitors seem to be able to block the electron flow, consequently inhibiting the bacterial growth. It is conceptually very interesting that MenA inhibitors can be developed as indirect ATP synthesis inhibitors. The assay data for the identified MenA inhibitors indicate that menaquinone biosynthesis is a unique and new antimycobacterial target. In addition, in tuberculosis, the key to shortening the long regimen of TB treatment lies in targeting the non-replicating persistence subpopulation (*vide supra*). Therefore, the discovery of molecules that kill non-replicating *Mtb* at lower concentrations than MIC against *Mtb* under aerobic conditions is of great significance. Moreover, over the last several years, we are unable to isolate resistant bacteria to the MenA inhibitors in the mutation frequency studies. Therefore, we concluded that unlike the other known bacterial molecular targets, menA shows extremely low mutation frequency.

In conclusion, robustness of our *in vitro* assay approaches to identify novel and selective MenA inhibitors against *Mtb* (summarized in **Figure 2-3**) has been demonstrated by the identification of a lead molecule (*R*)-**20** (MIC_{LORA}/MIC_{MABA} value of 0.37, SI >10).

CHAPTER 3. CELL WALL BIOSYNTHESIS INHIBITORS*

Introduction

The cell wall of *Mtb* offers many unique targets for drug development. *Mtb* cell wall is implicated in the virulence and macrophage survival of *Mtb* (101,139). Although, inhibitors of cell wall biosynthesis are included in TB treatment regimen, the significance of late stage cell wall biosynthesis as a drug development target against dormant *Mtb* is not well evaluated. There are reports of effectiveness of peptidoglycan biosynthesis inhibitors against non-replicating *Mtb*. Meropenem (a carbapenem) was effective in killing non-replicating *Mtb* in combination with clavulanate (a β -lactamase inhibitor) (92). A novel series of cephalosporins and feropenem were also reported to be effective against non-replicating *Mtb* (93,140). Although, a mechanism of action of their bactericidal effect against dormant *Mtb* cells is not known, these are few examples that cell wall biosynthesis inhibitors kill dormant form of *Mtb*. The mycolylarabinogalactan layers of the mycobacterial cell wall is significant for the dormant *Mtb* (101,102). The first step in the mycolyl arabinogalactan biosynthesis in *Mtb* is catalyzed by prenyl-phosphate-GlcNAc-1-phosphate transferase (WecA). Inhibition of WecA could block the entire biosynthesis of essential cell wall components of *Mtb* in both replicating and non-replicating states, and *wecA* enzyme could be a viable target for development of novel drugs. WecA and a peptidoglycan biosynthetic enzyme; MurX/MraY are members of P2HPT superfamily and are essential for *Mtb* growth (105). However, MurX/MraY inhibitors are effective in killing only replicating *Mtb* under aerobic conditions (109,141-143). Although, WecA inhibitors have the potential to be effective TB drugs that kill non-replicating *Mtb* under oxygen depleted conditions, only a few molecules are known to interfere with WecA and their effectiveness against non-replicating *Mtb* has been poorly characterized (90,144). In order to establish the significance of WecA inhibition against the dormant *Mtb*, it is crucial to evaluate selective WecA inhibitor molecules against dormant *Mtb*.

* Adapted with permission.

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Tunicamycin is a non-selective phosphotransferase inhibitor with both MurX and WecA inhibitory activity. Recently; Ishizaki et al. reported that caprazamycin (a MurX/MraY inhibitor) analog, CPZEN-45 inhibits mycobacterial WecA (90). Our group has reported novel muraymycin analogs (Muraymycin D1-amide and muraymycin D1-diamide) with both MraY and WecA inhibition (144). Much studies are required to understand WecA in dormant *Mtb*. MurX inhibitor such as capuramycin has a faster killing profile towards replicating *Mtb*, therefore, our strategy comprised of screening capuramycin analogs against MurX and WecA. A detailed screening for inhibitors of these enzymes requires a reliable and convenient assay. Therefore, we decided to develop convenient high throughput amenable assays to identify MurX and WecA inhibitor molecules routinely. The fluorescent probes, Park's nucleotide-*N*^E-C₆-FITC (**32**, for MraY/MurX) and UDP-glucosamine-C₆-FITC (**46**, for WecA) are developed. These fluorescent probes enable us to screen the polyprenyl-phosphate *N*-acetylhexosamine 1-phosphate transferase enzyme superfamily (e.g. MraY/MurX, WecA, AlgH, and DPAGT1) in high-throughput manner. A selective WecA inhibitor, UT-01320 (**45**) which kills non-replicating *Mtb* and intracellular *Mtb* was identified.

Development of a Convenient MurX Assay

Translocase I (MraY/MurX) catalyzes the transformation of UDP-MurNGlyc-pentapeptide and UDP-MurNAc-pentapeptide (Park's nucleotide) to the corresponding lipid I using decaprenyl (C₅₀) phosphate in *Mycobacterium spp* (**Figure 3-1**) (145,146). This process is believed to be a reversible process in which *E. coli* MraY catalyzes an exchange reaction between UMP and lipid I to form Park's nucleotide *in vitro* (147). Lipid I isolated from *Mtb* contains the C₅₀-prenyl unit that shows very poor water-solubility, and thus, this chemical characteristic of lipid I renders MurX enzyme assays impractical for screening.

Isolation and quantitation of Park's nucleotide and lipid I from *in vitro* MraY/MurX assay reaction mixtures are time-consuming processes (145). In addition, preparation of *Mtb* Park's nucleotide *via* semi-purified Mur enzymes is not amenable to multigram scale-up and the acquisition cost of enough decaprenyl phosphate for medium- to high-throughput screenings is very high. To date, several screening methods for MraY/MurX inhibitors have been reported that includes; 1) monitoring the transfer of phosphoryl-MurNAc-pentapeptide using fluorescent or radiolabeled Park's nucleotide and/or undecaprenyl phosphate (147), 2) measuring the exchange reaction between [³H]UMP to Park's nucleotide that requires separation of [³H]uridine after the treatment of alkaline phosphatase (148,149), 3) an indirect assay using a coupled MraY-MurG that requires biotinylated Park's nucleotide and [¹⁴C]UDP-GlcNAc (150), 4) an assay using HP20ss hydrophobic beads for isolating the generated radiolabeled lipid I (151), 5) a microplate-based assay using a radiolabeled-Park's nucleotide (152), and 6) a scintillation proximity assay using wheat germ agglutinin-coated beads to capture the lipid I from a radiolabeled-Park's nucleotide (153). Although a several assay methods were reported to be amenable to a HTS assay for MraY, in our hands, extraction of water-

Figure 3-1. Biosynthesis of lipid II in *Mtb*.

Translocase I (MurX) catalyzes the transformation from Park's nucleotide to lipid I.

insoluble lipid I derivative from assay media is essential (147,153,154). In our attempt at developing reliable *in vitro* MraY/MurX assay, we concluded that the reported assays need further optimization to be robust statistical methods that can identify MraY/MurX inhibitors routinely with IC₅₀ values. We established an efficient synthetic method for the generation of sufficient amount of fluorescent Park's nucleotide probes for HTS and tested the Park's nucleotide probes in MurX-catalyzed lipid I analog synthesis with decaprenyl and truncated prenyl phosphates (155,156). Surprisingly, under the optimized conditions the water-soluble lipid I-neryl (C₁₀) analog could be biosynthesized efficiently with the Park's nucleotide probes and neryl phosphate (35).

Most of the reported MraY inhibitors were competitive to Park's nucleotide but not to prenyl-phosphate. Because capuramycin is a well-characterized MraY/MurX inhibitor that showed activities focused against *Mycobacterium spp.* (157), we selected positive and negative controls from capuramycin derivatives to develop a convenient MurX assay method, and the new MurX assay developed here was planned to demonstrate by screen antimycobacterial uridyl-peptide library molecules.

N-Glycolylated muramic (MurNGlyc) acid predominates in *Mtb* and MurNGlyc is the major muramyl building blocks in mycobacterial cell walls (158,159). Thus, it is of importance to characterize specificity of MurX against N-glycolyl Park's nucleotide and N-acetyl Park's nucleotide for discovery of novel antimycobacterial MurX inhibitors. In order to understand the tolerance of MurX in the structure of Park's nucleotide, we have synthesized a series of Park's nucleotide analogs according to our established chemo-enzymatic or total chemical syntheses (145,155,156). We and other groups have demonstrated that the dansyl- or FITC-conjugated N-acetyl Park's nucleotides can be applied to MraY enzyme assays with the MraY obtained from Gram-negative and some Gram-positive organisms (159). In our preliminary studies, the membrane fraction containing MraY or MurX enzyme (P-60) obtained from *E. coli*, *S. aureus*, *M. smegmatis*, or *Mtb* could transfer the fluorescent-Park's nucleotide conjugates to the corresponding undecaprenyl lipid I analogs with undecaprenyl phosphate in 5-25% yields after 1h incubation (160). These results were in accordance with the data previously reported by several other groups (147,148). It was experimentally proven that MurX was also tolerated in the structure of the C₆-position (N^ε) of lysine moiety of Park's nucleotides.

In order to further study tolerance of MurX against the structure of the N-acyl moiety of muramic acid in Park's nucleotide, we examined time-course experiments of lipid I syntheses with N-glycolyl Park's nucleotide-N^ε-dansylthiourea and N-acyl Park's nucleotide-N^ε-dansylthiourea (**Figure 3-2**). MurX-catalyzed syntheses of lipid I analogs with **28** and **29** are summarized in **Table 3-1**. All reactions were performed in duplicate under the same reaction conditions except for the concentrations of decaprenyl phosphate and reaction time. The lipid I analogs (**30** and **31** of **Figure 3-2**) were chemically synthesized as reference molecules for HPLC studies (155,156). Direct comparison of the product yields for **30** and **31** revealed that Park's nucleotide-N^ε-dansylthiourea (**28** and **29**) were converted to the corresponding lipid I analogs **30** and **31**, respectively, by MurX

Figure 3-2. MurX-catalyzed syntheses of lipid I analogs from *N*-glycolyl- and *N*-acetyl-park's nucleotides.

Table 3-1. MurX-catalyzed syntheses of lipid I analogs from *N*-glycolyl- and *N*-acetyl-park's nucleotides.

Park's Nucleotide	Decaprenyl Phosphate (equivalents against 28 or 29)	Lipid I	Reaction time (h)	Yield (%) ^a
28	2	30	1	15-20
28	2	30	3	15-20
28	10	30	1	20-25
29	2	31	1	15-20
29	2	31	3	15-25
29	10	31	1	20-25

NOTE: Reaction conditions - Park's nucleotide (2 mM; 3.75 μL), MgCl_2 (0.5 M; 10 μL); KCl (2 M, 10 μL), Triton X100 (0.1%; 11.25 μL), Tris-buffer (pH = 8; 50mM, 5 μL), decaprenyl phosphate (10 mM, 2 or 10 equivalents against **28** or **29**), P-60 (15 μL), 26 $^\circ\text{C}$, 1 or 3h, ^a n-butanol extract was analyzed by HPLC (column: Kinetex 5u C8 100A, 150 $\times$ 4.60 mm, solvent - CH_3CN : 0.05 M aq. NH_4HCO_3 , flow rate - 0.5 mL / min.

without noticeable difference in reaction rate and product yield (**Table 3-1**). Increasing concentration of decaprenyl phosphate (from 2 to 10 equivalents against **28** or **29**) did not dramatically increase the product yield (**Table 3-1**). Kinetic studies revealed that N-glycolyl Park's nucleotide-N^ε-dansylthiourea and N-acyl Park's nucleotide-N^ε-dansylthiourea have a similar binding affinity towards MurX with the K_m values of 18.05 and 17.95 μ M, respectively (147). Although *Mtb* predominantly uses N-glycolyl Park's nucleotide for the biosynthesis of peptidoglycan through N-glycolyl lipid I, MurX recognizes N-acetyl Park's nucleotide equally (158). These structural tolerances of the Park's nucleotide binding domain of MurX implies the possibility of further simplification of Park's nucleotide for development of a convenient MurX/MraY assay.

Mycobacterium spp. uses decaprenyl phosphate (C₅₀-P) for the biosynthesis of lipid I (161). Due to the fact that C₅₀-lipid I does not dissolve in water media, thus, isolation and quantitation of the generated lipid I in the assay reaction mixtures are time-consuming processes (*vide supra*). In our hands, it is extremely difficult to develop medium- and high-throughput screening (HTS) for MurX/MraY assay using decaprenyl phosphate. We accomplished a practical chemical synthesis of neryl-lipid I and its fluorescent probes, and characterized their physicochemical properties. Neryl-lipid I-N^ε-dansylthiourea (**33** of **Figure 3-3A**) showed excellent water-solubility (>50 μ g/mL) and **33** can readily be analyzed *via* reverse-phase HPLC without gradient elution method (retention time: <9 min.) (**Figure 3-4**). MurX-catalyzed lipid I analog synthesis with Park's nucleotide-N^ε-dansylthiourea and neryl phosphate (5 equivalent against **29**) in the presence of 0.5% of triton X100 furnished neryl-lipid I-N^ε-dansylthiourea in 3-5% yield after 1h (157). Under the same reaction conditions, the yield of **33** was proportionated to the concentrations of neryl phosphate; the same reaction with 60 equivalents of neryl phosphate furnished **33** in greater than 50% yield in 1h (**Figure 3-3B**). As summarized in **Figure 3-3C**, effect of a phase transfer catalyst, triton X100 was observed in the transformation to **33** (162); 0.5% of triton X100 was determined to be the ultimate phase transfer catalyst of concentration for biosynthesis of lipid I-N^ε-dansylthiourea analog (**33** with **29**) and neryl phosphate. In short reaction times, application of higher concentrations of MurX enzyme (P-60) dramatically increased the reaction rate in biosynthesis of the lipid I analog (Fig 3-3D). However, Park's nucleotide-N^ε-dansylthiourea could be transformed to the neryl lipid I analog in greater than 90% yield even at lower concentrations of P-60 after 12h (**Figure 3-3E**). Among the other reaction parameters examined for MurX-catalyzed neryl-lipid I synthesis, the reaction temperatures did not noticeably affect the reaction rate within a temperature range between 22 and 37 °C. Thus, MurX assays can be performed conveniently at the ambient air temperatures. It is worth mentioning that all data obtained with P-60 membrane fraction from *Mtb* could be reproduced with that from *M. smegmatis*.

MurX/MraY Assays

The MraY-catalyzed transformation of lipid I from Park's nucleotide is believed to be a reversible process (147,163). On the contrary, under the optimized conditions

Figure 3-3. MurX-catalyzed biosynthesis of neryl lipid I analog.

