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REVIEW

Why are membrane targets discovered by phenotypic screens and genome sequencing in *Mycobacterium tuberculosis*?

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SUMMARY

High through put screening (HTS) was extensively used in attempts to discover new TB drugs from libraries of pure small molecule compounds many of which complied with the rule of five. Coupled with new methods for determining the target of lead compounds by resistance selection followed by genome sequencing, screening for growth inhibitors led to several recent reports of compounds linked to specific antitubercular targets. This systematic approach to drug discovery appears at present to select for small, hydrophobic molecules affecting the function of essential membrane proteins, for example DprE, MmpL3, AtpE, QcrB, and Pks13. All of these molecules possessed bactericidal activity *in vitro*. Mutations in GlpK were also selected with hydrophobic compounds identified by screening for growth inhibitors. The chemical properties of the compounds reported are considered in the context of uptake and possible mechanisms of inhibition of membrane bound targets based on other model systems (e.g. cardiovascular drugs affecting voltage-gated L-type calcium channels, daptomycin, telavancin, gramicidin S, and role of boundary lipids). The relationship between hydrophobicity, compound uptake, and mode of action are addressed. Compared to the average calculated logP for approved TB drugs of −1.0, the average for these hydrophobic compounds is 4.0 representing a major shift in hydrophobicity of 5 orders of magnitude. Furthermore several hydrophobic compounds in the Prestwick Chemical Library (FDA approved drugs) inhibit growth of *M. tuberculosis* at 10 μg/ml or less and have an average calculated logP of 5.7 signaling caution with respect to specificity. Key recommendations are made regarding follow-up of the hydrophobic leads recently discovered using phenotypic screening and target elucidation by genome sequencing. Consideration is also given to the properties of small molecule screening libraries, the types of molecules and targets recently discovered as antitubercular leads and compliance with the rule of 5.

1. Introduction

The quest to discover new antibacterials has undergone major paradigm shifts over the past 80 years. Technology has advanced from the early discovery of dyes and sulfa drugs, to discovery of natural products (e.g. penicillin and erythromycin) and then, in tandem with advances in biology and chemistry, to enzyme targeted and phenotypic screening of pure small molecule, fragment and combinatorial libraries. Each era experienced various degrees of success, but given the rapid selection and spread of resistant strains we are still in desperate need of new drugs to treat specific infections, e.g. multi-drug resistant tuberculosis (MDR-TB) and extensively-drug resistant tuberculosis (XDR-TB) including some strains resistant to most if not all antitubercular drugs [1,2]. Infections with drug sensitive TB are usually cured with a 6–9 month regimen of multiple antibiotics including isoniazid, rifampicin, ethambutol, and pyrazinamide. Unfortunately, our inability to deliver and monitor completion of therapy on a global level has led to the selection and spread of highly resistant strains over the past decades. In retrospect given the issues of counterfeit drugs and lack of patient compliance in completing therapy, the selection and spread of drug resistant strains is entirely consistent with our knowledge clinical microbiology and genetics [3]. The most recent estimates from the WHO indicate that between 220,000 and 400,000 MDR-TB cases occurred among global TB cases in 2011 and that only 20% of these case were enrolled in active treatment programs (http://www.who.int/tb/publications/MDRFactSheet2012.pdf). Furthermore, 9% of MDR strains possess resistance to two other classes of drugs, and thus are classified as XDR- TB. XDR-TB infections have been found in 84 countries. For a review of antitubercular drugs in preclinical and phase 2 trials see [4].

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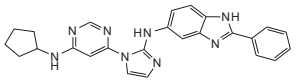
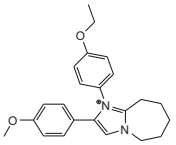
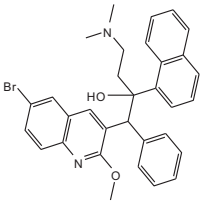
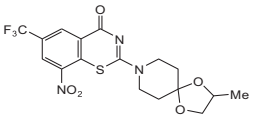
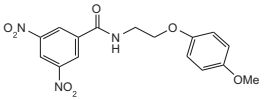
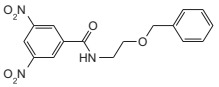
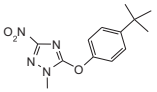
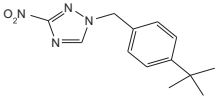
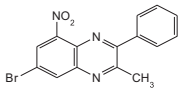
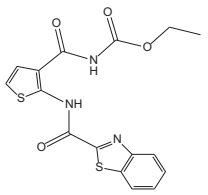
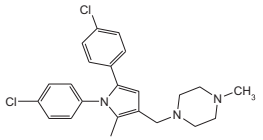
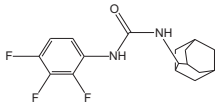
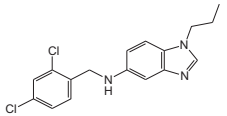
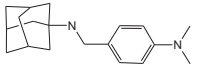
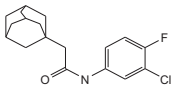
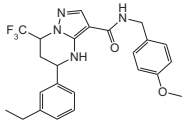
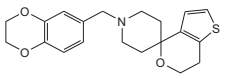
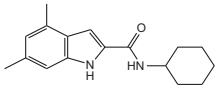
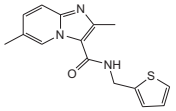
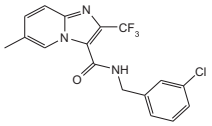
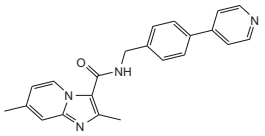
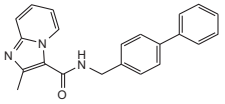
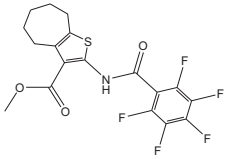
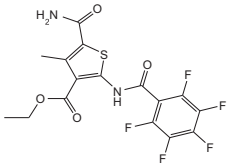
Table 1
Properties of new TB leads.

Target	Compound name	Structure	MIC (μM)*	clogP†	logP§	clogP**	clogP‡	Rule of five violations¶	Comment
GlpK: glycerol kinase	Compound 2		0.350	6.0	4.6	4.0	5.4	1	Not essential *in vivo* and when grown on alternative carbon sources; Orally bioavailable
GlpK	A039		1.500	1.4	NA‖	4.0	2.0	0	
AtpE: membrane ATP synthase	R207910, TMC207, bedaquiline/ SirturoTM		0.050	7.3	7.5	7.7	7.1	2	Early approval by FDA; Toxicity concerns; Continued Phase 3 with safety evaluations; Orally bioavailable; cidal
DprE1: enzyme subunit of the heteromeric decaprenylphosphoryl-β-D: -ribose 2′-epimerase	BTZ 043		0.002	3.0	NA	3.4	3.6	0	Covalent modification of C387; Trifluoromethyl group a key determinant of interaction with the enzyme; Active *in vivo* (mouse model); Orally bioavailable
DprE1	DNB1		0.200	3.0	2.4	2.7	2.7	0	Cidal
DprE1	DNB2		0.200	3.0	2.1	2.3	2.6	0	Cidal
DprE1	TAACF 339509		1.000	2.9	NA	3.6	3.5	0	Cidal
DprE1	TAACF 377790		0.500	2.7	NA	3.2	3.0	0	Cidal