A) Biosynthesis of neryl lipid I analog **33** from park's nucleotide analog **29** and neryl phosphate (**35**). B) Effect of concentrations of neryl phosphate in biosynthesis of **33**. C) Effect of a phase-transfer catalyst, triton x100 in biosynthesis of **33**. D) Effect of concentrations of MurX-containing membrane fraction (P-60). E) Time-course experiments of neryl-lipid I synthesis.

Figure 3-4. HPLC chromatogram of park's nucleotide- N^ϵ -fluorescent analogs (29 and 32) and neryl lipid I- N^ϵ -fluorescent analogs (33 and 34).

MurX-catalyzed neryl-lipid I synthesis from Park's nucleotide-N^ε-dansylthiourea is not an equilibrium reaction; **29** could be completely consumed to form **33** within 15h (**Figure 3-3E**). The neryl-lipid I analog **33** can readily be dissolved in water or the assay media. Without extraction of the MurX/MraY enzymatic product from the reaction mixtures, the assay media can be assayed directly *via* reverse-phase HPLC for quantitation. Separation of Park's nucleotide analog **29** and neryl-lipid I analog **33** could be performed *via* a C₈- or C₁₈-reverse phase column with a fixed solvent system (CH₃CN : 0.05 M aq. NH₄HCO₃ = 25 : 75) at flow rate of 0.5 mL/min. Under these assay conditions, the retention times of Park's nucleotide **29** and neryl-lipid I **33** were 4.0 min. and 7.9 min, respectively (**Figure 3-4**). On the other hand, separation of Park's nucleotide and decaprenyl-lipid I required a gradient method for HPLC analyses (147); the retention time of decaprenyl-lipid I (**31**) was 60 min under our optimized HPLC conditions (C₁₈-reverse phase column, solvent systems: (MeOH : 0.05 M aq. NH₄HCO₃ = 85 : 15 to 100% MeOH, flow rate: 2.0 mL/min). The K_m value for Park's nucleotide-dansylthiourea (**29**) was 18.29 μM at the concentrations of 450 μM of neryl phosphate; this was very similar to the K_m values obtained with decaprenyl or undecaprenyl phosphate (K_m: 18.05 μM) (147). The V_{max} for neryl-lipid I synthesis by MurX was determined to be 2.69 × 10⁻³ μM/sec through the Michaelis-Menten plot. As stated above, significant difference in MurX/MraY-catalyzed neryl-lipid I synthesis is that the transformation from Park's nucleotide **29** to neryl-lipid I **33** is not a reverse process and neryl-lipid I **33** is biosynthesized in greater than 80% yield in 8h (**Figure 3-3E**). Although the synthesis of neryl-lipid I **33** could be achieved with Park's nucleotide-N^ε-dansylthiourea (**29**) in over 50% yield within 1h *via* the purified MraY enzymes, unlike P-60 membrane fraction, the reactions did not attain over 65% yield even after 15h due probably to instability of the purified MraY under the assay reaction conditions. Thus, MurX/MraY assays have been conveniently performed using P-60 membrane fractions obtained from *Mtb* or *M. smegmatis*.

The fluorescence characteristics of the N^ε-dansylthiourea moiety in the enzymatic substrate and product can be applied to fluorescence-based analytical techniques in order to quantitate the inhibition of lipid I biosynthesis. The, signal-to-noise ratio of HPLC analyses using UV (350 nm) is excellent enough for quantitation of 10 μL of the assay mixtures. We have also demonstrated that the FITC-conjugated N-acetyl Park's nucleotides (Park's nucleotide-N^ε-C₆-FITC (**32**)) can be applied to MraY enzyme assays. Due to the wider applicability of fluorescein (FITC) for bioanalysis, **32** was utilized for the validation of MurX assay with antimycobacterial uridyl peptides. The range of linearity was established by injections (*via* an auto sampler) of six concentrations of Park's nucleotide-N^ε-C₆-FITC (**32**) and neryl-lipid I-N^ε-C₆-FITC (**34**) (r ≥ 0.9), and limit of detection was determined to be much lower than 1 μM concentrations.

Validation of MurX Assays with Antimycobacterial Uridyl Peptides

In order to determine usefulness of the MurX assay developed here, we examined several known MraY inhibitor molecules and negative controls, and demonstrated effectiveness of this MurX assay by screening our uridyl peptide library molecules (**Figure 3-5**). Known MraY inhibitors, capuramycin (**36**) and SQ641 (**37**) exhibited

Figure 3-5. Library of uridyl peptides utilized for screening against MurX enzyme.

strong enzyme inhibitory activity against MurX (entries 1 and 2 in **Table 3-2**); the IC₅₀ values of capuramycin (**36**) and SQ641 (**37**) were 0.152 and 0.109 μ M, respectively. Interestingly, these antimycobacterial *MraY* inhibitors **36** and **37** showed a 10-fold decrease in enzyme inhibitory activity against *E. coli* *MraY*. MurX enzyme inhibitory activity of tunicamycin (**38**) was determined to be the IC₅₀ value of 2.73 μ M in which the observed activity of tunicamycin was closely related to the data reported in the literatures (2.40-2.95 μ M) (147). In our screening of series of *MraY* inhibitors in enzyme and bacterial growth inhibitory assays, the MurX inhibitors that showed the IC₅₀ value of above 10 μ M did not exhibit significant bactericidal activity against *Mtb* (164-166). Thus, we set up an IC₅₀ threshold of 10 μ M to distinguish exploitable antimycobacterial MurX inhibitors from other antimycobacterial molecules. The analogs of Capuramycin (**36**); **39** and **40** have been used as negative controls in our program. These molecules did not show MurX inhibitory activity even at 100 μ M concentrations (entries 4 and 5 in **Table 3-2**). Although vancomycin showed the good activity in *MraY*-MurG coupled assays, it could be confirmed that vancomycin did not inhibit MurX even at 100 μ M concentrations. We evaluated over 50 synthetic uridine-glycosyl peptides and uridyl peptides that showed the MIC values of <25 μ g/mL against *Mtb* in the MurX assay screening at three to five different concentrations. Among the identified new MurX inhibitors two molecules UT-01326 (**41**) and UT-01309 (**42**) are worthwhile highlighting. The 2'-methyl isomer **41** and amino-benzodiazepinone analog **42** showed increased MurX inhibitory activity compared to capuramycin (entry 7 and 8 in **Table 3-2**). On the other hand, the pacidamycin analog **43** and muraymycin analog **44** did not exhibit MurX inhibitory activity even at 100 μ M concentrations (167,168). Thus, we concluded that dihydrouridyl analogs **43** and **44** exhibited antimycobacterial activity by targeting the other essential enzyme(s) for growth of *Mtb*. UT-01320 (**45**) (entry 11 in **Table 3-2**) showed no MurX inhibition and exhibited antimycobacterial activity.

Thus, the HPLC-based assay of MurX/*MraY* investigated here can be performed with standard analytical devices and will be adapted to medium- to high-throughput formats with close to ideal Z'-factors (0.5-1.0) (169). The Z'-factor was estimated from the data summarized in **Table 3-2**; an estimated Z'-factor was 0.84, and thus, the new MurX/*MraY* assay method described here is considered to be an excellent assay. We are currently generating a relatively large number of library molecules containing known MurX/*MraY* inhibitors to examine robustness of the described assay method.

Development of a Convenient WecA Transferase Assay

WecA-catalyzed reactions have been performed *via* UDP-[¹⁴C]GlcNAc, C₅₀-P (or C₅₅-P), and either purified WecA or crude membranes containing WecA. the reported assays require separation of the product by chromatography (90,170-172). These assays are inadequate to systematically characterize library molecules in a high-throughput manner (104,151). We identified new UDP-GlcNAc fluorescent probes, UDP-Glucosamine-C₆-FITC and UDP-Glucosamine-C₆-Dansyl, both of which can be recognized by the MurG transglycosylase, an essential peptidoglycan biosynthetic

Table 3-2. Assay of positive- and negative-controls, and a library of uridyl peptides against MurX.

Entry	Compound	MurX Inhibition (%) ^a					IC ₅₀ (μM) ^b
		0.01μM	0.1μM	1μM	10μM	100μM	
1	Capuramycin (36)	-	36.6	88.4	100	100	0.152 ± 0.0125 (0.150-0.0180) ^c
2	SQ 641 (37)	-	40	95	100	-	0.109 ± 0.00845 (0.0150-0.100) ^c
3	Tunicamycin (38)	-	9	42	72	72	2.73 ± 0.138 (2.40-2.95) ^c
4	39	-	0	0	0	0	ND
5	40	-	0	0	0	0	ND
6	Vancomycin	-	0	0	0	0	ND
7	UT-01326 (41)	3.8	81.2	100	100	-	0.105 ± 0.00330
8	UT-01309 (42)	-	40	95	100	-	0.0950 ± 0.00395
9	43	-	0	0	0	0	ND
10	44	-	0	0	0	0	ND
11	UT-01320 (45)	0	0	0	0	0	ND
12	DMSO	0	0	0	0	0	ND

NOTE: ^a Reaction conditions - Park's nucleotide-N^e-C₆-FITC (**32**) (75 μM; 3.75 μL), MgCl₂ (0.5 M; 10 μL); KCl (2 M, 10 μL), Triton X100 (0.1%; 11.25 μl), Tris-buffer (pH = 8; 50 mM, 2.5 μL), neryl phosphate **35** (10 mM, 45 μL), inhibitor molecule (0-100 μM in DMSO (2.5 μL)), P-60 (15 μL), 26 °C, 3h, The K_m 18.29 μM (Park's nucleotide **32**), ^b The IC₅₀ values were obtained 3 times and the standard error of the mean was calculated, ^c The IC₅₀ values in parentheses were reported in the literatures see, references (143,146,147,152,153) and/or were obtained *via* the assay conditions summarized in **Table 3-1**, The K_m 18.05 μM (Park's nucleotide **32**).

enzyme (173,174). Interestingly, under optimized conditions the water-insoluble decaprenyl-Glucosamine-C₆-FITC and decaprenyl-Glucosamine-C₆-Dansyl analogs could be biosynthesized with the WecA-containing membrane fractions obtained from *Mtb*, *M. smegmatis*, and *E. coli*.

Although extensive studies on assay developments of MurX/MraY-type phosphotransferases have been reported (141,150,152-154), there are a limited number of studies reported on practical WecA assays (90,151,172,175). The reported WecA assay protocols are not amenable to HTS and they require radiolabeled UDP-GlcNAc and chromatographic separation of the product for quantitation. To date, WecA-catalyzed reactions have been carried out under a variety of biochemical conditions in which the effects of pH, concentrations of divalent cation and detergent, and effects of additives (ATP, EDTA, etc) were evaluated. Moreover, specificity of WecA against the structure of polyprenyl phosphate was reported (105,176-181). After extensive optimization efforts, we could achieve the high-yielding WecA-catalyzed reactions with UDP-Glucosamine-C₆-FITC or UDP-Glucosamine-C₆-Dansyl and exogenous decaprenyl phosphate (C₅₀-P) that significantly increases reliability of WecA assays.

Specificity of WecA against Nucleotide Substrate and Optimization of Reaction Conditions

While developing high-throughput screens for GlcNAc transferase such as MurG, we identified that UDP-Glucosamine-C₆-FITC is recognized by *Mtb* MurG to form the decaprenyl-P-P-GlcNAc-MurNAc-(pentapeptide) (lipid II) analog. We sought to explore whether these modified UDP-GlcNAc derivatives could be applied for WecA assays. To facilitate the screening for WecA inhibitors, specificity of WecA against UDP-Glucosamine-C₆-FITC (**46**) and UDP-Glucosamine-C₆-Dansyl (**47**) was examined using the membrane fraction (P-60) prepared from *M. smegmatis* and exogenous decaprenylphosphate (C₅₀-P). Because WecA and MurX/MraY use different UDP-2-deoxy-2-N-acetyl-D-hexosamine donor substrates, we first applied the optimized reaction conditions established for MurX/MraY (50 mM MgCl₂, 0.5% Triton-X, 50 mM Tris-HCl buffer at pH 8.0) to the WecA-catalyzed transformation from **46** and **47** to C₅₀-P-P-Glucosamine-C₆-FITC **48** (or C₅₀-P-P-Glucosamine-C₆-Dansyl (**49**)) (141). The results show that P-60-catalyzed phosphotransfer reactions of **46** and **47** with C₅₀-P resulted in the formation of **48** and **49** with 7.5–10% yields in 2 h. The generated WecA products **48** and **49** could be separated by partition with n-butanol (n-BuOH). In this process, majority of **46** and **47** remained in the water phase. The consumption of **46** and **47** (water phase) and generation of **48** and **49** (n-BuOH phase) could be monitored *via* reverse-phase HPLC (solvent: CH₃OH:0.05 M aq. NH₄HCO₃ = 40: 60 to 80: 20, UV: 485 or 350 nm, flow rate: 0.5 mL/min). Under these conditions, the retention times of UDP-Glucosamine-C₆-FITC (**46**) and C₅₀-P-P-Glucosamine-C₆-FITC (**48**) were 11.6 min and 21.2 min, respectively (**Figure 3-6**). The HPLC retention times of **48** and **49** were identical to those of the chemically synthesized molecules. Because of wider applicability of fluorescein (FITC) for bioanalysis, we decided to utilize UDP-Glucosamine-C₆-FITC towards the development of a WecA assay.

Figure 3-6. WecA-catalyzed biosynthesis of C₅₀-P-P-Glucosamine-C₆-fluorescent analogs 48 and 49.

A) UDP-GlcNAc donor analogs **46** and **47** can be recognized by WecA. B) Fluorescence detection-based HPLC at the endpoint of the reaction.

Although the synthesis of C₅₀-P-P-Glucosamine-C₆-FITC (**48**) could be achieved with 7.5–10% yield in 2 h using the UDP-GlcNAc fluorescent probe, C₅₀-P, and P-60, optimization of reaction condition is important to improve assay efficiency and to reduce false-positive and false-negative errors in high-throughput screening (HTS) assays. The effects of adding ATP, EDTA, detergent, and co-factors have been previously reported for WecA-catalyzed reactions by using radiolabeled UDP-GlcNAc and undecaprenyl phosphate (C₅₅-P). In general, conversion of over 20% or more of reactions provides reliable analyses in fluorescence-based assays for screening; enzyme inhibitory assays of conversion of 10% or below produce high standard errors by false-positive and false-negative assay data.