DprE	VI-9376		3.100	4.2	4.9	4.1	4.3	0	Cidal
DprE1	TCA1		0.520	3.3	3.1	2.7	1.8	0	Dual targeting of DprE1 and MoeW; orally active in mouse model of TB infection; cidal
MmpL3: transmembrane protein transports trehalose monomycolate across the plasma membrane	BM212		3.600	7.4	5.3	5.2	5.1	1	Active in macrophages; cidal; may have second target
MmpL3	AU 1235		0.300	5.1	3.3	4.8	4.6	0	Cidal
MmpL3	C215		16.000	5.8	4.2	5.1	4.9	0	Cidal
MmpL3	TAACF 356596 (DA5)		5.000	4.3	3.7	4.8	4.5	0	Cidal
MmpL3	TAACF 391844 (DA8)		10.600	6.7	4.3	5.5	5.1	1	Cidal
MmpL3	THPP1		0.300	4.2	3.7	4.8	4.5	0	Cidal
MmpL3	Spiro2		0.300	2.7	3.0	4.2	3.2	0	Cidal

(*continued on next page*)

Table 1 (*continued*)

Target	Compound name	Structure	MIC (μM)*	clogP†	logP§	clogP**	clogP‡	Rule of five violations¶	Comment
MmpL3	Compound 3		0.900	4.5	3.3	5.3	4.3	0	Tentative inhibitor of MmpL3, supporting data not yet published
QcrB: subunit of the electron transport ubiquinol cytochrome C reductase.	IP1		0.200	3.3	3.0	4.2	2.7	0	CIdal
QcrB	IP2		0.200	3.9	4.2	5.5	4.3	0	CIdal
QcrB	IPA04		0.010	3.4	3.8	5.0	3.3	0	CIdal
QcrB	IPA05		0.005	4.2	4.8	5.6	4.2	0	CIdal
Pks13: polyketide synthase 1 family involved with mycolic acid synthesis	TP2 (TAACF 311032)		1.000	5.2	4.8	5.8	4.6	0	CIdal
Pks13	TP4 (TAACF 343948)		0.500	3.4	2.1	3.1	2.7	0	CIdal

* MIC data using Mtb strain H37Rv.
† logP estimated by Chemdraw (http://www.cambridgesoft.com/support/DesktopSupport/Documentation/ChemDrawPlugin/).
§ clogP calculated by Chemdraw (http://www.cambridgesoft.com/support/DesktopSupport/Documentation/ChemDrawPlugin/).
** clogP calculated by KowWin (http://esc.syrres.com/esc/est_kowdemo.htm).
‡ clogP calculated Molinspiration (http://www.molinspiration.com/cgi-bin/properties).
¶ Rule of five violations determined at http://www.molinspiration.com.
‖ NA specific program did not calculate a value.

Methods for discovering new classes of antibacterial compounds are constantly evolving, including development of new methods for high throughput screening (HTS) of large number of pure small molecules in libraries designed for diversity, or focused libraries if a specific pharmacophore is used as the lead structure in optimization or screening. Hundreds to perhaps thousands of HTS projects covering all disease targets (targets for human diseases and infectious disease targets) were run during the first decades of HTS activity but the results were overall disappointing for various reasons. 1) Compounds active on purified enzyme targets *in vitro* did not always enter cells, leading to a discussion of the benefits of enzyme vs. whole cell screens. One approach to this issue was the design of reporter based whole cell assays to capture transport and target information. 2) Many lead compounds identified were nonspecific inhibitors [5–12] and thus of little value. Screening libraries were then purged of most nonspecific inhibitors. 3) Compounds were not always adequately bioavailable orally based on physical chemistry properties. This led to analysis of the chemical properties of successful drugs and development of specific parameters that screening libraries adopted to bias toward discovery of leads compounds more likely to yield viable drug candidates. The rule of five was developed [13], which predicts poor absorption is more likely when there are more than 5 H-bond donors, 10 H-bond acceptors, the molecular weight is greater than 500 and the calculated log P is greater than 5. Several of these issues were recently discussed for TB drugs [14].

Advances in molecular genetics over the past years led to new methods for identifying essential genes and for cloning and sequencing of resistance determinants in *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis. The latter advances led to a renewed interest in HTS for growth inhibitors (capturing compounds that either enter the cell or act on or outside the plasma/outer membrane) and allowed rapid target identification by selection of resistant strains and genome sequencing for identification of the genetic alteration and thus putative drug target/metabolic pathway. Genetically constructed strains over or under expressing a target protein have also been used to confirm target identification. Historical methods of testing for inhibition of specific macromolecular synthesis pathways (e.g. DNA, RNA, protein, and cell wall synthesis) using pulse labeling can and did lead to target identifications when followed up with pathway specific analysis. While precise identification of a drug target is not essential for development, lacking this information can severely impede a project and lead to target drift during the search for more potent analogs.

2. High throughput screening for growth inhibitors and target identification

2.1. Unexpected findings

The discovery of growth inhibitors by whole cell screening and subsequent genetic identification of the bacterial target will be discussed in the context of the chemical properties of the lead compounds and the specific targets identified. While the number of studies is not large, there is a clear indication of a possible bias in the type of inhibitors and targets discovered to date. This latter topic will be covered in detail in the context of the physical chemical properties of the libraries used, compounds identified, and targets discovered. We will see that there is a bias for membrane localized targets and that this is consistent with compound hydrophobicity.

Several HTS programs to identify inhibitors of Mtb growth were pursued over the past several years [15–24] with some unexpected findings. For example, pyrimidine–imidazole (PI) compounds with MIC_{50} values ranging from 0.11 to >20 μM [23] and calculated logPs from 3.5 to 5.3 were discovered to have glycerol dependent activity using *Mycobacterium bovis* following identification of growth inhibitors from a small molecule library (properties and numbers screened not described). Compound 2, logP = 4.0 to 4.6, (Table 1) was synthesized as part of an analog program with MIC = 0.44–1.21 μM on four Mtb strains. While orally bioavailable no efficacy was observed in a mouse model of TB infection. Spontaneous mutants were selected in Mtb and sequenced, identifying mutations in *glpK* (one SNP, causing change G187V, and several clones with frame-shift mutation 191GGT to GGGT). Deletion of *glpK* rendered bacteria resistant to compound 2 *in vitro* and sensitivity was regained upon reintroduction of a functional *glpK* gene. The mode of action *in vitro* was perturbation of glycerol metabolism leading to accumulation of toxic metabolites that inhibit growth. The most active compound, 5, had the highest logP (5.3) while the least potent compound, 9, had the lowest logP (3.5) [23]. Thus novel pyrimidine-imidazoles were identified which lacked *in vivo* activity even after generation of analogs with favorable pharmacokinetics, because the glycerol pathway is not required during *in vivo* infection. An active substituted imidazole (A039, logP = 1.4 to 4, Table 1) was also identified as a growth inhibitor dependent on the presence of glycerol [22] and selection of resistance yielded a frame shift mutation in the *glpK* gene (glycerol kinase) due to a single base pair insertion at codon 191 (191GGT > 191GGGT). Glycerol kinase action is tightly coupled with glycerol transport via the glycerol facilitator GlpF and a direct interaction at the membrane between the glycerol facilitator protein and glycerol kinase that stimulates kinase activity was postulated based on experimental data [25], analogous to the hexokinase- and glycerol kinase–porin interactions in mitochondria. Thus the kinase may reside, at least transiently, near the membrane in contact with GlpF explaining the action of a very hydrophobic compound such as A039. Screening in the absence of glycerol was suggested to preclude identification of glycerol dependent compounds.

2.2. Calculation of logP values

LogP values in Tables 1–4 were calculated/estimated logP values as designated in the footnotes to the Tables. For currently used TB drugs (Table 3), data from Ksaim et al. [26]. was included. While these programs calculate logP values based on various algorithms the role of ionization states are not accurately reflected. Except for R207910 (see below) the logP values reported in the original papers cited below are all calculated values. Given the membrane location of the targets and likelihood of uptake by passive diffusion, hydrophobicity is likely the driving force behind their modes of action. Rule of five violations were determined at http://www.molinspiration.com.

3. New inhibitors targeting membrane proteins

3.1. AtpE

The next example is the discovery of the diarylquinoline, R207910 (also called TMC207 and bedaquiline/Sirturo™) and recently approved by the FDA via a rapid approval mechanism (however, not with out some concern over toxicity, see Box Warnings on possible QT prolongation (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm333695.htm). The lead compound was discovered by screening for growth inhibitors of *Mycobacterium smegmatis* using a library of about 70,000 prototype compounds representing selected chemical series [27]. Scaffolds were selected based on chemical diversity properties, not the rule of five (Koen Andries personal

communication). The lead compound breached 2 of the rule of five stipulations (MW and logP) as did the analog chosen for development (R207910). Subsequent chemical optimization led to a series of active compounds with minimum inhibitory concentrations (MIC) below 0.5 μg/ml against Mtb. R207910 (1-(6-bromo-2-methoxy-quinolin-3-yl)-4-dimethylamino-2-naphthalen-1-yl-1- phenyl-butan-2-ol) was identified as the most active compound in the series with molecular weight 555.51 and logP of 7.1–7.7. Resistance studies were conducted to determine not only the frequency of resistance but also to isolate resistant strains for the identification of the putative target. Resistance colonies were selected at frequencies of 5×10^{-7} to 1×10^{-8} depending on the drug level used in selection. Genome sequencing revealed point mutations in *atpE*, a part of the F0 subunit of ATP synthase, and several follow up studies have confirmed targeting the ATPase [28–31]. The two point mutations identified, (D32V) for *M. smegmatis* and (A63P) for Mtb were both within the *membrane-spanning domain region of the protein*. This later finding is of significant interest to the conclusions arrived at in the present analysis (see sections below). The logP for R207910 is 7.5, which is consistent with its targeting a site on a membrane protein within the membrane-spanning region (see further discussion below). This compound violates the rule of five based on hydrophobicity and MW, but is orally active. The compound was active in mouse models of TB infection and was subsequently developed for treating TB in humans. Bedaquiline was also active on non-replicating Mtb via disruption of ATP homeostasis [31]. *M. smegmatis* was used as the screening strains leading to the discovery a successful new TB drug but others report the use of *M. smegmatis* severely limits the detection of compounds active on Mtb. For example, only 20% of the Mtb active compounds discovered from screening 20,000 small molecules were active on *M. smegmatis* [22], a surrogate strain used in screening for new antitubercular compounds. In another study [32] 50% of compounds active against Mtb were inactive on *M. smegmatis*. Similar results were found with the large scale NIH screens conducted by the NIH supported contract to Southern Research Institute and via the NIH Molecular Libraries Screening Centers Network (MLSCN) [15,20] program (see http://pubchem.ncbi.nlm.nih.gov) wherein only 50% of the Mtb actives identified were active on *M. smegmatis* (unpublished results). These data indicate that whenever possible Mtb should be used for screening for new TB drugs, even though *M. smegmatis* screening did lead to discovery of bedaquiline.

3.2. DprE1

The third example is discovery of inhibitors of DprE1 [33,34] an essential [35] membrane-associated enzyme subunit of the heteromeric decaprenylphosphoryl-β-D: -ribose 2′-epimerase [36–40]. This heteromeric decaprenylphosphoryl-β-D-ribose 2′-epimerase encoded by the *dprE1* (Rv3790) and *dprE2* (Rv3791) genes is involved in the biosynthesis of D-arabinose (transformation of decaprenylphosphoryl-D-ribose to decaprenylphosphoryl-D-arabinose, a substrate for arabinosyltransferases in the synthesis of arabinogalactan and liporabinomannan of mycobacteria). Arabinogalactan is a fundamental component of the mycobacterial cell wall that covalently binds the outer layer of mycolic acids to peptidoglycan.

Nitrobenzothiazinones were discovered by screening a series of sulfur-containing heterocycles synthesized as a possible source of new antibacterials and antifungals, and found to possess potent and specific antibacterial and bactericidal activity for mycobacteria [41]. Library compliance with the rule of five was not discussed. The sulfur atom and the nitro groups at positions 1 and 8 were critical for activity as assessed by structure activity relationships. BTZ043 (Table 1, logP = 3.0–3.6), with an MIC on Mtb of 1 ng/ml (2.3 nM), was selected for follow up studies and found active on MDR-TB and XDR-TB strains. It was bactericidal, reducing viability *in vitro* >1000-fold in <72 h but appeared less active on non-replicating bacteria. Bactericidal activity appeared to involved cell lysis. DprE1 was identified as the putative target by using cosmids and selection and analysis of resistant mutants. A change at C387 (replaced by S or G) was found in all drug-resistant mutants. BTZ043 was active in a mouse model of TB infection and found to inhibit transformation of decaprenylphosphoryl ribose (DPR) to decaprenylphosphoryl arabinose (DPA) in cell extracts. Extracts from resistant mutants were not inhibited, further substantiating DprE1 as the target.

High content screening using automated confocal fluorescent microscopy to monitor intracellular growth of GFP- expressing Mtb H37Rv in Raw264.7 macrophages was used in another study. Screening a library of 57,000 small molecules identified active compounds [17]. The library was purchased from Timtec (26,500 molecules), Cerep (10,484) and ChemBridge (20,000) and each sub-library consisted of a selection of molecules based on their chemical diversity and drug-like properties. Most of the compounds (80%) were consistent with the rule of five. Benzamides, representing a cluster of 24 active molecules identified in the initial screen, were used as the starting point for synthesis of analogs based on the 2-nitro, 4-methoxyl benzamide series. This led to identification of two compounds, N-(2-(4-methoxyphenoxy) ethyl)-3,5-dinitrobenzamide (DNB1, logP = 2.4–3.0) and N-(2-(benzyloxy) ethyl)-3,5-dinitrobenzamide (DNB2 logP = 2.1 to 3.0. Table 1), for further mechanistic studies and target identification. Potent activity was restricted to the actinomycetes with the most potent activity observed against Mtb (sensitive, MDR and XDR strains) with MICs of about 75 ng/mL (0.2 μM). Both compounds were bactericidal and inhibited the heteromeric decaprenyl-phospho-ribose epimerase encoded by the rv3790c (*dprE1*)/rv3791c (*dprE2*) genes in Mtb H37Rv in cell extracts. Resistant mutants were selected at frequencies of 1.2×10^{-6} to 1.0×10^{-8} on agar containing 2–16 times the MIC of DNB1 or DNB2. Subsequently, DNB1 and DNB2 were tested for activity on *M. smegmatis* strains possessing the C387G or C387S and *M. bovis* possessing the C387S mutations in DprE1 with cross resistance observed.

Interestingly two classes of compounds, benzothiazinones (BTZs) and dinitrobenzamides (DNBs) derivatives, inhibit DprE1 by a similar mechanism. Resistance to DNBs and BTZ [42–44] can be due to a change at C394 of DprE1 or to increased expression of NfnB nitroreductase that can bioreduce the nitro groups rendering the compounds inactive. Recently the crystal structure of DprE1 was solved [45] and the structural basis of the interaction with and inhibition of DprE1 elucidated [46,47].