WecA-catalyzed synthesis of C₅₀-P-P-Glucosamine-C₆-FITC (**48**) with UDP-Glucosamine-C₆-FITC (**46**) and decaprenyl phosphate (3 equivalents against **46**) in the presence of 0.5% of triton X (optimized concentrations for MurX/MraY) furnished **48** in 7.5–10% yields after 2 h. As summarized in **Figure 3-7A**, the effect of surfactant, CHAPS was observed in the transformation from **46** to **48**; it was determined that 1% of CHAPS is the ultimate concentration of the surfactant (103,181). Under the conditions with 1% of CHAPS, the optimum MgCl₂ concentration was 40 mM (**Figure 3-7B**); the same reaction furnished **9** in 46% yield at 37 °C (pH 8.0) after 2 h (103,172). In the time-course experiments, application of higher concentrations of WecA enzyme (P-60) increased the product yield of **48**. However, saturation of the product formation was observed at the concentration of P-60 at 15 µL (1 mg P-60/µL) (**Figure 3-7C**). Under the optimized conditions, the synthesis of **48** was increased in a time-dependent manner; WecA-catalyzed transformation of **46** with decaprenyl phosphate (3 equivalents) furnished **48** in 57.5% yield after 6 h (**Figure 3-7D**). Among the other reaction parameters examined for *M. smegmatis* WecA-catalyzed synthesis of **48**, the reaction temperatures (30 vs. 37 °C) and pH (8.0 vs. 8.3) did not noticeably affect the reaction rate. Although the other groups reported effects of ATP or EDTA in WecA-catalyzed GlcNAc-P-P-C₅₅ formation (90,151,172), we concluded that these additives show no effect in the transformation from **46** to **48** with the P-60 obtained from *M. smegmatis* (**Figure 3-7E**). It is worth mentioning that the active P-60 membrane consumed UDP-Glucosamine-C₆-FITC in the absence of exogenous C₅₀-P but formed **46** by using endogenous polyprenyl phosphate(s). As illustrated in **Figure 3-7F**, the time-course study demonstrated that the consumption of **46** was well correlated to the generation of **48**; in this reaction, **48** was formed in 5–10% yield after 2–12 h. The optimized conditions established with the P-60 membrane fraction from *M. smegmatis* were effective in the same reaction with the other sources of P-60 prepared from *Mtb* and *E. coli*. At the concentration of P-60 [15 µL (1 mg P-60/µL)] optimized in **Figure 3-7C**, the P-60 prepared from a WecA overexpressed *E. coli* strain (*E. coli* B21wecA) dramatically improved the transformation from **46** to **48** compared to that with the P-60 from a wild type *E. coli*. (**Figure 3-7D**). Quantitations of the remaining **46** and generated **48** in each reaction mixture were performed *via* reverse-HPLC. Signal-to-noise ratio of HPLC analyses using UV (485 nm) is excellent for quantitation of 25 µL of the assay mixtures. The range of linearity was established by injections (*via* an auto sampler) of six concentrations of chemically synthesized **46** and **48** ($r \geq 0.9$), and limit of detection was

Figure 3-7. WecA-catalyzed biosynthesis of C₅₀-P-P-Glucosamine-C₆-FITC. A) Effect of the phase transfer catalyst CHAPS. B) Effect of concentrations of MgCl₂. C) Effect of concentrations of P-60 obtained from *M. smegmatis*. D) Time-course experiments of biosynthesis of **48** with different sources of membrane fractions. E) Effect of additives (ATP and EDTA). F) Time-course experiments of biosynthesis of **48** in the absence of exogenous C₅₀-P.

determined to be much lower than 0.1 μM concentrations. The K_m value for UDP-Glucosamine- C_6 -FITC was 194.7 μM at concentrations of 140 μM of exogenous decaprenyl phosphate; this was similar to the K_m values obtained with undecaprenyl phosphate (K_m : 195.5 μM). The V_{max} for C_{50} -P-P-Glucosamine- C_6 -FITC synthesis by the crude membrane containing *M. smegmatis* WecA (P-60) was determined to be 0.404 $\mu\text{M}/\text{min}$ through the Michaelis-Menten plot. Significant difference in WecA-catalyzed phosphoryltransfer reactions is that the transformation between UDP-Glucosamine- C_6 -FITC and C_{50} -P-P-Glucosamine- C_6 -FITC is not a reverse process, whereas the recent studies have concluded that *Thermotoga maritima* WecA-catalyzed reaction shows equilibrium between UDP-GlcNAc and C_{55} -P-P-GlcNAc (182). Similarly, MraY/MurX-catalyzed phosphoryltransfer of polyprenyl phosphate to Park's nucleotide is known to be an equilibrium reaction (141).

Development of UV/Vis Spectroscopy-based Assay for WecA

C_{50} -P-P-Glucosamine- C_6 -FITC is readily extracted with n-BuOH and UDP-Glucosamine- C_6 -FITC remains in water media. Taking advantage of the hydrophobic character of **48**, the fluorescence in n-BuOH extract of WecA reaction was monitored *via* ultraviolet-visible (UV-vis) spectrometry (excitation of 485 nm, emission of 528 nm); the UV-vis-based assay was performed at a sufficiently high concentration of **46** (22.5 μM) for UV-vis spectrometry and enough concentrations of C_{50} -P that fulfill the K_m value. Progress of the WecA-catalyzed reaction of **46** was determined periodically and monitored for 12 h. As shown in **Figure 3-8B**, an increase in fluorescence signal was observed in a time-dependent manner that was well-correlated to the yield curve obtained *via* the HPLC method (**Figure 3-8A**). A new assay method developed here was validated by demonstrating the inhibition of WecA activity by a known MurX/WecA inhibitor, tunicamycin; generation of **48** was inhibited by tunicamycin in a dose-dependent manner (**Figure 3-8C**). The IC_{50} value of tunicamycin against *M. smegmatis* WecA and *E. coli* WecA was determined to be 0.59 $\mu\text{g}/\text{mL}$ and 0.12 $\mu\text{g}/\text{mL}$ by constructing dose-response curves *via* UV-vis spectrometry (**Figure 3-8B**). **Figure 3-9** shows WecA inhibitors identified in this assay. The data summarized in **Table 3-3** were obtained from at least three independent experiments and the standard deviation was less than 10%. It was determined that DMSO did not inhibit the WecA assays at 5% (v/v) concentrations. However, inhibition of the reactions was started at 10% (v/v) of DMSO; approximately 50% of the enzyme activity was reduced at this concentration (**Figure 3-8D**).

Specificity of WecA against the Structures of Prenyl Phosphate

We identified that neryl phosphate (*cis*- C_{10} -P) and (2Z,6E)-farnesyl phosphate (*cis*- C_{15} -P) could be recognized by the MurX/MraY-type phosphotransferases to form the corresponding lipid I analogs, whereas, *trans*- C_{10} -P and - C_{15} -P, and *cis*- and *trans*-phytols (C_{20} -P) were not recognized by MurX/MraY enzymes (141,183). To investigate the specificity of WecA against truncated prenyl phosphates, we evaluated WecA-catalyzed

Figure 3-8. Validation of WecA assays *via* uv-vis.

A) Time-course experiments of biosynthesis of **48** monitored *via* HPLC. B) Time-course experiments of biosynthesis of **48** monitored *via* UV-vis. C) Dose-response curves of tunicamycin against *E. coli* and *M. smegmatis* WecA. D) Effect of DMSO on the WecA activity.

Figure 3-9. WecA inhibitors identified in this assay.

Table 3-3. Assays against a library of capuramycin analogs against the WecA enzyme (P-60 from *E. coli* B21WecA).

Compound	WecA Inhibition (%) ^a			IC ₅₀ (μg/mL) ^b
	0.02μg/mL	2μg/mL	20μg/mL	
Tunicamycin (38)	36.0 (38.5) ^c	97.0 (100)	100 (100)	0.120 ± 7.80
Capuramycin (36)	-	-	-	-
UT-01309 (42)	-	-	-	-
SQ 641 (37)	-	-	-	-
UT-03347 (50)	11.0 (15.5)	23.0 (29.0)	80.5 (84.5)	ND
UT-03328 (51)	0 (0)	23.0 (28.0)	60.5 (75.5)	ND
UT-03325 (52)	30.0 (35.0)	35.0 (45.0)	55.0 (60.5)	ND
UT-01320 (45)	42.0 (45.0)	100 (100)	100 (100)	0.0354 ± 9.14
DMSO	0 (0)	0 (0)	0 (0)	-

NOTE: ^a Reaction conditions - UDP-Glucosamine-C₆-FITC (**46**) (1, 0.56 μL), MgCl₂ (0.5 M; 4 μL), CHAPS (5%; 10 μL), β-mercaptoethanol (50 mM, 5μL)Tris-buffer (pH = 8.0, 50 mM, 9.74 μL), C₅₀-P (4 mM, 1.7 μL), P-60 (*E. coli* B21WecA) (18 μL), 37 °C, 2 h, The K_m :194.7 μM (for **46**), ^b Each experiment was performed three times, and the average IC₅₀ values were summarized, ^c WecA inhibitory activity in parentheses were obtained *via* HPLC, ND - Not determined.

transformations of UDP-Glucosamine-C₆-FITC with a series of synthetic prenyl phosphates. Due to the fact that endogenous decaprenyl phosphate (C₅₀-P) that exists in the *M. smegmatis* P-60 membrane could be utilized in the WecA-catalyzed transformation of **48**, generations of prenyl-P-P-Glucosamine-C₆-FITC analogs with the truncated lipid-P were determined by comparison of the retention times of the chemically synthesized analogs *via* HPLC. The generated products were also quantitated by HPLC. Unlike MurX/MraY enzymes, C₁₀-P, C₁₅-P, and C₂₀-P were not incorporated by WecA; the corresponding prenyl-P-P-Glucosamine-C₆-FITC derivatives were not formed (**Figure 3-10**). In these experiments, C₅₀-P-P-Glucosamine-C₆-FITC was generated in 5–10% yield. Undecaprenyl phosphate (C₅₅-P) was equally effective to C₅₀-P in WecA catalyzed transformation from **46** to **48** using P-60 prepared from *M. smegmatis*.

Screening a Library of Capuramycin-based Analogs *via* the New UV-VIS- based WecA Assay

To identify WecA inhibitors, we applied the UV–vis-based WecA assay to screen an optimized library of 50 capuramycin analogs in duplicate with 384-well plates. In these screening, the P-60 membrane fraction isolated from *E. coli* B21WecA was applied. All compounds were screened at three different concentrations (0.02, 2.0, and 20 µg/mL). Each plate contained four control wells: the first one with the denatured P-60 (heated at 100 °C for 5 min), the second one without P-60, the third one addition of 30% (v/v) of DMSO and the fourth one with tunicamycin at 20 µg/mL. Under the assay conditions, four molecules were identified as WecA inhibitors (**50**, **51**, **52** and **45**). The identified WecA inhibitors were confirmed by the HPLC-based assays at 0.02, 2.0, and 20 µg/mL concentrations. A dose-response curve of UT-01320 (**45**) against WecA obtained *via* UV–vis spectrometry determined the IC₅₀ value of UT-01320 (**45**) to be 35.4 ng/mL (**Figure 3-11**). The observed inhibition of the WecA enzyme by **45** is more potent than that of tunicamycin (IC₅₀ 0.12 µg/mL). It is important to note that the inhibitory activities of WecA inhibitors identified using P-60 from *E. coli* B21wecA was well-correlated to those against the WecA containing membranes from *M. smegmatis* ATCC607 (**Figure 3-8C**).

Mycobactericidal Activity of UT-01320

A 2'-methylated capuramycin analog, UT-01320 (**45**) did not exhibit MurX enzyme inhibitory activity even at 400 µg/mL concentrations; however, it was noticed that UT-01320 inhibited WecA with the IC₅₀ values of 35.4 ng/mL (*E. coli* B21WecA) and 50.7 ng/mL (*M. smegmatis* wecA), respectively (103,141). While screening of new capuramycin analogs against replicating and nonreplicating (dormant) *Mtb*, it was found that **45** killed both replicating and nonreplicating *Mtb* in MABA, LORA and LOEBEL assays, respectively (91). To the best of our knowledge, it is the first observation that a capuramycin analog exhibited bactericidal activity against nonreplicating *Mtb* at low concentrations.

Figure 3-10. Comparison of specificity of MurX and WecA against prenyl phosphates.

A) Specificity of MurX against C₅₅-P, C₅₀-P, *cis*-C₁₅-P and *cis*-C₁₀-P. B) Specificity of *M. smegmatis* WecA against C₅₅-P, C₅₀-P, *cis*- and *trans*-C₂₀-P, *cis*-C₁₅-P and *cis*-C₁₀-P.

Figure 3-11. Dose-response curves for the WecA inhibitory activities of UT-01320 and tunicamycin.

WecA containing membrane fraction (P-60) was prepared from *E. coli* B21WecA and applied to the inhibition studies.

The novel MurX and WecA assays were useful in studying the structure–activity relationship of Capuramycin towards improving efficacy of its antimycobacterial activity *in vitro* and *in vivo* (143). Daiichi-Sankyo and Sequella reported several capuramycin analogs in which *in vitro* MraY enzyme and antimycobacterial activity could be improved *via* the modification of the carboxylic group of the capuramycin biosynthetic intermediate, A-500359 (142,143,184,185). Our group has synthesized new capuramycin analogs *via* our total synthetic scheme, in which all analogs are structurally different from the reported molecules and they are difficult to access from A-500359 (164). To date, we have synthesized Capuramycin (**36**), SQ 641 (**37**), and over 100 new capuramycin analogs in which their structures were diversified with optically active amines (R3) and the four uridyl templates, 3'O-methyl (natural form), 2'O-methyl, 2',3'O-dimethyl and 3'O-demethylated (unnatural forms) uridine-mannopyranuronate derivatives (**Figure 3-12**). The syntheses of capuramycin analogs with these templates were accomplished in our lab *via* the synthetic scheme reported previously (164,165). As the results of *in vitro* evaluation of these molecules in MurX enzyme inhibition and *Mtb* (H₃₇Rv) growth inhibitory activities against replicating and nonreplicating (dormant) *Mtb* *via* the MABA and LORA assays, it was identified that UT-01309 (**42**) possessing 3-amino-1,4-benzodiazepine-2-one exhibited increased MurX enzyme and *Mtb* growth inhibitory activity (MIC: 2.5 µg ml⁻¹ vs 12.0 µg ml⁻¹ for capuramycin, IC₅₀ 0.095 µM vs 0.127 µM for capuramycin) (47,136,141,186). The analogs synthesized from the 3'O-methyluridine-mannopyranuronate (natural form) did not exhibit bactericidal activity against nonreplicating *Mtb* even at 400 µg ml⁻¹ concentrations. The isocapuramycin (2'O-methyl) analogs (R₁=Me and R₂=H in **Figure 3-12**) showed equal MurX inhibitory and antimycobacterial activities to the corresponding analogs derived from the natural form (R₁=H and R₂=Me in **Figure 3-12**). The antimycobacterial MurX inhibitors, isocapuramycin analogs (2'O-methyluridine-mannopyranuronate derivatives) were not capable of killing nonreplicating *Mtb* in the LORA assay. The 3'O-demethyl capuramycin analogs (R₁, R₂=H in **Figure 3-12**) did not exhibit activities in MurX enzyme inhibitory and antimycobacterial assays. Remarkably, 2'O-methylated capuramycin, UT-01320 (**45**) killed both replicating and nonreplicating *Mtb* at low concentrations with the MIC_{LORA/MABA} value of 1.72, whereas the MIC_{LORA/MABA} value for rifampicin was 7.35 (**Table 3-4**). The MIC_{LORA/MABA} value close to 1 or below 1 is considered to be ideal TB drug lead molecules in our programs. **45** was also tested against nutrient deficient model of *Mtb* (LOEBEL) (187). In both models of non-replicating *Mtb*, no CFU was observed after 15 days' incubation of the *Mtb* cultures treated with UT-01320 at 100 µg ml⁻¹, 10 µg ml⁻¹ and 2.58 µg ml⁻¹, respectively (**Figure 3-13**). Cytotoxicity (IC₅₀) of **45** was evaluated *in vitro* using Vero monkey kidney cells and HepG₂ human hepatoblastoma cells. As observed for other capuramycin analogs, UT-01320 did not show cytotoxicity even at 400 µg ml⁻¹ concentrations (**Table 3-4**); the selectivity index (IC₅₀ in mammalian cells/MIC against *Mtb*) of **45** was determined to be >266.