Inhibitors of DprE1 A were also identified in a third screening report [22]. The initial leads were found during the sponsored screening of compounds for inhibitors of Mtb growth conducted by Southern Research [15,20,48]. This library (100997 compounds) was purchased from ChemBridge and compounds were selected to comply with the rule of five and for minimal numbers of potentially non-specific inhibitors. Validated hits from this screen (about 1113 compounds) were sent to several TB labs for follow up analysis and two of these compounds were identified as inhibitors of DprE1 [22]. Two nitro-substituted triazoles were identified (Table 1), TAACF 339509 (logP = 2.9–3.6) and 377790 (logP = 2.7–3.2) with MICs of about 1.0 and 0.5 μM respectively. SAR analysis focusing on compounds 339509 and 377790 indicated that the nitro group was essential for activity, since analogs lacking the nitro group or with substitutions in place of the nitro group were significantly less active. Resistant mutants were selected using TAACF 339509 and

377790 and the genomes of 4 independently generated resistant mutants (2 independent clones from each analog) were sequenced. Mutations in *dpreE1* were found in all mutants resulting in the amino acid change C387S. Strains over expressing DprE1 were resistant to 339509 and 377790 *in vitro*, similar to the findings with benzothazinones.

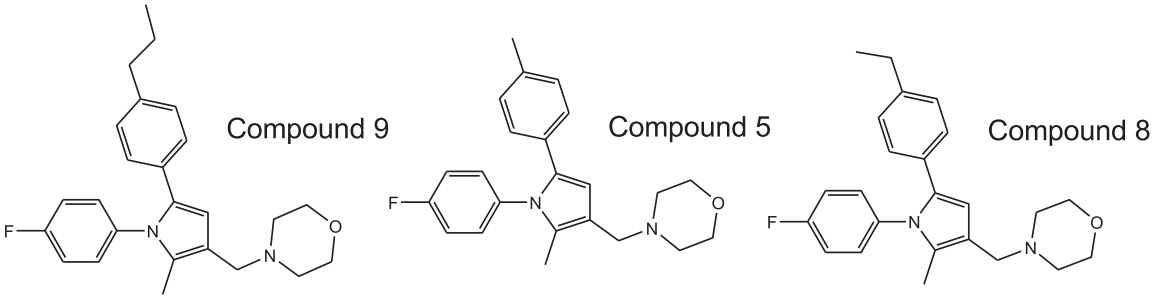
Compound TCA1 was discovered using *M. smegmatis* in a biofilm HTS from a diversity library of library of 70,000 heterocycles (further details not discussed) [193]. TCA1 was active on Mtb (MIC 0.19 μg/ml) and inhibited DprE1. TCA1 is distinguished from other DprE1 inhibitors by its bactericidal activity on both growing and non-replicating Mtb due possession of a secondary target. TCA1 was bactericidal against nonreplicating Mtb at a concentration of 7.5 μg/mL reducing cfu by 3 logs in 3 weeks. TCA1 was active against Mtb in macrophages with an MIC_{50} value of 0.6 μg/ml and in a mouse model of TB infection via oral administration. Spontaneous resistant mutants of *M. smegmatis* and Mtb to TCA1 (at 10^{-8} to 10^{-9}) were isolated. Whole-genome sequencing demonstrated single-point mutations in *dprE1* giving Y321C in *M. smegmatis* and Y314C in Mtb. TCA1 inhibited DprE1 in membrane and cell envelope fractions.

TCA1 blocked benzothiazinone (see above) binding to DprE1 demonstrating some overlap in binding sites. However, TCA1 does not have an active nitro-moiety, and the Tyr314Cys mutant strain that is resistant to TCA1 is sensitive to BTZ, suggesting that the binding mechanism of TCA1 is different from these nitro-heterocycles. The structure of the TCA1:DprE1 complex was determined by X-ray crystallography. TCA1 bound to the central cavity of the enzyme, adjacent to the isoalloxazine ring of FAD, in a boomerang-like conformation, with the thiophene moiety inserted at the bottom of the active site. Two key pieces of information indicate that TCA1 may have more than one target in Mtb: 1) TCA1 is still active on non-replicating Mtb possessing the Y314C mutation and 2) TCA1 potentiates the action of INH or RIF on a DprE1-over expressing strain. An affinity based target capture system was used to pull down a possible secondary target for TCA1. Mass spectrum analysis identified MoeW, an enzyme involved in the biosynthesis of the molybdenum cofactor (MoCo). Consistent with this observation, TCA1 also blocked synthesis of MoCo in growing Mtb. MoCo is an important cofactor for the functioning of nitroreductases, and nitroreductases appear to be involved in the response of Mtb to hypoxia.

Finally, 12,000 compounds from a kinase inhibitor library were screened in whole cell assays with *Corynebacterium glutamicum* and Mtb, and a target-based assay with protein kinase PknA. The library was organized based on 108 core structures and >500 scaffolds, conforming to ATP-binding site inhibitors and a subset of compounds with documented kinase inhibition. Two hits were specific for growth inhibition of Mtb. VI-18469 (benzoquinoxaline pharmacophore), and VI-9376 (structurally related to the benzothiazinones). VI-9376 (7-bromo-2-methyl-5-nitro-3phenylquinoxaline, see Table 1 (logP = 4.1 to 4.9, MIC 3.1 μM) was cross-resistant when tested against BTZ043 resistant strains (*M. smegmatis*, *M. bovis* and *C. glutamicum*) demonstrating that the target is the essential enzyme decaprenylphosphoryl-β-D-ribose 2′-epimerase, DprE1, required for arabinan synthesis [24]. The nitro group at the fifth position of the quinoxaline scaffold was required for activity, consistent with targeting DprE1.

3.3. MmpL3

A forth example is the identification of a set of compounds that inhibit the function of MmpL3, an essential transmembrane protein that appears to be involved in trehalose monomycolate transport across the plasma membrane [49–56]. The pyrrole derivative BM212 (logP = 5.1 to 7.4, Table 1) was identified as active against Mtb following screening of library of azole compounds (containing imidazole, pyrrole, toluidine, or methanamine groups) for growth inhibitory activity [54]. It was active against MDR-TB clinical isolates and Mtb residing within macrophages. MmpL3 was identified as the cellular target of BM212 [50]. *M. smegmatis* strains resistant to BM212 were selected at a frequency of 3.7×10^{-7} with MIC values ranging from 12.5 to 25 μg/ml, 4- to 8-fold higher than for the parent strain. Resistant mutant were also isolated in *M. bovis* BCG and Mtb H37Rv with MICs ranging from 3.12 to 20 μg/ml, 4- to 25-fold higher than the parent strain. Mutants were isolated at frequencies of 1.7×10^{-8} for *M. bovis* and 2×10^{-9} for *M. tuberculosis*. MmpL3 was identified as the site of mutations causing resistance (whole genome sequencing) with various site substitutions identified. Changes A326T (*transmembrane domain*, see http://tuberculist.epfl.ch/tmhmm/Rv0206c.html), V689G (*transmembrane domain*), and V197M (cytoplasmic domain) were found in *M. smegmatis*. All resistant *M. bovis* BCG isolates possessed a change L320P (*transmembrane helix*). Resistant Mtb strains possessed the change L215S in the predicted third transmembrane segment. Thus most amino acid changes were mapped within the first *six transmembrane segments of the predicted topology*. Again, these findings are of interest in the context of possible mode of action (see discussion below). Resistance due to efflux or overexpression of Mmpl3 was ruled out by experimentation.

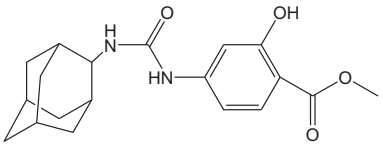


Analogs of BM212 were synthesized to identify derivatives with improved activity including activity in a mouse model of TB infection [56]. Compound 9 (MIC 0.12 μM, logP = 5.1 to 7.1, with one R of 5 violation) was orally bioavailable and active in a mouse model of TB infection. Compound 5 (MIC 0.3 μM, logP = 4.3–6.1) and compound 8 (MIC 0.2 μM, logP = 4.7 to 6.6 were also used to select for resistant mutants in *M. bovis* BCG and *M. tuberculosis*

H37Rv. Mtb strains resistant to compounds 5 and 8 were isolated at a frequency of about 3×10^{-7} and while no *M. bovis* mutants were isolated to compound 5 they were obtained for compound 8. While the pattern of resistance was complex, specific mutations gave rise to changes in MmpL3 with a V681I change (*within a presumed transmembrane helix*, see http://tuberculist.epfl.ch/tmhmm/Rv0206c.html) causing the higher level of resistance in Mtb. Mutation G253E (*also within a presumed transmembrane helix*) was also identified (G253E was also found in strains resistant to AU1235, see below). Mutation Q40R in Mtb gave lower level resistance and mapped to a *predicted internal loop* of MmpL3. *M. bovis* strains resistant to compound 8 revealed changes L320P or L320R (also found in resistance to C215, see below). L320 maps to a predicted *transmembrane domain*. Of interest, the 1,5-diphenyl pyrroles possessed some activity against non-replicating bacteria suggesting that another target may exist.

A second report [49] described the identification of a novel urea derivative [1- (2-adamantyl)-3-(2,3,4-trifluorophenyl)urea]. Compound AU1235 (logP = 3.3–5.1) was discovered by screening a library of 12,000 compounds from LeadScreenTM (preformatted screening set commercialized by Tripos; St Louis, MO) at a concentration of 10 μg/ml against Mtb H37Rv. The MIC/MBC was 0.1 μg/ml (0.3 μM). AU1235 was also active against MDR clinical isolates of Mtb displaying resistance to isoniazid, rifampicin, and pyrazinamide in addition to streptomycin, fluoroquinolones, and/or ethambutol suggesting a new mode of action unrelated to current antitubercular drugs. AU1235 at 10 μg/ml was inactive against nonreplicating Mtb in an anaerobic model. AU1235 was inactive against other Corynebacterineae such as *C. glutamicum*. AU1235 had minimal effects on RNA, DNA, protein, and polysaccharide synthesis but did cause a concentration-dependent decrease in trehalose dimycolates (TDM) and all three forms of cell wall-bound mycolic acids (α-, methoxy- and keto-). While trehalose monomycolate (TMM) synthesis was apparently unaffected, the subcellular distribution of TMM was altered. Less TMM was shed into the culture filtrate and more remained cell associated as the concentration of AU1235 increased, suggesting that translocation of TMM to the membrane of Mtb was inhibited. Resistant mutants were selected at 2–4 times the MIC and analyzed genetically to identify the putative target. Spontaneous mutants were isolated at a frequency of 4×10^{-9} (4× MIC) to 1×10^{-7} (2× MIC). Genome sequencing identified a single mutation in the *mmpL3* gene. In all cases the change observed was G253E. *Residue G253 maps at the end of one of the predicted membrane spanning domains of MmpL3*. Efflux was ruled out as the mechanism of resistance.

Adamantyl ureas (with high logP values) were found to inhibit human soluble epoxide hydrolase (hsEH) and Mtb epoxide hydrolases [51]. Thus a library of 1600 ureas (mostly adamantyl ureas), synthesized for the purpose of increasing the bioavailability of inhibitors of hsEH, was screened for activity against Mtb. 1-Adamantyl-3-phenyl ureas with a polar para substituent retained moderate activity against Mtb and one (compound 6, logP = 3.2–5.8) was orally bioavailable in mice. However, neither this compound nor a closely related analog was active in a mouse model of TB infection. No correlation between *in vitro* potency against Mtb and the hsEH inhibition was found supporting the concept that activity against hsEH and Mtb can be separated. No correlation was found between logP and inhibition of Mtb growth. Most importantly for the purposes of this paper, members of two classes of adamantyl ureas containing polar substituents (to increase their bioavailability) lacked efficacy against Mtb but were taken up by bacteria, suggesting that such modifications altered the interaction with the target but did not affect cellular uptake. This finding is consistent with the hypothesis that binding to the membrane target is not required for partitioning into the cell membrane (see discussions below). A follow up study from this group reported on new analogs synthesized to improve selectivity, pharmacokinetics, and potency while maintaining MmpL3 target inhibition [57]. The role of adamantyl groups in drug design/properties is discussed in the literature [58,59].

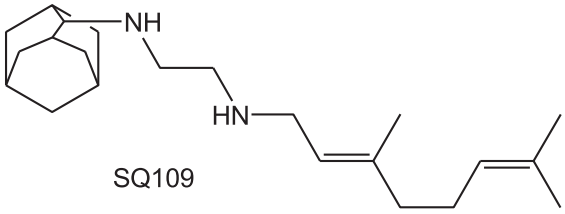


Compound 6

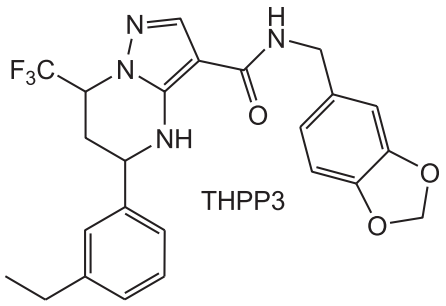
MmpL3 was also identified as the target of a benzimidazole that inhibited Mtb growth [22]. A high throughput screen, using constitutive GFP fluorescence as a reporter, was use to screened library of 20,000 compounds preselected for maximal chemical diversity, and consisted of commercially available compounds, natural products, and compounds generated by Diversity Oriented Synthesis and other synthetic compounds (see Chembank at http://chembank.broadinstitute.org/). A benzimidazole (C215 logP = 4.2 to 5.8, see Table 1) was identified. Four independent resistant mutants were generated with about ~2 fold resistance. *Rv0206c* (MmpL3) *was* the only gene with mutations in all four resistant mutants, suggesting that mutation of *Rv0206c* confers resistance to C215. A single mutation in *mmpL3* (G253E) was identified in all AU1235 resistant mutants (see above). In contrast, a different mutation in *mmpL3* was identified in each of the C215 resistant mutants (L320P, T667A, V684A, V51A). Changes at positions 320 and 684 occur in *predicted transmembrane helices* while position 667 is near the *start of a transmembrane domain* (inside segment) and position 51 may be near the inside segment between the first two predicted *transmembrane domains* (analysis by http://www.cbs.dtu.dk/services/TMHMM/, TMHMM Server v. 2.0 Prediction of transmembrane helices in proteins). A dose dependent decrease in cell wall-bound mycolic acids (α, methoxy, and keto) upon treatment of cells with C215 confirmed that MmpL3 is the likely target of C215.