Effectiveness of UT-01320 against Intracellular *Mtb*

Activity of UT-01320 **45** against intracellular *Mtb* was evaluated. Murine macrophage cell J774A.1 infected by a transformant *Mtb* CDC1551 containing tdTomato

Figure 3-12. Structures of capuramycin (36), SQ 641 (37), UT-01320 (45), and UT-01309 (42) and summary of SAR obtained from a 100-membered library.

Table 3-4. MICs of selected compounds against *Mycobacterium spp.*

Compounds	IC ₅₀ MurX μM	MIC (MABA) μg/mL	MIC (LORA) μg/mL	IC ₅₀ (Vero Cells) μg/mL ^a
Capuramycin (36)	0.127	12.5	>100	>400
SQ 641 (37)	0.098	1.56	>100	>400
UT-01320 (45)	>100	1.50	2.58	>400
UT-01309 (42)	0.095	2.50	>100	>400
RIF	-	0.20	1.47	>400
INH	-	0.04-0.10	>100	>400
EMB	-	0.78	>100	>400

NOTE: MABA - Microplate alamar blue assay, LORA - Low oxygen recovery assay,

^a Cytotoxicity against Vero monkey kidney cells was > 400 μg/mL.

A. Wayne Model

B. LOEBEL Model

Figure 3-13. Bactericidal activity of UT-01320 against non-replicating *Mtb* evaluated in Wayne and nutrient deficient models.

UT-01320 (45) was tested at MIC, 4x MIC and 40x MIC in comparison with RIF, INH and ETB at 6x MIC. RIF – Rifampicin, INH – Isoniazid.

(MOI = 10) were treated with **45** (at x2 and x4 MIC_{LORA}). After 24, 48, and 72 h of incubation, the relative intensity of the fluorescence was measured (emission wavelength (581 nm)) *via* UV–vis spectroscopy (**Figure 3-14A and B**). Alternatively, the lysates were tenfold serially diluted in 7H10-S broth and inoculated on 7H11-S plates to determine the number of viable cell-associated *Mtb* to confirm the bactericidal effect of UT-01320 against intracellular *Mtb* in 72 h. **45** killed *Mtb* in infected macrophages at x2 MIC_{LORA} concentrations within 72 h. **Figure 3-14A and B** showed clearly that **45** kills intracellular *Mtb* as effectively as capreomycin (a 2nd line TB drug). Bactericidal effect of **45** against the intracellular *Mtb* was distinguished from that of rifampicin in 72 h. The intracellular activity of **45** was visualized *via* fluorescence confocal microscopy; significant reduction of intracellular *Mtb* was observed in 48–72 h compared to untreated control cultures (**Figure 3-14C**).

Rapid Antimycobactericidal Activity of UT-01320

Because of alteration of molecular target, we were interested in knowing whether a WecA inhibitor **45** remains rapidly bactericidal against replicating *Mtb* under aerobic conditions. The time-kill experiments were performed at two and fourfold the MIC of capuramycin, **45** and a few first-line TB drugs (RIF and INH)). Viable cell counting was performed at every 24 h for 14 days. CFUs were counted after 15 days of incubation at 37 °C. The rate of killing of **45** against *Mtb* was compared directly with the reference molecules and the time-kill assessments at 2x and 4x MIC concentrations are shown in **Figure 3-15**. The MurX inhibitor, capuramycin yielded log₁₀ CFU ml⁻¹ reduction of 3.0 or greater for *Mtb* in 1 day and capuramycin killed *Mtb* completely in 2 days at 24.0 µg ml⁻¹, albeit, RIF and INH required over 7 days to kill 50% of *Mtb* at 0.4 µg ml⁻¹ and 1.0 µg ml⁻¹, respectively (**Figure 3-15A**) (91,188). **45** killed >50% of *Mtb* within 3 days and 100% within 5 days at 3.0 µg ml⁻¹ (2x MIC), and killed 100% of *Mtb* within 3 days at 6.0 µg ml⁻¹ (4x MIC) (**Figure 3-15B**). Thus, the fast killing profile of **45** was similar to that of capuramycin; **45** killed *Mtb* faster than RIF and INH, but was less effective than capuramycin.

Synergistic Effect of UT-01320 with MurX Inhibitors

The synergistic or antagonistic activities of MurX inhibitors (capuramycin or SQ 641) and WecA inhibitor, **45** were assessed *in vitro* *via* micro dilution broth checkerboard technique (189,190). In the checkerboard analyses of a combination of SQ 641 and **45**, the FIC index range of 0.06 to 4 was observed for 47 combinations of two molecules out of 64 different concentrations. **Table 3-5** summarizes the results of FIC index analyses for a combination of SQ 641 plus **45** and capuramycin plus **45** that showed synergistic combination (ΣFIC<0.5). It was demonstrated that **45** exhibited synergy with SQ 641 and capuramycin that killed *Mtb* at much lower concentrations than the MIC_A and MIC_B. The structure of **45** is closely related to the MurX inhibitor SQ 641. The PK/PD parameters of **45** may be correlated with those of SQ 641, and thus, a combination of two capuramycin

Figure 3-14. Effect of UT-01320 and representative TB drugs against intracellular *Mtb* CDC1551-tdTomato (a transformant *Mtb* CDC1551 containing tdTomato) in macrophages (J774A.1 cells).

A) Time-kill curve for intracellular *Mtb* at 2x MIC concentration. B) Time-kill curve for intracellular *Mtb* at 4x MIC concentration. C) Fluorescence confocal microscopy of J774A.1 murine macrophages infected with tdTomato (red) expressing *Mtb* after the treatment of 45 (at 4xMIC). Images were obtained for the treated and untreated cells for 72 h. Fixed cells were stained with a blue fluorescent stain, DAPI (4',6-diamidino-2-phenylindole) to visualize nuclei.

Figure 3-15. *In vitro* time-kill assessment of capuramycin, 45 and the first-line TB drugs (RIF and INH).

All the compounds were tested at 2x MIC and 4x MIC. RIF – Rifampicin, INH – Isoniazid.

Table 3-5. Fractional inhibitory concentration of a combination of two inhibitor molecules.

Combination of A and B	MIC _A (µg/mL) MIC _B (µg/mL) ^a	MIC (µg/mL) C _A and C _B ^b	ΣFIC ^{c,d}
A: SQ 641 (37)	1.56	0.10	0.12
B: UT-01320 (45)	1.50	0.08	
A: SQ 641 (37)	1.56	0.10	0.17
B: UT-01320 (45)	1.50	0.16	
A: SQ 641 (37)	1.56	0.10	0.28
B: UT-01320 (45)	1.50	0.32	
A: SQ 641 (37)	1.56	0.10	0.49
B: UT-01320 (45)	1.50	0.645	
A: SQ 641 (37)	1.56	0.10	0.92
B: UT-01320 (45)	1.50	1.29	
A: Capuramycin (36)	12.50	1.563	0.18
B: UT-01320 (45)	1.50	0.08	
A: Capuramycin (36)	12.50	1.563	0.23
B: UT-01320 (45)	1.50	0.16	
A: Capuramycin (36)	12.50	0.78	0.28
B: UT-01320 (45)	1.50	0.32	
A: Capuramycin (36)	12.50	0.78	0.49
B: UT-01320 (45)	1.50	0.645	
A: Capuramycin (36)	12.50	0.78	0.92
B: UT-01320 (45)	1.50	1.29	

NOTE: ^a MIC_A and MIC_B are the MIC value of molecule A or B against *Mtb*, ^b C_A and C_B are concentrations of A and B in combination, ^c ΣFIC index for the wells at growth–no growth interface, ^d ΣFIC is the sum of fractional inhibitory concentration calculated by the equation ΣFIC = FIC_A + FIC_B = C_A/MIC_A + C_B/MIC_B.

analogs that have distinct drug targets is a great interest in evaluation of their synergistic interactions. These forward-looking biological characteristics for **45** promise that new TB drug leads will be discovered by extensive structure–activity relationship studies of 2'O-methylated capuramycin analogs.

Spectrum of Activity of UT-01320

As summarized in **Table 3-6**, **45** has a very narrow spectrum of activity; in growth inhibitory assays against Gram-positive and -negative bacteria including *Mycobacterium spp.*, **45** killed *Mtb* strains selectively. It is interesting to note that unlike other capuramycin analogs, **45** did not kill a fast-growing *M. smegmatis* at $>200\text{ }\mu\text{g ml}^{-1}$ concentrations (for example, MICs $6.5\text{ }\mu\text{g ml}^{-1}$ and $25\text{ }\mu\text{g ml}^{-1}$ for capuramycin and UT-01309 respectively).

Conclusions

We have discovered a novel capuramycin analog, UT-01320 (**45**); with WecA inhibitory activity that kills non-replicating *Mtb*. This was achieved by screening a library of capuramycin analogs against MurX and WecA enzymes. Convenient high-throughput amenable screening methods for assay of WecA and MurX enzymes were developed. We have demonstrated MurX/MraY-catalyzed synthesis of neryl-lipid I-N^e-C₆-fluorescent analogs (**33** and **34**) from Park's nucleotide-N^e-fluorescent analogs (**29** and **32**) with neryl phosphate. Biosynthesis of neryl-lipid I analogue was achieved, for the first time, in excellent yield with the MurX-containing membrane fraction (P-60). Similarly, neryl-lipid I-N^e-dansylthiourea could be biosynthesized *via* the different sources of MraY enzymes such as *M. smegmatis*, *E. coli*, and *S. aureus*. However, the purified MraY enzymes seem to be denaturing under the assay conditions developed for P-60 membrane fractions. A water-soluble lipid I generated in MurX assay media could be quantitated conveniently *via* reverse-phase HPLC without sophisticated extraction procedures. Signal-to-noise ratio of HPLC analyses of **29** and **33** is significantly high without using fluorescence detector. Furthermore, difference in the retention times between Park's nucleotide-N^e-dansylthiourea (**29**) and neryl-lipid I-N^e-dansylthiourea (**33**) was more than 3.5 min and each assay analysis could be completed within 10 min. We developed convenient methods for preparation of MurX/MraY enzymatic substrates, **29** and neryl phosphate (**35**), and thus, the substrates for the assays are available to screen a relatively large number of molecules in our laboratory. In order to determine usefulness of the MurX/MraY assay protocols developed here, we screened a 50-membered library including new uridyl peptides, and known positive- and negative-controls. MurX enzyme inhibitory activity of all positive-controls showed approximately equal to the IC₅₀ values obtained with the previously reported methods. The negative-control molecules did not exhibit MurX inhibitory activity even at high concentrations. Because the assay method described here quantitates the MurX/MraY substrate and product simultaneously in each assay vial without extraction or separation, the errors in qualitative analyses of assay caused by quantitation of single-molecule (remaining molecule or converted molecule)

Table 3-6. Spectrum of activity of UT-01320.

Species and Strain	MIC (µg/mL) ^a		
	UT-01320 (45)	RIF	INH
<i>Mtb</i> H ₃₇ Rv	1.50	0.01	0.57
<i>Mtb</i> H ₃₇ Rv INHr ^b	2.50	0.10-0.20	>8.0
<i>Mtb</i> H ₃₇ Rv RFPr ^c	2.45	>4.0	0.05
<i>M. smegmatis</i> ATCC607	>200	-	-
<i>S. aureus</i> ATCC6538D-5	>200	-	-
<i>E. faecium</i> ATCC 349	>200	-	-
<i>E. coli</i> ATCC 25019	>200	-	-
<i>K. pneumonia</i> ATCC 8047	>200	-	-
<i>P. aeruginosa</i> ATCC 27853	>200	-	-

NOTE: ^a The microplate alamar blue assay method was used, ^b INH-resistant *Mtb*, ^c RFP-resistant *Mtb*, RIF - rifampicin, INH - isoniazid.

and/or by complicated work-up procedures are diminished. In the screening of a small library of molecules using the described method, to date, a false positive or false negative result has not been identified.

We have been studying the molecular target for antimycobacterial non-MurX/MraY inhibitors identified in this program. High reliability for the MurX/MraY assay protocols described here will be a variable asset to identify selective MurX inhibitor molecules for development of new antibacterial agents.

We have developed convenient methods for preparation of WecA enzyme substrates and an assay amenable to high- and medium- throughput screens. We have validated the assay by screening a library of 50 capuramycin analogs and identifying **45** as a selective WecA inhibitor with bactericidal activity against replicating and non-replicating *Mtb*, as well as *Mtb* in macrophages. The efficacy of **45** against non-replicating bacteria is especially significant, suggesting that the WecA enzyme is necessary to maintain viability of *Mtb* in a latent state (102,191).

Methylation at the C2'-hydroxyl group of capuramycin (R_1 in **Figure 3-12**) alters the molecular target of capuramycin. **45** did not interfere with MurX, but exhibited bactericidal activity against nonreplicating (dormant) *Mtb* with the MIC_{LORA}/MIC_{MABA} value of 1.72 (MIC_{LORA}/MIC_{MABA} = 7.35 for RIF). In preliminary assay screening of UT-01320 against the potential drug targets for non-replicating *Mtb*, it was observed that, UT-01320 did not inhibit electron transport systems, menaquinone biosynthesis, *Mtb* serine/threonine kinases (PKnA and B) and DNA gyrase.