Two 2-adamantan-1-yl derivatives were also identified a MmpL3 [52] inhibitors from among the validated hits (~1113 compounds) from the sponsored screening of 316103 compounds for inhibitors of Mtb growth conducted by Southern Research [15,20,48]. This library set mostly complied with the rule of five. The *iniBAC* promoter [60] in mycobacteria is induced by exposure to cell wall inhibitors but not kanamycin, ciprofloxacin or other stress inducers (e.g. hydrogen peroxide, heat shock, acidosis). The set of 1113 compounds was screened using the *iniBAC* promoter construct expressing a reporter and several compounds were found to induce. Two compounds TAACF 356596 (logP = 3.7–4.8) and TAACF 391844 (logP = 5.1–6.7) identified as inducers (Table 1) were used in subsequent studies to determine the mode of action. Interestingly, these two lead compounds (similar in structure to SQ109, a compound in clinical trials) were the vehicle that assisted in identification of the target and mode of action of SQ109 [52]. SQ109 is hydrophobic with logP = 4.3 to 6.8. The amino-adamantyl group of SQ109 is present in TAACF 356596 and 391844. The precise target of SQ109 was unknown and no resistant mutants could be isolated. However, TAACF 391844 and 356596, DA8 and DA5, respectively in were successfully used to select resistant mutants for sequencing. Resistant mutants were isolated at a frequency of about 1×10^{-7}. Two DA5-resistant and three DA8-resistant mutants were isolated, and all five were cross-resistant to SQ109. Genomes of three were sequenced and all possessed single nucleotide transition mutations leading to replacement polymorphisms in *mmpL3*, leading to amino

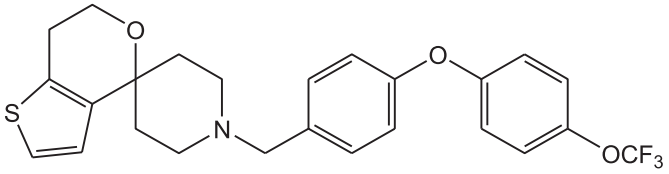
acid changes A700T (*start of a transmembrane helix*) in one strain and Q40R (*predicted inside loop*) in the two other strains. Further sequencing of *mmpL3* from other DA5- and DA8-resistant mutants further confirmed that single nucleotide polymorphisms in this gene were associated with resistance, with one carrying a novel mutation leading to a L567P amino acid change in a *predicted transmembrane helix*. Inhibition of MmpL3 function was verified by treating cells with compound and observing inhibition of trehalose dimycolate (TDM) production and failure to attach mycolates to the cell wall arabinogalactan. Since total mycolate levels were unaffected these effects were not due to inhibition of mycolate synthesis, but instead resulted in the accumulation of trehalose monomycolate (TMM), the precursor of TDM and cell wall mycolates, consistent with inhibition of MmpL3.



SQ109



THPP3



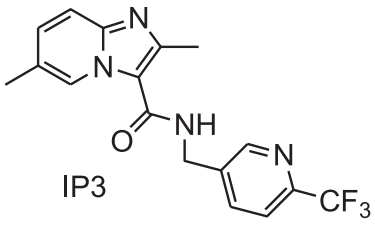
Spiro4

Inhibitors of MmpL3 were also discovered [61] following phenotypic screening of the GSK corporate compound collection of 2M compounds [62] against *M. bovis* BCG as an *M. tuberculosis* surrogate. The specifics of the library design were not discussed but for follow up of hits, the authors comment on looking for compounds that reside comfortably within the bounds occupied by marketed drugs, and that the Mtb hits were more lipophilic. Of note from this study [62]: the highly potent clusters were hydrophobic ranging from logP = 3.0 to 4.9. Also only 55% of the BCG positives were able to inhibit H37Rv at 10 μM and the percentage increased to 86% when tested at 25 μm again indicating that some compounds could be missed when using a surrogate strain. Tetrahydropyrazolo[1,5-a]pyrimidine-3-carboxamides (THPP) and *N*-benzyl-6′,7′-dihydrospiro[piperidine-4,4′-thieno[3,2-c]pyran]s (Spiro) analogues were identified as active against Mtb [61]. THPP1 (logP = 3.7–4.8) and Spiro2 (logP = 2.7–4.2) see Table 1, gave MICs of 0.3 μM on Mtb. Spontaneous resistant mutants were isolated and whole-genome sequencing analysis detected single nucleotide polymorphisms in *mmpL3*. THPP1 gave changes A249P, A677V, F644C, and V713M, while Spiro2 gave changes F255L, Y252C, F255V I292S, Ile292Thr, and S591I. All isolated mutants were susceptible to moxifloxacin, ethambutol, and linezolid. A complex series of cross-resistance patterns was observed between THPP and Spiro compounds series suggesting specific analogs act via compound specific interactions with the proposed target. Most of the mutations identified in MmpL3 mapped to the first *six transmembrane segments*. The authors noted that most of the amino acid changes are located within *predicted transmembrane segments* and comment on the hydrophobicity of the THPP and Spiro series of compounds vs. the membrane target. They further speculated that if these amino acids do interact directly with THPP and Spiro they might inhibit the ability of MmpL3 to act as a TMM transporter, which was observed following treatment of bacteria with THPP1 or Sipro2. Analogs THPP3 (logP = 3.7–4.8, MIC 0.16 μM) and Spiro4 (logP = 6.2–7.3, MIC 0.06 μM) were synthesized and were active via oral administration in a mouse model of TB infection.

One preliminary report [63] described the identification of compounds that inhibit the growth of Mtb from a library of 6800 compounds that fall mainly within the rule of five stipulations. This work identified an indole-2-carboxamide scaffold (hit compound 3, MIC 0.9 μM, logP = 3.3–5.3, Table 1). Preliminary studies indicate that this compound targets MmpL3 (assessed by the isolation and sequencing of resistant mutants), however specific data on mutant isolation and sequencing were not divulged.

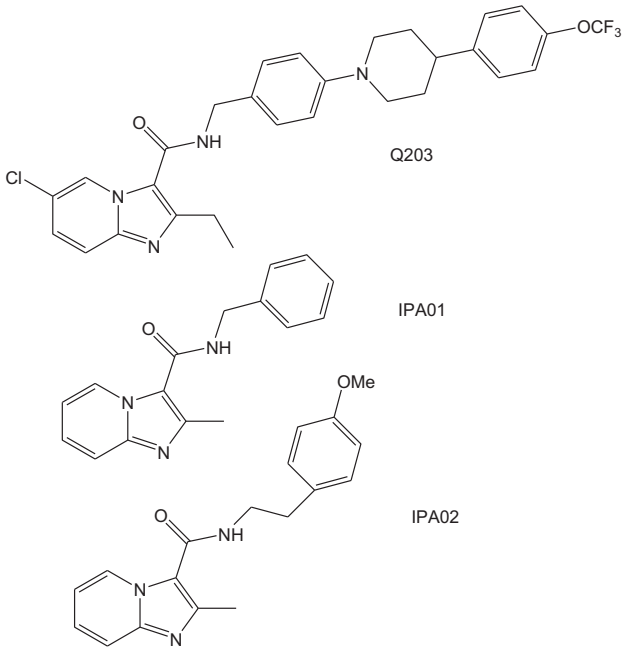
3.4. QcrB

A fifth final example is the discovery of inhibitors of the *qcrB* gene product, which encodes the β subunit of the electron transport ubiquinol cytochrome C reductase. QcrB is a putative ubiquinol cytochrome c reductase (subunit b), an integral membrane protein and member of the bc1 complex of the respiratory electron transport chain [64] and appears to be an essential protein [65,66]. A HTS campaign was run for growth inhibitors of *M. bovis* BCG but the properties of the library used were not specified. However the screen did identify imidazo[1,2-*a*]pyridines that also inhibited growth of *M. tuberculosis* H37Rv with MICs of 0.03–0.2 μM. Two of these, IP1 (logP = 2.7–4.2) and IP2 logP = 3.9–5.5, Table 1) are quite hydrophobic. Spontaneous resistant mutants were generated in *M. bovis* BCG at 5× MIC and resistant mutants selected at a frequency of 10^{-8}. After sorting out SNPs that likely arose over time in the lab strain, a single base change in *qcrB* (^{937}ACC >^{937}GCC) conferring a predicted amino acid alteration (T313A) in the protein sequence was identified. Residue 313 is predicted to be in an *outside loop between two transmembrane helices* (http://www.cbs.dtu.dk/services/TMHMM/, TMHMM Server v. 2.0 – Prediction of transmembrane helices in proteins). Mutants were cross-resistant. Strains over-expressing QcrB were resistant, with MIC increasing from 0.5 μM to >8 μM (>16 fold), further confirming QcrB as the target. Since the original leads suffered from low stability in mouse microsomal fractions more soluble compounds were synthesized. IP3 was thus identified and found active in a mouse model of acute TB infection via oral administration at 300–500 mg/kg. IP3 is hydrophobic with logP = 2.5–3.7.



IP3

Screening commercial chemical libraries (121,156 compounds) for compounds inhibiting mycobacterial growth in infected macrophages [67] identified imidazopyridine amides IPA01 (logP = 2.4–3.9, MIC_{50} = 2.03 μM) and IPA02 (logP = 2.7–4.4 and MIC_{50} = 2.63 μM). The rationale for the compound library properties was not stated. Analogs IPA04 (logP = 3.3–5.0, MIC_{50} = 10 nM) and IPA05 (logP = 4.2–5.6, MIC_{50} = 5 nM) see Table 1, were synthesized and used to select spontaneously resistant mutants that were cross-resistant to other IPAs, including analog Q203 (logP = 6.3–8.2, MIC_{50} = 2.7 nM). A single amino acid substitution was found in the cytochrome *b* subunit (*qcrB*, Rv2196) by genome sequencing causing a change at T313 to either A or I. The spontaneous mutation frequency was about 2.4×10^{-8}. Selecting resistance to Q203 directly yielded strains with T313A in QcrB. The location of T313 was analyzed using structural data from crystal structures of *bc1* from the soil bacteria *Paracoccus denitrificans*, *Rhodobacter sphaeroides* and *Saccharomyces cerevisiae*. T313 appears to lie within the ubiquinol Q_P site. Treatment of bacteria with Q203 caused a reduction in intracellular ATP, consistent with inhibition of QcrB. Q203 was efficacious in a mouse model of TB infection via oral administration and is being considered for clinical development base on its preclinical profile.



Another set of hydrophobic antibiotics (myxothiazol, mucidin, and stigmatellin, logPs 3–5) is known to inhibit the ubiquinol-cytochrome *c* oxidoreductase, bc_1 complex from *Rhodobacter capsulatus* [68], and yielded resistant mutants with a functional bc1 complex. This relevant finding further supports the findings in Mtb, i.e. hydrophobic compounds can target membrane processes and give rise to a functional, resistant phenotype.

3.5. Pks13

Pks13 plays a key a role in mycolic acid biosynthesis [69,70] and is essential in Mtb [35,66,70,71]. Pks13 (Rv3008c) appears associated with the cell membrane [37–40] and may contain a transmembrane segment near the carboxy-terminus (http://tuberculist.epfl.ch/tmhmm/Rv3800c.html). Compounds that inhibit growth of Mtb in culture (1113 publically available compounds) identified by the NIH/MLSCN [15,20,48] were tested for their ability to induce the *iniBAC* promoter construct (see above), and several thiophenes were identified as inducers [72]. This library mostly conformed to the rule of five. MICs ranged from 0.5 to 20 μM with TP2 (TAACF 311032, logP = 4.6–5.2) and TP4 (TAACF 343948, logP = 2.1–3.4) see Table 1, being among the most active and causing the greatest induction of *iniBAC*. Synthesis of mycolic acid methyl esters was inhibited when cell were treated with TP2 or TP4 with accumulation of fatty acid methyl esters. Spontaneously resistant mutants were obtained using TP2 and showed cross-resistance to TP4. A T236C single-nucleotide polymorphism in the *pks13* (*Rv3800c)* gene was identified causing a F79S substitution in the protein sequence. While overexpression of Pks13 led to a 2-fold level of resistance, overexpression of the resistant homolog (F79S) led to 128-fold resistance to TP2. Resistance to TP2 did not change the potency of other standard TB drugs. Interestingly, the combination of INH and TP2 or 4 led to synergistic killing of Mtb in culture. TP2 and 4 kill *M. bovis* BCG residing in J774A.1 macrophages. Molecular modeling suggests that thiophenes bind to a neutral, *hydrophobic groove* in Pks13 with van der Waals contacts with Phe79, consistent with resistance studies. The inhibitors, targets, and pathways discussed above are diagramed in Figure 1.

A recent report [62] described the results of HTS for growth inhibitors of *M. bovis*. These data also support the contention that Mtb should be used as the screening strain if at all possible since only 55% of the *M. bovis* actives were active on Mtb at 10 μM (increase to 86% at 20 μm). Compounds were profiled by lipophilicity, size, cytotoxicity, and active against intracellular organisms. Seven classes of compounds were identified and representative hits from the seven classes had logP values of 3.0–4.2. The authors indicate that preliminary studies of the modes of action of the most promising hits are likely to be electron transport and cell wall synthesis, a result not inconsistent with the concepts present in this review.

4. Newly discovered compounds targeting membrane proteins are hydrophobic

The present analysis is limited to HTS campaigns that yielded hits with identification of the target via genome sequencing. Of note none of the resistant mutants/targets identified were in transport proteins for the drug itself, supporting passive diffusion as the mode of uptake, and consistent with their hydrophobicity. Resistant mutants sometimes contain compensatory mutations in pathways indirectly associated with the true drug target. However, for almost all of the studies discussed above the putative target identified was also validated by additional biochemical or structural studies: GlpK inhibitors cause accumulation of a toxic metabolite related to glycerol metabolism. R20710 and QcrB inhibitors deplete cellular ATP. DprE1 inhibitors interact with DprE covalently or reversibly (also supported by structural studies). When bacteria are treated with MmpL3 inhibitors, trehalose monomycolate transport across the plasma