CHAPTER 4. NOVEL PLEUROMUTILIN ANALOGS AS PROTEIN BIOSYNTHESIS INHIBITORS

Introduction

Pleuromutilin (**53**), a strong binder of the 50S ribosomal subunit, was first isolated in 1952 from two *Basidiomycete spp.* Pleuromutilin analogs, tiamulin (**54**) and valnemulin (**55**) are FDA-approved drugs for use in veterinary medicine (192-194). Although azamulin (**56**) was developed for human use, due to its strong inhibition of cytochrome 450, it did not progress beyond phase I clinical trials. Retapamulin (**57**) (GSK) was approved by FDA in 2007 for topical (skin) use in humans (117,195). An experimental drug, lefamulin (**58**) (Nabriva Therapeutics), entered Phase II clinical studies for systemic human use (196,197). Strong antibacterial activity of pleuromutilins against Gram-positive bacteria has been known since 1952; however, the effectiveness of pleuromutilin analogs against *Mycobacterium spp.* was not reported until 2003 (198). Long et al. reported moderate activity of valnemulin against *M. smegmatis* (MIC 8-16 µg/mL) (133). To date, a large array of reported pleuromutilin derivatives are analogs whose structures are modified at the C14-ester side chain. Dong et al. reported *N*-benzylamine substituted pleuromutilin analogs with moderate anti-*Mtb* activity (60-100% inhibition at 20 µM) (199). Lotesta et al. reported a tiamulin analog (**59**) which displayed moderate activity against *Mtb* (MIC ~12.5 µg/mL) (200). Ascher et al. filed a patent for several antimycobacterial pleuromutilin analogs whose structures are diversified at the C2, C4, C6, C7, C12, or C14 positions (**Figure 4-1**) (201).

Pleuromutilins interfere with the peptidyl transferase center (PTC) of the 50s bacterial ribosomes. The co-crystal structure of *Deinococcus radiodurans* 50s ribosome with tiamulin (**54**) revealed that pleuromutilins bind two ribosomal key sites known as the 'A' site and 'P' site of the 50S subunit (118), resulting in the inhibition of peptide elongation, and thus, the cessation of bacterial growth. On the other hand, oxazolidinone antibacterial agents occupy solely the 'A' site of 50s ribosomes (112). Consequently, it is concluded that there is a lower probability of cross resistance between pleuromutilin and linezolid (198). **Figure 4-2** illustrates the binding sites of pleuromutilin derivatives and linezolid on the bacterial 50S ribosomal subunit.

We have identified several pleuromutilin analogs that exhibit bactericidal activity against both replicating and non-replicating forms of *Mtb*. UT-800 (**64**) displayed the MICs of < 1.5 µg/mL in MABA and LORA assays. Unlike previously reported analogs, UT-800 is a narrow spectrum bactericidal molecule that showed focused activity against *Mycobacterium spp.* We have evaluated UT-800 in basic pharmacological *in vitro* experiments towards *in vivo* studies using the *Mtb* mouse infection models.

Figure 4-1. Representative structures of pleuromutilin analogs.

^a The anti-*Mtb* compound reported by Lotesta et al, ^b Dong et al, ^c Ascher et al.

Figure 4-2. Binding sites of pleuromutilin derivatives and linezolid on 50s ribosome.

Pleuromutilin derivatives interfere with both the A- and P- sites of bacterial 50s ribosome; **a** - Pleuromutilin binding site, **b** - Linezolid binding site.

Results and Discussion

Screening of Pleuromutilin Analogs against *Mtb*

To identify new pleuromutilin analogs with activity towards *Mtb*, we have generated over 50 analogs whose structures contained the methoxyamine-oxime (**Figure 4-3A**), hydroxylamine-oxime (**Figure 4-3B**), or 3-hydroxy (**Figure 4-3C**) core structure. These structures were further diversified by the reduction of the double bond (C16 position), amide formations or reductive aminations. The generated molecules were evaluated in the growth inhibitory assays against *Mtb* (H₃₇Rv). We identified eight pleuromutilin analogs which exhibited the MIC value <12.5 µg/mL against *Mtb* (**Figure 4-3D**). Out of the eight molecules UT-800 (**64**), UT-810 (**65**) and UT-815 (**66**) exhibited the lowest growth inhibitory activities against *Mtb* (MIC 0.78-1.56 (MABA) and 1.04-1.98 (LORA)).

Synthesis of UT-800, UT-810 and UT-815

In order to confirm anti-*Mtb* activity of three potent compounds from the screenings, these molecules were resynthesized in our group. Their syntheses are illustrated in Scheme 1 (**Figure 4-4**). The C3-carbonyl group of pleuromutilin (**53**) was reduced with NaBH₄ to form the *secondary* alcohol with the *R*-configuration exclusively. The *primary* alcohol of the C14-side chain was tosylated with TsCl and DMAP to afford **72** in **Figure 4-4** in 90% overall yield. Thioether formation of **72** with 1-amino-2-methylpropane-2-thiol was accomplished according to a reported condition, yielding the free amine **73** in **Figure 4-4** in 90% yield (202,203). Amide-forming reaction of **73** with Boc-D-Leu-OH under glycerolacetone-Oxyma, EDCI, and NaHCO₃ in DMF-H₂O followed by reduction with H₂/Pd-C in MeOH-EtOAc. Deprotection of the Boc group with 4*N* HCl afforded UT-800 (**64**) (204-206). Compounds **65** and **66** were synthesized by tosylation of the *primary* alcohol of the C14-side chain with TsCl and DMAP to afford **74** in **Figure 4-4**, and the corresponding thioether formation of **74** with 1-amino-2-methylpropane-2-thiol was accomplished, yielding the free amine **75**. Corresponding amide formation with Boc-D-Leu-OH and subsequent Boc-deprotection yielded UT-810 (**65**). The Boc protected derivative was reduced with H₂/Pd-C in MeOH-EtOAc followed by Boc-deprotection yielded UT-815 (**66**). The analogs synthesized in Scheme 1 were purified *via* reverse-phase HPLC.

Effectiveness of UT-800, 810 and 815 against Replicating and Non-replicating *Mtb* *via* MABA and LORA Assays

The MIC values of the analogs were determined against *Mtb* H₃₇Rv in both aerobic and anaerobic conditions *via* MABA and LORA assays, respectively. The analogs **64**, **65** and **66** exhibited MIC_{MABA} of 0.78-1.46 µg/mL and MIC_{LORA} of 1.04-1.98 µg/mL (**Table 4-1**). The ratio of MIC_{LORA}/MIC_{MABA} for the three compounds was

Figure 4-3. General structures of pleuromutilin derivatives and Anti-*Mtb* molecules.

The structures were diversified by the reduction of the double bond (C16 position), amide formations and reductive aminations at C14-side chain and reduction at C3 position.

Figure 4-4. Scheme 1: syntheses of pleuromutilin analogs UT-800, UT-810 and UT-815.

Conditions - a) NaBH_4 , MeOH, 0 °C. b) TsCl , DMAP, CH_2Cl_2 , 0 °C. c) 1-amino-2-methylpropane-2-thiol, 1M NaOH, nBu_4NBr , THF/ H_2O (1 : 1), 50 °C. d) Boc-D-Leu-OH, Glyceroacetone-Oxyma, EDCI, NaHCO_3 , DMF- H_2O (9/1). e) H_2 , Pd/C, MeOH-EtOAc. f) HCl, Dioxane.

Table 4-1. Anti-*Mtb* activity and cytotoxicity of pleuromutilin analogs.

Entry	Compounds	MIC (µg/mL) against <i>Mtb</i>		IC ₅₀ (µg/mL) against Vero Cells
		MABA ^a	LORA ^b	
1	UT-800 (64)	0.83	1.20	10.45
2	UT-810 (65)	1.56	1.98	13.96
3	UT-815 (66)	0.78	1.04	5.26
4	UT-522 (67)	3.06	3.46	25
5	UT-490 (68)	6.25	-	45.3
6	UT-820 (69)	5.81	2.76	5
7	UT-825 (70)	8.18	-	3.13
8	UT-910 (71)	12.5	-	5.26
9	Valnemulin (55)	3.13	-	15.59
10	RIF	0.01-0.2	0.05	102.3
11	INH	0.57	50.24	>200
12	Tunicamycin (38)	12.5	-	0.18

NOTE: ^a The microplate alamar blue assay method was used, ^b Low oxygen recovery assay.

between 1.27 – 1.45, which is closer to the ideal value of 1 or <1. The identified molecules are analogs of valnemulin and they exhibited a superior inhibitory profile against *Mtb* H₃₇Rv compared to valnemulin.

In order to obtain insights into potential toxicity of identified inhibitor molecules, all antimycobacterial pleuromutilin analogs were evaluated in *in vitro* cytotoxicity assays against Vero monkey kidney cells. The toxicity profile of **64**, **65** and **66** was similar to that of valnemulin (**55**). We are currently evaluating the acute toxicity (LD₅₀ value) of **64** via ICR female mice (4 weeks old).

Effectiveness of UT- 800, 810 and 815 against Intracellular *Mtb*

The activity of the three analogs against intracellular *Mtb* was evaluated. Murine macrophage cell J774A.1 infected by a transformant *Mtb* CDC1551 containing tdTomato (MOI=10) were treated with UT-800, -810 and -815 (at x2 and x4 MIC_{LORA}). After 24, 48, and 72 h of incubation, the relative intensity of the fluorescence was measured (emission wavelength (581 nm)) via UV–vis spectroscopy (**Figure 4-5A and B**). Alternatively, the lysates were ten-fold serially diluted in 7H10-S broth and inoculated on 7H11-S plates to determine the number of viable cell-associated *Mtb* to confirm the bactericidal effect of the analogs against intracellular *Mtb* in 72 h. The analogs killed *Mtb* in infected macrophages at x2 MIC_{LORA} concentrations within 72 h. **Figure 4-5A and B** shows that **64**, **65** and **66** kill intracellular *Mtb* better than rifampicin. INH did not kill intracellular *Mtb* at x2 and x4 MIC.

Rapid Antimycobactericidal Activity of UT-800, 810 and 815

The time-kill experiments were performed at two and four-fold the MIC of **64**, **65**, **66** and two first-line TB drugs (RIF and INH). Viable cell counting was performed at every 24 h for 16 days. CFUs were counted after 15 days of incubation at 37 °C. The rate of killing of analogs against *Mtb* was compared with the reference molecules, and the time-kill assessments at x2 MIC concentrations are shown in **Figure 4-6**. The killing profile of the new analogs was similar to that of RIF and INH. RIF and INH required 7 days to kill 50% of *Mtb* at 0.4 µg/ml (x2 MIC) and 1.0 µg/ml (x2 MIC), respectively.

Spectrum of Activity of UT-800, 810 and 815

As summarized in **Table 4-2**, the spectrum of activity of the three pleuromutilin analogs (**64**, **65** and **66**) was determined by assaying against 15 bacterial strains including Gram-positive, -negative bacteria and *Mycobacterium spp.* We concluded that **64**, **65** and **66** kills *Mycobacterium* species selectively and are especially effective in killing *Mtb* at low concentrations.

Figure 4-5. Effect of pleuromutilin analogs and representative TB drugs against intracellular *Mtb* CDC1551-tdTomato (a transformant *Mtb* CDC1551 containing tdTomato) in macrophages (J774A.1 cells). A) Time-kill curve for intracellular *Mtb* at 2x MIC concentration. B) Time-kill curve for intracellular *Mtb* at 4x MIC concentration.

Figure 4-6. *In vitro* time-kill assessment of valnemulin (55), 64, 65, 66 and the first-line TB drugs (RIF and INH).

Table 4-2. Spectrum of activity of UT-800, UT-810 and UT-815.

Species and Strain	MICs (µg/mL) ^a							
	UT-800 (64)	UT-810 (65)	UT-815 (66)	VAL (55)	RIF	INH	CPR	LIN
<i>Mtb</i> H ₃₇ Rv	0.83	1.46	0.77	3.13	0.2	0.57	3.13	1.61
<i>M. smegmatis</i> ATCC 607	25	>25	25	25	>25	-	25	0.8
<i>S. aureus</i> ATCC 25923	>25	>25	>25	>25	>25	-	>25	>25
<i>S. aureus</i> BAA 2094	>25	>25	>25	>25	0.2	-	>25	>25
<i>S. aureus</i> BAA 1683	>25	>25	>25	>25	0.2	-	>25	25
<i>S. aureus</i> BAA 44	>25	>25	>25	>25	25	-	>25	>25
<i>E. faecium</i> ATCC 349	>25	>25	>25	>25	>25	-	>25	>25
<i>E. faecalis</i> ATCC 19433	12.5	25	25	12.5	1.56	-	>25	25
<i>S. pneumoniae</i> ATCC 6301	>25	>25	>25	>25	>25	-	>25	>25
<i>S. salivarius</i> ATCC 7073	>25	>25	>25	>25	>25	-	>25	>25
<i>B. subtilis</i> ATCC 6051	>25	25	25	25	0.4	-	>25	6.25
<i>E. coli</i> ATCC 10798	>25	>25	>25	>25	25	-	>25	>25
<i>K. pneumonia</i> ATCC 8047	25	>25	>25	25	3.13	-	>25	>25
<i>P. aeruginosa</i> ATCC 27853	>25	>25	>25	>25	-	-	-	-
<i>A. baumannii</i> ATCC 19606	3.13	25	12.5	6.25	3.13	-	>25	>25

NOTE: ^a The microplate alamar blue assay method was done, VAL – Valnemulin, RIF – Rifampicin, INH – Isoniazid, CPR – Capreomycin, LIN – Linezolid.

***In vitro* Metabolic Stability of UT-800, UT-810, and UT-815**

In our study of *in vitro* metabolism of the three potent pleuromutilin analogs in rat liver microsomes, we observed a striking difference in half-life ($t_{1/2}$) between C3-hydroxyl compound **64** and C3-carbonyl compounds **65** and **66**. The half-life of **64** was determined to be > 60 min, on the other hand, $t_{1/2}$ of Ut-810 and -815 were 1.29 min. As such, *in vitro* half-life was significantly extended by reduction of the C3-carbonyl group of valnemulin (**Figure 4-7**). We have also observed the same trend of increased *in vitro* half-life by reducing the C3-carbonyl group of valnemulin to hydroxyl group in our anti-*Acinetobacter baumannii* analog, UT-490 (**68**) (207).

Prediction of Intestinal Permeability of UT-800 via Caco-2 Permeability Assay

The suitability of **64** for oral dosing was evaluated by using a bidirectional Caco-2 permeability assay by Cyprotex to predict *in vivo* intestinal permeability and to investigate drug efflux. This established *in vitro* model predicts the *in vivo* absorption of drugs across the gut wall by measuring the rate of transport across the Caco-2 cell line that resembles the intestinal epithelial cells (208). **64** was added to the apical side of the membrane and the transport of the compound across the monolayer was monitored over a 2 h time period. The transport of **64** in the apical to basolateral direction (A-B) as well as the basolateral to apical direction (B-A) was assessed to determine an efflux ratio which provides an indicator as to whether **64** undergoes active efflux. The permeability coefficient (P_{app}) was determined to be 3.39×10^{-6} cm/s from A-B and 18.3×10^{-6} cm/s from B-A (**Figure 4-8**). Efflux ratio ($P_{app}(B-A)/P_{app}(A-B)$) was determined to be 5.44. Ranitidine was used as a low permeability control ($P_{app} < 1$) and warfarin was used as a high permeability control ($P_{app} > 1$). Talinolol, a known P-gp substrate, was screened as a control compound to confirm that the cells are expressing functional efflux proteins ($P_{app}(B-A)/P_{app}(A-B) = 76.8$). A P_{app} rate coefficient > 1 and efflux ratio approx 5 indicates that **64** has permeability across the membrane with moderate levels of efflux (an efflux ratio greater than two indicates that drug efflux is occurring).

Genetic Analysis of UT-490 to Study the Resistance Mechanism

It is well established that the pleuromutilin derivatives target the peptidyl transferase center of the 50S ribosomal protein L3 (rplC), inhibiting protein biosynthesis (118,119,198,209-213). Previously, other groups reported multiple mutations in *rplC* of *S. aureus* that can define a region of *rplC* capable of causing decreased susceptibility of the pleuromutilin derivative in *S. aureus* (118,210,211). We have evaluated an anti-*Acinetobacter baumannii* analog, UT-490 (**68**) for its potential mechanism of resistance. UT-490 has also displayed moderate activity against *Mtb* (Entry 5 in (**Table 4-1**) (207). In order to identify a potential mechanism of resistance to UT-490 (**68**), we isolated the chromosomal DNA from the resistant mutant (UT-490^R, 16xMIC) and its parental wild-type control *A. baumannii* (ATCC19606). The *rplC* gene fragment was amplified using

Figure 4-7. *In vitro* metabolic stability of pleuromutilin analogs (64, 65, and 66). Verapamil was utilized as a control compound, and the half-life ($t_{1/2}$) of verapamil was determined to be 10 min.

Figure 4-8. *In vitro* Caco-2 permeability assay to predict intestinal permeability of UT-800 (64).

A) Experimental data for Caco-2 assay. B) **64** and control compounds were plotted against the permeability rate coefficient (P_{app} (10^{-6} cm/s).

A. baumannii *rplC* specific primers and sequenced (214,215). The DNA sequencing results were blasted against *rplC* DNA sequence of *A. baumannii* in the NIH genetic sequence database. The DNA sequence alignment revealed a C456A single nucleotide mutation, which corresponded to N152K mutation in the protein sequence of RplC (**Figure 4-9**). UT- 800 (**64**) is structurally similar to **68**; the D-valine moiety at the C14 side chain is replaced by D-leucine and the C16 vinyl moiety is saturated (**Figure 4-3**). The mode of action of **64** is believed to be the same as that of **68**; the transcription/translation coupled luciferase reporter assays showed that the both molecules inhibited protein biosynthesis by 55-67% at 0.1 µg/mL concentration.

Conclusions

We identified a new pleuromutilin derivative UT-800 (**64**) that displays a strong antimycobacterial activity against *Mtb*. UT-800 kills *Mtb* in both replicating and non-replicating forms with 0.83 and 1.20 µg/mL concentration, respectively, and it effectively kills the intracellular *Mtb*; the time-killing profile of **64** is similar to that of RIF. Interestingly, spectrum of activity of UT-800 is focused against *Mycobacterium* spp. that is an attractive characteristic to progress anti-TB drugs. UT-800 is a C3-reduced analog of pleuromutilin that significantly extends the *in vitro* half-life ($t_{1/2}$ > 60 min) with rat liver microsomes. The gene analyses of *A. baumannii* strain that is resistant to **68** revealed a single mutation in the *rplC* gene, suggesting that an *Mtb* mutant against **64** may exhibit a similar resistant mechanism.

The pleuromutilin analog, **64** exhibited permeability across Caco-2 epithelial monolayers (P_{app} rate coefficient > 1) with moderate levels of efflux (an efflux ratio of approximately 5). These Caco-2 permeability data predict an oral bioavailability for **64** *in vivo*. Pharmacokinetics, oral bioavailability and *in vivo* toxicity of **64** are currently being studied in our group using preclinical animal models.

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Query  367  FGGVKRWNFRTQDATHGNSVSHRVLGSTGQQTPGRVFKGKKMAGHLGDERVTVOGLEI
          FGGVKRWNFRTQDATHGNSVSHRVLGSTGQ  QTPGRVFKGKKMAGHLGDERVTVOGLEI
Sbjct  121  FGGVKRWNFRTQDATHGNSVSHRVLGSTGQQTPGRVFKGKKMAGHLGDERVTVOGLEI

```

Figure 4-9. The amino acid alignment of the 50S ribosomal protein L3 (RplC) from a UT-490 (**68**)-Resistant *A. baumannii* strain (**68R**).

68^R (Query) and wild-type control (Sbjct): the red color represents the site mutation in RplC. (Reprinted with permission. Siricilla, S., Mitachi, K., Yang, J., Eslamimehr, S., Lemieux, M. R., Meibohm, B., Ji, Y., and Kurosu, M. (2017). A new combination of a pleuromutilin derivative and doxycycline for treatment of multidrug-resistant *Acinetobacter baumannii*. *J. Med. Chem. American Chemical Society*. Article ASAP)

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The efforts to control and prevent TB are seriously threatened by the emergence of MDR, XDR and TDR strains of *Mtb*. The current regimen to treat MDR- and XDR-TB takes several years. Treatment of TDR-TB requires completely novel therapeutic strategy. Drugs that shorten the treatment time of TB chemotherapy is extremely important. *Mtb* is known to survive by entering a non-replicating (dormant) state in the host. *Mtb* can exist in the dormant state for many years in the host, causing the latent infection. To discover drugs that kill *Mtb* in non-replicating state is believed to be a promising strategy to shorten the current TB chemotherapy. This dissertation discusses the development of novel antimycobacterial drug leads effective against both replicating- and non-replicating *Mtb* at low concentrations. These antimycobacterial agents summarized here have the potential to shorten the treatment for TB.

New antimycobacterial molecules that kill non-replicating *Mtb* were identified by screening libraries of synthetic natural products and their analogs (**Figure 5-1**). Screening of a 400-membered library of aurachin RE analogs resulted in the discovery of UT-317, (*R*)-**20**. UT-317 is a selective MenA inhibitor which ultimately inhibits biosynthesis of the electron carrier, vitamin K₂. UT-317 exhibits an antimicrobial spectrum focused against *Mtb*, and effectively kills both replicating and non-replicating *Mtb*.

In our ongoing programs of discovery of new drug leads against bacterial membrane-associated proteins, novel fluorescent probes, Park's nucleotide-*N*^E-C₆-FITC (**32**, for MraY/MurX) and UDP-glucosamine-C₆-FITC (**46**, for WecA) were developed. These probes enable us to perform medium- to high-throughput screening. Preliminary screening of a 50-membered library of capuramycin analogs against translocase I (MraY/MurX) and WecA identified a selective WecA inhibitor, UT-01320 (**45**) that kills both replicating and non-replicating *Mtb*. WecA catalyzes the first step of mycolyl-arabinogalactan conjugate biosynthesis of *Mtb* cell wall. The efficacy of UT-01320 against non-replicating bacilli is especially important, suggesting that some cell wall biosynthesis is necessary to maintain viability of *Mtb* in a latent state.

Screening of a 50-membered library of pleuromutilin derivatives resulted in discovery of UT-800 (**64**) that displays a strong antimycobacterial activity. UT-800 is a first-in-class pleuromutilin analog that kills non-replicating *Mtb* under anaerobic conditions and exhibits efficacy against intracellular *Mtb*.

Future Directions

It would be interesting to see the *in vivo* efficacy of the identified MenA inhibitors using the mouse infection models. A new MenA inhibitor UT-321 (structure not shown) has demonstrated effectiveness against *Clostridium difficile* *in vivo*. Thus, a

Figure 5-1. Drug leads identified against dormant *Mtb* and novel fluorescent probes for the assay of phosphotransferases.

pharmacological proof-of-concept of a MenA inhibitor was demonstrated for the first time. Similarly, a MenA inhibitor having an appropriate ADMET profile is expected to demonstrate *in vivo* efficacy in the *Mtb* infection animal models.

UT-01320 (**45**) is the only selective WecA inhibitor identified to date. The *in vitro* activity, intracellular activity and narrow spectrum of activity towards *Mtb* demonstrated for **45** is excellent. UT-01320 did not exhibit toxicity in *in vitro* assays. In order to establish a pharmacological proof-of-concept, the PK/PD characteristics of **45** need to be studied. Our lab is currently optimizing the synthesis of **45** that enables us to generate over four gram-quantity as a pure form. WecA is required for the synthesis of mycolyl-arabinogalactan, an essential component of cell wall for *Mtb* to survive in macrophages. Genetic and microbiological studies of UT-01320 escape mutants will validate the unique mode of action of UT-01320 and WecA target for dormant *Mtb*.

UT-800 (**64**) has demonstrated its metabolic stability ($t_{1/2}$ > 60 min). Our group has initiated the pharmacokinetics, oral bioavailability and *in vivo* toxicity studies of UT-800 using preclinical animal models.

CHAPTER 6. MATERIALS AND METHODS

General Information

All antimycobacterial molecules were synthesized in our laboratory except for control compounds. The control compounds were obtained from sigma. All molecules were diluted with DMSO/H₂O/H₂O:PEG (1:2) mixture to be the concentration of 1 mg/100 μ L (stock solution). Difco Middlebrook 7H10 agar, Middlebrook 7H9 broth, Tryptic soy agar, Tryptic soy broth, MOPS, tris(hydroxymethyl)aminomethane, β -mercaptoethanol, triton-X 100, CHAPS, DMSO, resazurin, methylene blue, tween 80, menaquinone (VK₂) were purchased from Sigma-Aldrich. ADC, OADC enrichment, MTT, EMEM and FBS were purchased from Fisher Scientific. Magnesium chloride and potassium chloride were obtained from VWR. *E. coli* circular DNA assay kit (L1020) for transcription / translation coupled assay was purchased from Promega Corporation.

Bacterial Strains

Mycobacterium tuberculosis (H₃₇Rv, H₃₇Rv INHr and H₃₇Rv RFPr) were obtained through BEI Resources, NIAID/NIH. *Mycobacterium smegmatis* (ATCC 607 and ATCC 14468), *Mycobacterium bovis* (BCG), *Staphylococcus aureus* (ATCC 25923, BAA 2094, BAA 44, and BAA 1683), *Enterococcus faecium* (ATCC 349), *Enterococcus faecalis* (ATCC 19433), *Streptococcus pneumoniae* (ATCC 6301), *Streptococcus salivarius* (ATCC 7073), *Bacillus subtilis* (ATCC 6051), *Clostridium difficile* (), *Klebsiella pneumoniae* (ATCC 8047), *Pseudomonas aeruginosa* (ATCC 27853), *Acinetobacter baumannii* (ATCC 19606), and *Escherichia coli* (ATCC 10798) were obtained from ATCC. *E. coli* B21 wecA strain was obtained from Dr. McNeil (Colorado State University).

Bacterial Culture Conditions

A single colony of each *Mycobacterium* strain (H₃₇Rv, H₃₇Rv INHr, H₃₇Rv RFPr, *M. smegmatis* ATCC 607, ATCC 14468 and *M. bovis* (BCG)) was obtained on a Difco Middlebrook 7H10 nutrient agar enriched with 10% oleic acid, albumin, dextrose and catalase (OADC for *Mtb*), albumin, dextrose and catalase (ADC for *M. smegmatis* and *M. bovis*). Tryptic Soy agar was used for all other strains except *C. difficile*. A single colony of *C. difficile* was obtained on modified reinforced clostridial agar that was prepared anaerobically.

Seed cultures of each *Mycobacterium* strain were obtained in Middlebrook 7H9 broth enriched with OADC (for *Mtb*), ADC (for *M. smegmatis* and *M. bovis*). Tryptic Soy broth was used for all other strains except *C. difficile*. *C. difficile* was cultured in modified reinforced clostridial broth that was prepared anaerobically in an anerobic chamber under 10% H₂, 5% CO₂, and 85% N₂ conditions. Flasks containing each

bacterial strain was incubated to mid-log phase in their respective culture media, in a shaking incubator at 37 °C with a shaking speed of 200 rpm and cultured to mid-log phase (Optical density - 0.5). The optical density was monitored at 600 nm using a microplate reader (Biotek Synergy XT). All experiments involving *Mtb* were performed in a BSL3 facility.

Screening and Assay Development Procedures

Minimum Inhibitory Concentration Assays

Microplate Alamar Blue Assay (MABA). These were performed according to the published protocol (134). Bacterial cultures at 0.5 optical density, was treated with serial dilutions of inhibitors in aerobic conditions and incubated at 37 °C for 15 days for *Mtb* (H₃₇Rv, H₃₇Rv INHr and H₃₇Rv RFPr) and *M. bovis* (BCG). Incubation time was 48h for *M. smegmatis* (ATCC 607and 14468). All other bacteria were incubated for 24 h. After incubation, 20 µL of resazurin (stock-0.02%) was added and incubated on a shaking incubator at 37 °C for 4 h for *Mtb* and *M. bovis*. For all other bacteria 20 µL was added from a 0.08% resazurin stock solution and left for 1 h. The lowest concentration at which the color of resazurin was completely retained as blue was read as the MIC₁₀₀ (Pink = Growth, Blue = No growth). The absorbance measurements were also performed using a Biotek Synergy XT (Winooski, VT, USA), 96 well plate reader at 570 nm and 600 nm. Assay plates for *C. difficile* was incubated for 24 h in the anaerobic conditions and the optical density was measured at the end of incubation and wells with no visible growth was considered MIC.

Luminescence-based Low-oxygen-recovery Assay (LORA). These assays were performed according to the reported procedures in the facility of Illinois TB Research Institute (47). In brief, *Mtb* H₃₇Rv cells were transformed by mixing at least 1 µg of the purified plasmid, pFCA-luxAB and incubating at room temperature for 30 min, followed by electroporation (216). *Mtb* pFCA-luxAB strain cultured was diluted in Middlebrook 7H12 broth, and sonicated for 15s. The cultures were diluted to obtain an A₅₇₀ of 0.03 to 0.05 and 3,000 to 7,000 RLUs per 100 µl. Twofold serial dilutions of antimicrobial agents were prepared in black 96-well microtiter plates (100 µl), and 100 µl of the cell suspension was added. The microplate was placed under anaerobic conditions (oxygen concentration, less than 0.16%) by using an Anoxomat model WS-8080 (MART Microbiology) and three cycles of evacuation and filling with a mixture of 10% H₂, 5% CO₂, and 85% N₂. Incubation was continued for 10 days, and transferred to an ambient gaseous condition (5% CO₂-enriched air) incubator for a 28h “recovery.” 100 µl culture was transferred to white 96-well microtiter plates for determination of luminescence.

MIC on Nutrient Starved *Mtb* (Loebel Model Assay). *Mtb* H₃₇Rv was grown in conical flasks with air filter, containing Middlebrook 7H9 medium supplemented with

OADC at 37 °C according to the procedure established by Loebel. Exponentially growing bacilli at an OD₆₀₀ of 0.2 were transferred to PBS supplemented with 0.025%, Tween 80 and diluted to a final OD₆₀₀ of 0.1. 50 ml of this suspension was transferred into a conical flask and starved for 14 days at 37 °C (137,187,217). 100 µL of the culture was transferred to a 96 well plate and MIC experiment was performed according to the procedure described (*vide supra*).

***E. coli* Growth Inhibitory Assays under Anaerobic Conditions**

E. coli was grown with inhibitor molecule (5µg/mL) under anaerobic conditions in the modified *E. coli* growth media and the growth curve was monitored photometrically by reading the optical density at 600 nm (218,219) (**Figure 6-1**). *E. coli* growth rescue studies were performed by supplementing VK₂ (50 µM).

Oxygen Consumption Assays

In sterile glass vial 1.5 mL of *Mtb* or *M. smegmatis* (grown to 0.5 OD value) was placed. Inhibitor molecule (80 µL, total concentration ranged from 5–100 µg/mL) and 0.01% methylene blue (100 µL) was added into the culture solution. The culture vials were kept at 37 °C for 22 h. The effective concentration of the molecule to inhibit the electron transport systems was determined by comparing the color intensity with the control vial (no inhibitor) (**Figure 6-2**).

Preparation of Membrane Fraction

Mtb H₃₇Rv, *M. smegmatis* (ATCC 607), *S. aureus* (ATCC 25923) and *E. coli* (wild type; ATCC 10798 and BL21WecA) were grown to mid-log phase in their respective culture conditions described (*vide supra*) and the cells were harvested by centrifugation at 4 °C followed by washing with 0.9% saline solution (thrice) through centrifugation. ~5 g of pellet (wet weight) was collected. The washed cell pellets were suspended in homogenization buffer (containing 50 mM MOPS of pH = 8, 10 mM MgCl₂ and 5 mM 2-mercaptoethanol) and disrupted by probe sonication on ice (10 cycles of 60s on and 90s off). The resulting suspension was centrifuged at 1,000 ×g for 10 min at 4 °C to remove unbroken cells. The supernatant was centrifuged at 25,000 ×g for 40 min at 4 °C (3 to 4 times). All pellets in each tube were pooled and a second sonication was performed (10 cycles of 60s on and 270s off). The lysate was centrifuged once at 25,000 ×g for 1h and the supernatant was subjected to ultracentrifugation at 60,000 ×g for 1h at 4 °C. The supernatant was discarded and the membrane fraction containing MenA, MurX, and WecA enzymes (P-60) was suspended in the TRIS-HCl buffer (pH 7.5, containing 2-mercaptoethanol) (160,220). Total protein concentrations are about 8~10 mg/mL (221). Aliquots were stored in eppendorf tubes at –80°C.

Figure 6-1. *E. coli* growth inhibitory assays under anaerobic conditions.

A) *E. coli* was grown under anaerobic conditions using the growth media. B) Anaerobic conditions were maintained by using a mBraun LABstar anaerobe chamber and the O₂ levels were maintained below 50 ppm.

Figure 6-2. Oxygen consumption assays.

A) Oxidized form of methylene blue is blue in color while the reduced form is colorless. B) Color intensity of methylene blue in vials with varying concentrations of inhibitor was monitored.

MenA Enzyme Inhibitory Assay (IC₅₀)

The substrate 1,4-dihydroxy-2-naphthanoic acid (DHNA) (500 μ M; 20 μ L), MgCl₂ (5 μ M; 20 μ L); CHAPS (0.1%; 20 μ L), Tris-buffer (pH = 8; 20 μ L), membrane fraction containing Men A (100 μ L), inhibitors (0–60 μ g/mL of DMSO) and farnesyl pyrophosphate (200 μ M, 40 μ L) were mixed together and incubated for 2h at 37 °C. After the incubation, the reaction mixture was quenched with 0.1 M AcOH in MeOH and the reaction mixture was extracted with hexanes (thrice). The organic portions were then concentrated and diluted with MeOH (300 μ L). From each set of enzymatic reaction mixture 20 μ L was injected into the HPLC (CH₃CN:0.05%TFA in H₂O = 90:10, UV: 325 nm, flow rate: 1.5 mL/min) and the area of the peak for DMMK was quantified to obtain the IC₅₀ value for different inhibitor molecules (**Figure 6-3**).

MurX Assay

MurX assay substrates, Park's nucleotide-N^ε-dansylthiourea (**29**), Park's nucleotide-N^ε-FITC (**32**), and neryl phosphate (**35**), were chemically synthesized according to the reported procedures (141,145,155,156,183). **29** or **32** (2 mM stock solution; 3.75 μ L (75 μ M)), MgCl₂ (0.5 M; 10 μ L (50 mM)), KCl (2 M, 10 μ L (200 mM)), triton X100 (0.5%; 11.25 μ L), tris-buffer (pH = 8; 50mM, 2.5 μ L), neryl phosphate (10 mM, 45 μ L), and inhibitor (0-100 μ M, in DMSO (2.5 μ L)) were placed in a 500 μ L Eppendorf tube. To a stirred reaction mixture, P-60 (15 μ L) was added (the total volume of the reaction mixture: 100 μ L). The reaction mixture was incubated for 1h at room temperature (26 °C), and quenched with CHCl₃ (200 μ L). Two phases were mixed *via* vortex and centrifuged at 25,000 $\times$ g for 10 min. The upper aqueous phase was assayed *via* reverse-phase HPLC. The water phase (10 μ L) was injected into HPLC (solvent: CH₃CN: 0.05 M aq. NH₄HCO₃ = 25: 75, UV: 350 nm, flow rate: 0.5 mL/min, Column: Kinetex 5u C8 100Å, 150 $\times$ 4.60mm), and the area of the peak for lipid I-neryl derivative was quantified to obtain the IC₅₀ value. The IC₅₀ values were calculated from plots of the percent product inhibition versus the inhibitor concentration.

The MurX assays developed here were tolerated to 2.5% of DMSO concentrations in total volume of the reaction solution (100 μ L). The maximum tolerated concentration for DMSO in MurX-catalyzed neryl lipid I-N^ε-dansylthiourea has not been determined.

WecA Assay

UDP-Glucosamine-C₆-FITC [2 mM stock solution; 0.56 μ L (22.5 μ M)], MgCl₂ [0.5 M; 4 μ L (40 mM)], CHAPS (5%; 10 μ L (1%)), β -mercaptoethanol [50 mM; 5 μ L (5 mM)], tris-buffer (pH = 8.0; 50 mM, 9.74 μ L), decaprenyl phosphate dissolved in Tris-HCl (50 mM, pH 8): PEG: DMSO (5: 1: 2) [4 mM, 1.7 μ L (140 μ M)], and inhibitor [0–100 μ M, in DMSO (1 μ L)] were placed in a 1.5 mL Eppendorf tube. P-60 (18 μ L) was added (the total volume of the reaction mixture: 50 μ L) to the reaction mixture. The

Figure 6-3. MenA assay.

A) MenA assay conditions. B) HPLC chromatogram of MenA assay depicting the retention times and concentration dependent change in peak area of substrate (DHNA) and MenA enzyme product (DMMK). C) A representative MenA inhibition curve with different concentrations of (R)-20.

reaction mixture was incubated for 2 h at 37 °C, and quenched with n-butanol (150 µL). Two phases were mixed *via* vortex and centrifuged at $10,000 \times g$ for 3 min. The upper butanol phase was assayed *via* reverse-phase HPLC or UV–vis BioTek Synergy HT plate reader. For HPLC analyses, the butanol phase (25 µL) was injected into HPLC (solvent: CH₃OH:0.05 M aq. NH₄HCO₃ = 85: 15, UV: 485 nm, flow rate: 0.5 mL/min, Column: Kinetex 5µ C8 100 Å, 150 × 4.60 mm); the area of the peak for decaprenyl-P-P-Glucosamine-C₆-FITC was quantified to obtain the IC₅₀ value. For the UV–vis-based assay, the n-butanol phase (20 µL) was transferred to a 384 well black plate and fluorescence was measured at an excitation of 485 nm and emission of 528 nm. The IC₅₀ values were calculated from plots of the percent product inhibition versus the inhibitor concentration.

Kill-curve Graph: Determination of Colony Forming Units per Milliliter

Mtb cultures at mid-log phase (OD=0.5) were diluted to OD=0.25 and treated with inhibitor molecules at MIC, x2 MIC and x4 MIC. Each culture well was diluted 10, 100, 1000 and 10,000 fold every 24 h and 20µL from each dilution was plated on 7H10 agar plates supplemented with OADC enrichment. Plates were incubated for 15 days in a static incubator at 37 °C and colonies were counted.

Determination of Cytotoxicity (IC₅₀) in Vero and HepG₂ Cells

Cytotoxicity assays were performed using Vero monkey kidney (ATCC CCL-81) and HepG₂ human hepatoblastoma cell (ATCC HB-8065) lines. Vero or HepG₂ cells were subcultured in 75 cm² cell culture flasks using ATCC-formulated eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum and penicillin-streptomycin at a subcultivation ratio of 1:3.

Cells were transferred to a 96 well cell culture plate and serially diluted aliquots of each test compound at concentrations ranging from 1-25 x of the MIC for *Mtb* H₃₇Rv were added to the cells. Control compounds with known toxicity such as rifampicin, INH, tunicamycin or atovaquone were included on each plate. The plates were incubated for 72 h and cytopathic effects were determined *via* the MTT assay. Cell viability was assessed on the basis of cellular conversion of MTT into a purple formazan product (**Figure 6-4**). The absorbance of the colored formazan product was measured at 570 nm by BioTek Synergy HT Spectrophotometer. Selectivity index (SI = IC₅₀/MIC) of SI >10 was considered to be significant in this program.

Kinetic Parameter Evaluation

MurX/MraY Activity Assay. Evaluation of kinetic parameters were performed through *M. smegmatis* MurX/MraY-catalyzed lipid I synthesis. The MurX/MraY activity

Figure 6-4. Cytotoxicities (IC_{50}) of selected compounds with activity against *Mtb* H37Rv in Vero monkey kidney cells and HepG2 cells.

A) Cytotoxicities (IC_{50}) of (R)-19, (R)-20, atovaquone, and rifampin in HepG2 cells. B) Cytotoxicities (IC_{50}) of UT-01320 (45), tunicamycin and rifampin in Vero cells. C) Cytotoxicities (IC_{50}) of UT-800 (64), UT-810 (65), UT-815 (66), valnemulin, tunicamycin and rifampin in Vero cells. All plotted data are an average of three experiments.

was assayed with various concentrations of Park's nucleotide-N^ε-dansylthiourea while keeping the concentration of Neryl PO₄ constant (4.5 mM). K_m and V_{max} were determined by Michaelis-Menten enzyme kinetics. The correlation (Michaelis-Menten plot) between the concentrations of Park's nucleotide- N^ε-dansylthiourea (x axis) and rate (V) of lipid I formation (y axis) was obtained using GraphPad Prism Software (174,222).

WecA Activity Assay. Evaluation of kinetic parameters was performed through *E. coli* (B21 WecA) P-60-catalyzed WecA synthesis. The WecA activity was assayed with various concentrations of UDP-GlcNAc-C₆-FITC while keeping the concentration of undecaprenyl PO₄ constant (140 μM). K_m and V_{max} were determined by Michaelis-Menten enzyme kinetics. The correlation (Michaelis-Menten plot) between the concentrations of UDP-Glucosamine-C₆-FITC (x axis) and rate (V) of decaprenyl-Glucosamine-C₆-FITC (y axis) formation was obtained using GraphPad Prism Software.

Preparation of Polyprenyl Phosphates for MurX and WecA Assays

Decaprenyl phosphate (C₅₀-P) and undecaprenyl phosphate (C₅₅-P) were prepared as follows: Leaves of *Magnolia kobus*, (*Rosaceae*) were collected in the Botanical Garden of Polish Academy of Sciences, Powsin, dried in open air, pulped using mortar and pestle and extracted with acetone/hexane (1:1, v/v) for 48 h at r.t. After filtration and evaporation of the solvents, the crude mixture of lipids containing polyprenols was dissolved in hexane and purified by silica gel chromatography (hexanes to 1:1 hexanes/Et₂O) (223,224). The crude polyprenol mixture was purified by C₁₈-silica gel chromatography (LiChroprep RP-18, 40–63 μm, Merck, solvent: MeOH to acetone:MeOH = 10/1). Fractions containing pure decaprenol and undecaprenol were pooled, evaporated, dissolved in hexane and stored at –20 °C. Polyprenyl phosphates were synthesized *via* the procedures reported by Danilov et al. (225). Stirred solution of decaprenol in CHCl₃ was added Cl₃CN and nBu₄NH₂PO₄. After 30 min at room temperature, all volatiles were evaporated and the residue was dissolved in THF. Aqueous ammonia was added to the mixture and kept overnight at 4 °C. The supernatant was collected, evaporated and dissolved in CHCl₃/MeOH (2:1, v/v) containing 3% of water and loaded onto DEAE-Sephadex A-25 column. Unreacted decaprenol was eluted with CHCl₃/MeOH (2:1) and the decaprenyl phosphate was eluted with ammonium acetate (0–100 mM) in CHCl₃/MeOH (2:1). Purified decaprenyl phosphate was dissolved in CHCl₃/MeOH (2:1) containing 3% of NH₃ and stored at - 20 °C. Undecaprenyl phosphate was synthesized by the same method described for decaprenyl phosphate.

Synergistic Effect of UT-01320 (45) with SQ 641 (37) or Capuramycin (36)

The synergistic or antagonistic activities of MurX inhibitors (capuramycin or SQ 641) and UT-01320 were assessed *in vitro* via micro dilution broth checkerboard

technique (189,226) (**Figure 6-5**). The FIC index was calculated to according to the following equation. $FIC = FIC_A + FIC_B = C_A/MIC_A + C_B/MIC_B$ where, MIC_A and MIC_B : MIC of drugs A and B, C_A and C_B =concentrations of drugs A and B used in combination. In these interaction studies, ΣFIC of less than 0.5 represents synergistic activity (**Table 6-1**).

Killing Effect against Intracellular *Mtb*

J774A.1 cells were seeded at 2.5×10^5 cells/well in 24-well dishes or 1×10^5 cells/well in 8-well chamber slides and incubated overnight at 37 °C in DMEM. A transformant *Mtb* CDC1551 expressing tdTomato was grown in 7H9 Middlebrook medium supplemented with OADC. The *Mtb* cells were harvested at an optical density of 0.5, washed and re-suspended in saline. J774A.1 cells were maintained in cell culture medium and were infected by *Mtb* (10^6 bacteria in 0.2 mL of media): a multiplicity of infection (167) of ≈ 10 (bacteria/cell). The extracellular bacteria were removed by washing with PBS. The infected macrophages were treated with antibacterial agents at x2 and x4 MIC concentrations and the relative intensity of the fluorescence was measured [emission wavelength (581 nm)] via UV-vis spectroscopy in 24, 48, and 72 h for inhibition of intracellular bacterial growth. Surviving *Mtb* cells were confirmed by CFU method (227).

Microsomal Stability

Pooled Sprague-Dawley rat liver microsomes were purchased from Corning Life Sciences (Oneonta, NY, USA). Microsomes ((20 mg/mL) were thawed on ice and diluted using phosphate buffer (100 mM, pH: 7.4), resulting in a protein concentration of 1 mg/mL. Stock solutions (10 mg/L) of UT-800, UT-810, UT-815, valnemulin and verapamil (positive control) were prepared in DMSO (50%). A final concentration of 500 ng/mL was used for incubation with microsomes. NADPH (final concentration: 1 mM) was used as a co-factor. All the above solutions except NADPH were added to individual wells (12-well) in triplicate and were allowed to equilibrate for 5 min at 37°C. NADPH was then added. 50 μ L aliquots in triplicate were drawn from the incubation mixture at 0, 5, 10, 20, 30, 45 and 60 min and immediately the reaction was quenched by addition of ice-cold methanol (4 volumes) (228). The samples containing methanol was lyophilized to remove all volatiles. The residue was dissolved in 1N HCl aq. (10 μ L) and MeOH (40 μ L). The resulting solution (20 μ L) was injected to LC-MS. MS solvent 90:10 acetonitrile/0.05% formic acid in water. Flow rate: 0.5mL/min.

Gene Analyses of Resistant Strain of *A. baumannii* against UT-490 (68)

Spontaneous resistant mutants of *A. baumannii* ATCC 19606 with decreased sensitivity towards **68** were isolated by sub-culturing bacteria on agar plates with concentrations of UT-490 at MIC and above. Resistant mutants were isolated at 16 x

A SQ 641 ($\mu\text{g/mL}$)								
6.24	4.05	4.11	4.21	4.43	4.86	5	7.44	10.88
3.12	2.05	2.11	2.21	2.43	2.86	3	5.44	8.88
1.56 (MIC)	1.05	1.11	1.21	1.43	1.86	2	4.44	7.88
0.78	0.55	0.61	0.71	0.93	1.36	1.50	3.94	7.38
0.391	0.30	0.36	0.46	0.68	1.11	1.25	3.69	7.13
0.2	0.18	0.23	0.34	0.56	0.99	1.13	3.57	7.01
0.1	0.12	0.17	0.28	0.49	0.92	1.06	3.50	6.94
0.05	0.09	0.14	0.26	0.46	0.89	1.03	3.47	6.91
	0.08	0.15	0.32	0.645	1.29	1.50 (MIC)	5.16	10.32
UT-01320 ($\mu\text{g/mL}$)								
B Capuramycin ($\mu\text{g/mL}$)								
50	4.05	4.11	4.21	4.43	4.86	5	7.44	10.88
25	2.05	2.11	2.21	2.43	2.86	3	5.44	8.88
12.5 (MIC)	1.05	1.11	1.21	1.43	1.86	2	4.44	7.88
6.25	0.55	0.61	0.71	0.93	1.36	1.50	3.94	7.38
3.125	0.30	0.36	0.46	0.68	0.11	1.25	3.69	7.13
1.56	0.18	0.23	0.34	0.56	0.99	1.13	3.57	7.01
0.78	0.12	0.17	0.28	0.49	0.92	1.06	3.50	6.94
0.391	0.08	0.14	0.24	0.46	0.89	1.03	3.47	6.91
	0.08	0.16	0.32	0.645	1.29	1.50 (MIC)	5.16	10.32
UT-01320 ($\mu\text{g/mL}$)								

Figure 6-5. Checkerboard combinations of UT-01320 with SQ641 or Capuramycin.

A) Checkerboard combinations of SQ641 (MIC = 1.56 $\mu\text{g/mL}$) and UT-01320 (MIC = 1.50 $\mu\text{g/mL}$). B) Checkerboard combinations of Capuramycin (MIC = 12.5 $\mu\text{g/mL}$) and UT 320 (MIC = 1.50 $\mu\text{g/mL}$). Shaded wells indicate growth and white wells indicate no growth. Each well shows calculated ΣFIC values.

Table 6-1. Σ FIC index for the wells at growth – no growth interface.

Drug	MIC (Alone -μg/mL)	C_A and C_B (μg/mL)	ΣFIC
SQ 641 (Drug A)	1.56	0.1	0.12
UT-01320 (Drug B)	1.50	0.08	
SQ 641 (Drug A)	1.56	0.1	0.17
UT-01320 (Drug B)	1.50	0.16	
SQ 641 (Drug A)	1.56	0.1	0.28
UT-01320 (Drug B)	1.50	0.32	
SQ 641 (Drug A)	1.56	0.1	0.49
UT-01320 (Drug B)	1.50	0.645	
SQ 641 (Drug A)	1.56	0.1	0.92
UT-01320 (Drug B)	1.50	1.29	
SQ 641 (Drug A)	1.56	0.05	1.03
UT-01320 (Drug B)	1.50	1.50	
Capuramycin (Drug A)	12.5	1.563	0.18
UT-01320 (Drug B)	1.50	0.08	
Capuramycin (Drug A)	12.5	1.563	0.23
UT-01320 (Drug B)	1.50	0.16	
Capuramycin (Drug A)	12.5	0.78	0.28
UT-01320 (Drug B)	1.50	0.32	
Capuramycin (Drug A)	12.5	0.78	0.49
UT-01320 (Drug B)	1.50	0.645	
Capuramycin (Drug A)	12.5	0.78	0.92
UT-01320 (Drug B)	1.50	1.29	
Capuramycin (Drug A)	12.5	0.391	1.03
UT-01320 (Drug B)	1.50	1.50	

NOTE: C_A and C_B are concentrations of Drug A and Drug B in combination.

MIC of **68**. Spontaneous resistant mutants with decreased sensitivity against rifampicin were isolated at 32 x MIC of rifampicin (control). The chromosomal DNAs from the resistant mutant (**68^R**) and wild-type *Acinetobacter baumannii* ATCC19606 were isolated. The *rplC* gene fragment was amplified using *A. baumannii* *rplC* specific primers (AbrplCupPrimer 5' CTTTGGGTTA AGGCTTTCGG 3' and Abrplcdn 5'CAACAGCAGAGCCG GAAACAG 3'), purified, and DNA sequenced (214,215). The DNA sequencing result was used to blast against *rplC* DNA sequence of *A. baumannii* in NIH Genome database (**Figure 6-6**).

A TTAACAAAGATGCGCGTGACCAGTACGAAATCCGCACCTACAAACGTTTGATCGACATCG
TTCAACCTACAGATAAAACCTGTTGATGCATTGATGAAGTTAGATCTTGCAGCTGGTGTG
ATGTTTCAGATCGCTTTGGGTTAAGGCTTTTCGGTTAATTAACGATTAGTTAATTAGGCCGC
TTTTTTAGAGGTTTATGCACATGGCTATTGGTTTAGTCGGTCGCAAATGTGGTATGACTC
GCATCTTTACAGATGCTGGTGTTCCTGTACCTGTTACAGTCATCGAAGTCGATCCAAACC
GCATTACGCAAATCAAACACTTGAACCTGATGGTTATCAAGCTGTTCAAGTAACTACTG
GCGAACGTCGCGAGTCTCGCGTAACTAACGCTCAAAAAGGTCACCTTCGCTAAAGCGGGTG
TTGCTGCTGGTCGTTTAGTTAAAGAGTTTCGTGTTACTGAAGCTGAGCTTGAAGGCCGTG
AAGTTGGCGGTACTATTGGCGTTGATTTGTTACAGTTGGTCAAATGTTGACGTAACCTG
GTCAATCAAAGGTAAAGGTTTCCAAGGTGGTGTAAACGTTGGAATTTCCGTACCCAAG
ATGCTACTCACGGTAACTCTGTTTCTCACCGTGTTCCTAGGTTCTACAGGTCAAACCAAA
CTCCTGGACGCGTGTCAAAGGCCAAAAAATGGCTGGTCACCTTAGGTGATGAACGCGTAA
CAGTACAAGGTCTTGAATCGTATCTGTTGACACTGAACGTTTCAAGTTTGGTTGTTAAGG
GTGCAATTCCTGGTGCAACTGGCGGTGACGTTATCGTACGTCCTACCATCAAGGCCCTGAG
GGGAAATACCGTGAATTTAAAAAAGTGTTCCTGGCTCTGCTGTTGAATTGCTGAAGTAGC
TTTCGGACGTGAATTTAAACGAAGCTCTTGTACACCAAGTTGTTACTGCTTACTTAGCAGG
TGGTCGTCAAGGTACTCGTGTCTACAAATCACGTGCAGACGTTTCTGGCGGTGGTAAAAA
ACCATTCGGTCAAAAAGGT

B ABrpICupPrimer
5' CTTTGGGTTAAGGCTTTTCGG
AbrpICdn
5' CAACAGCAGAGCCGGAACAG

C ATGGCTATTGGTTTAGTCGGTCGCAAATGTGGTATGACTCGCATCTTTACAGATGCTGG
TGTTTCTGTACCTGTTACAGTCATCGAAGTCGATCCAAACCGCATTACGCAAACACTTG
AAACTGATGGTTATCAAGCTGTTCAAGTAACTACTGGTGAACGTCGCGAGTCTCGCGTA
ACTAACGCTCAAAAAGGTCACCTTCGCTAAAGCGGGTGTTGCTGCTGGTCGTTTAGTTAA
AGAGTTTTCGTGTTACTGAAGCTGAGCTTGAAGGCCGTGAAGTTGGCGGTACTATTGGCG
TTGATTTGTTTACAGTTGGTCAAATGTTGACGTAACCTGGTCAATCAAAGGTAAAGGT
TTCCAAGGTGGTGTAAACGTTGGAATTTCCGTACCCAAGATGCTACTCACGGTAACTC
TGTTTCTCACCGTGTTCCTAGGTTCTACAGGTCAAAAACAACTCCTGGACGCGTGTTC
AAGGCAAAAAAATGGCTGGTCACCTTAGGTGATGAACGCGTAACAGTACAAGGTCTTGAA
ATCGTATCTGTTGACACTGAACGTTTCAAGTTTGGTTGTTAAGGGTGCAATTCCTGGTGC
AACTGGCGGTGACGTTATCGTACGTCCTACCATCAAGGCCCTGAGGGGAAATACCGTGAA
TTTAA

D Query 7 MAIGLVGRKCGMTRIFTDAGVSVPTVIEVDPNRITQIKTLETGQYQAVQVTTGERRESR 186
MAIGLVGRKCGMTRIFTDAGVSVPTVIEVDPNRITQIKTLETGQYQAVQVTTGERRESR
Sbjct 1 MAIGLVGRKCGMTRIFTDAGVSVPTVIEVDPNRITQIKTLETGQYQAVQVTTGERRESR 60

Query 187 VTNAOKGHFAKAGVAAGRLVKEFRVTEAELEGREVGGTIGVDLFTVGQIVDVTGQSKGKG 366
VTNAOKGHFAKAGVAAGRLVKEFRVTEAELEGREVGGTIGVDLFTVGQIVDVTGQSKGKG
Sbjct 61 VTNAOKGHFAKAGVAAGRLVKEFRVTEAELEGREVGGTIGVDLFTVGQIVDVTGQSKGKG 120

Query 367 FQGGVKRWNFRTQDATHGNSVSHRVLGSGTQOTPGRVFKGKKMAGHLGDERVTVOGLEI 546
FQGGVKRWNFRTQDATHGNSVSHRVLGSGTQOTPGRVFKGKKMAGHLGDERVTVOGLEI
Sbjct 121 FQGGVKRWNFRTQDATHGNSVSHRVLGSGTQOTPGRVFKGKKMAGHLGDERVTVOGLEI 180

Query 547 VSVDTERSVLVKGAIIPGATGGDVIVRPTIKA 642
VSVDTERSVLVKGAIIPGATGGDVIVRPTIKA
Sbjct 181 VSVDTERSVLVKGAIIPGATGGDVIVRPTIKA 212

Figure 6-6. Identification of resistant mechanism of *A. baumannii* to 68.

A) Sequence of *A. baumannii* 50S ribosomal subunit protein L3 (rplC). B) Primers. C) *rplC* Sequence of 68^R Resistant Mutant (68^R) of *A. baumannii*. D) Blast against Wild type and Mutant *A. baumannii*. (Reprinted with permission. Siricilla, S., Mitachi, K., Yang, J., Eslamimehr, S., Lemieux, M. R., Meibohm, B., Ji, Y., and Kurosu, M. (2017). A new combination of a pleuromutilin derivative and doxycycline for treatment of multidrug-resistant *Acinetobacter baumannii*. *J. Med. Chem. American Chemical Society*. Article ASAP).

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VITA

Shajila Siricilla was born in 1981 in Kerala, India. She completed her Bachelor's degree (BPharm) in Pharmacy in 2003 from JSS College of Pharmacy, Dr. MGR Medical University of Chennai, India. In 2007, She earned her Post Graduate Diploma (Master's degree) in Business Administration from the Symbiosis Center of Distance Learning, Pune, India. From 2004 to 2008 she worked for Juggat Pharmaceuticals, India (QC Chemist), Kaya Skin Clinic, India (Client Coordinator) and Knight Frank, India (Management Coordinator). In August 2011, she joined Pharmaceutical Sciences PhD Program at University of Tennessee Health Science Center, Memphis under the supervision of Dr. Michio Kurosu.

She has been elected for membership of the American Association of Pharmaceutical Scientists (AAPS), the American Chemical Society (ACS) and the American Association for the Advancement of Science (AAAS). During her graduate school, she obtained a patent related to her dissertation research, published ten peer-reviewed articles in scientific journals and was invited to share her scientific findings at many scientific conferences in the United States. Shajila was a recipient of AAPS Drug Discovery and Development Interface Travel Award (2016) and UTHSC CGHS John Autian Student Enrichment Travel Award (2016). During her time at UTHSC she enjoyed her research assistant and teaching assistant jobs for the College of Pharmacy at UTHSC. She has mentored many PharmD, summer students and junior PhD students on research projects. During her PhD, she studied development of new tuberculosis drug leads that are effective in killing dormant *Mycobacterium tuberculosis* and development of novel *in vitro* assays for screening and validation of selective inhibitors of molecular targets.