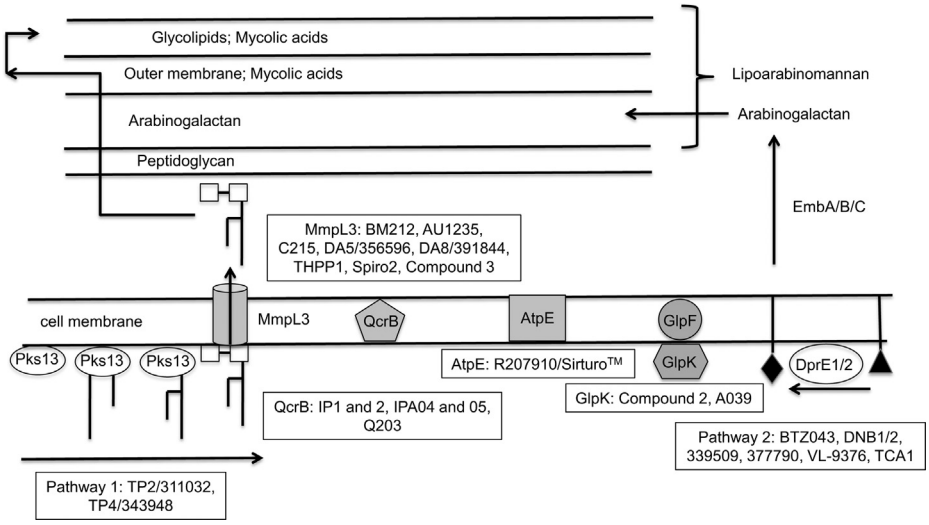


Figure 1. Outline of the metabolic pathways inhibited by compounds listed in Table 1. Pathway one represents the processing of mycolic acids by Pks13: α-meroacyl-AMP and 2-carboxyl-C26-S-CoA are transferred to the N-terminal and C-terminal PPB domains, respectively. The meroacyl derivative is then transferred to the condensing reaction site followed by condensation and reduction to yield the final α-mycolate. Newly formed mycolic acids are transferred to man-P-heptaprenol and then to trehalose 6-phosphate to give TMM-P. The recently identified TP2/311032 and TP4/343948 thiophenes inhibit Pks13 possibly by binding near the ACP-domain and blocking the loading reaction [72]. MmpL3 is the likely transporter for TMM since inhibitors BM212, AU1235, C215, DA5/356596, DA8/391844, THPP1, Spiro2, and Compound 3 inhibit trehalose dimycolate (TDM) production resulting in the accumulation of trehalose monomycolate (TMM), the precursor of TDM, and cell wall mycolates fail to attach to the cell wall arabinogalactan. AtpE is a component of the proton translocating ATPase and is inhibited by R207910/Sirturo™. GlpK (glycerol kinase) is inhibited by Compound 2 and A039. Glycerol kinase activity is tightly coupled with glycerol transport via the glycerol facilitator GlpF and a direct interaction at the membrane between the glycerol facilitator protein and glycerol kinase that stimulates kinase activity was observed [25]. Pathway 2 represents the processing of decaprenyl-P-ribose (triangle) to decaprenyl-P-D-arabinofuranose by the DprE1/2 complex inhibited by BTZ043, DNB1/2, 339509, 377790, and VL-9376. QcrB encodes the β subunit of the electron transport ubiquinol cytochrome C reductase and is inhibited by IP1 and 2 and IPA01 and 02. Specific analogs of the primary hits also inhibit the respective targets (see text).

membrane is blocked, causing accumulation of trehalose monomycolate (TMM), the precursor of TDM and cell wall mycolates. Finally, when bacteria were treated with Pks13 inhibitors synthesis of mycolic acid methyl esters was inhibited with accumulation of fatty acid methyl esters.

Overall, the compounds listed in Table 1 are hydrophobic with an average logP of 4.0 and there is experimental evidence that they target essential membrane proteins, a finding consistent with their hydrophobicity (see Table 2 for a summary of logP data). This is in striking contrast to currently used TB drugs where the average logP is 1.0 (Table 3), thus representing a difference of 5 orders of magnitude. Several key questions are raised by the above studies. 1) What can we learn from other drugs that target membrane function? 2) What is the role of the lipid bilayer in the mechanism of action and what are key aspects of the mode of action of these compounds? 3) How do such hydrophobic compounds enter cells? 4) What role might the design of screening libraries play? 5) Why have these recent HTS efforts identified hydrophobic molecules that target membrane protein? 6) Will the trend continue and what should we be concerned about when moving such compounds forward in development?

Table 2
Comparison of calculated logP values.*

	Current TB drugs		New TB leads		Prestwick actives	
	Average	Median	Average	Median	Average	Median
ChemDraw logP	−1.4	−0.7	4.1	3.7	5.8	6.1
ChemDraw clogP	−1.4	−0.7	3.6	3.5	5.3	5.3
Molinspiration clogP	−1.0	−0.2	3,8	4	5.5	5.6
KowWin clogP	−1.3	−0.5	4.4	4.2	6.0	5.9
Kasin et. al 26	−0.3	0.6	NA†	NA	NA	NA

* Values from Tables 1, 3, and 4 used to calculate averages and medians.
† NA not applicable.

4.1. Inhibition of membrane protein function

A central theme in biology is the evolution of systems that enhance the interaction of reversibly interacting species. One of the mechanisms used is the restriction of interacting species to a cellular compartment such as the cell membrane. Cooperative binding was recently discussed in terms of biological complexity and drug discovery [73]. The restriction of reversibly interacting species to a cell membrane substantially reduces relative freedom of motion while still allowing diffusion and rotation within the membrane. Cooperative interactions via cell membrane localization was considered to enhance interactions by reducing the entropy of unbound states, thus enhancing binding compared to the same molecules in solution by reduction of relative freedom of motion, a theme traceable to the pioneering work of Adams and Delbruck 1968 [74]. This type of cooperative interaction between reversibly interacting species can also enhance the interaction of multi-subunit receptors (e.g. activation of cytokine and growth factor receptors via ligand-induced dimerization/oligomerization), and the interaction of drugs with specific membrane bound targets.

The literature on perturbation of membrane protein targets in other fields can offer some potential answers to some of the questions posed above. Drugs can affect membrane function by binding to a membrane protein, affecting boundary lipids, affecting the structure of lipid domains, lateral pressure, or membrane curvature. Drugs can also cause nonspecific inhibition of membrane function. The mode of action of calcium channel blockers appears quite relevant [75–82] and may offer some valid approaches to understanding the mode of action profile of the hydrophobic drugs described above. Studies on the mode of action of calcium channel blockers began with X-ray and neutron diffraction studies to elucidate the structure of sarcoplasmic reticulum membrane, wherein the water and lipid profile structures were determined at

Table 3
Properties of current TB drugs.

Drug	Target	Uptake	clogP*	logP†	clogP§	clogP**	clogP‡	Rule of five violations¶
Isoniazid	Cytoplasm	Passive transport	−0.7	−0.67	−0.81	−1.0	0.6	0
Rifampicin	Cytoplasm	Passive diffusion	2.7	NA‖	4.2	2.6	3.7	3
Ethambutol	Membrane	Passive diffusion	−0.6	0.12	−0.41	0.4	0.06	0
Pyrazinamide	Cytoplasm	Active transport	−1.3	−0.68	−0.53	−0.7	−1.4	0
Kanamycin	Cytoplasm	Complex	−6.3	−5.2	−6.7	−5.7	−7.9	2
Cycloserine	Cytoplasm	Active transport	−0.9	−1.2	−1.72	−1.9	−1.6	0
Moxifloxacin	Cytoplasm	Passive diffusion	1.68	−0.3	0.95	0.4	2	0
PAS	Cytoplasm	Active transport	0.89	1.1	0.98	0.9	1	0
Capreomycin	Cytoplasm	Unknown	−9.6	−5.68	−9.56	−5.5	NA	3
Ehtionamide	Cytoplasm	Passive diffusion (tentative based on similarity to isoniazide)	1.4	1.7	1.52	1.5	0.7	0

* logP estimated by Chemdraw (http://www.cambridgesoft.com/support/DesktopSupport/Documentation/ChemDrawPlugin/).
† clogP calculated by Chemdraw (http://www.cambridgesoft.com/support/DesktopSupport/Documentation/ChemDrawPlugin/).
§ clogP calculated by KowWin (http://esc.syrres.com/esc/est_kowdemo.htm).
** clogP calculated Molinspiration (http://www.molinspiration.com/cgi-bin/properties).
‡ clogP: estimated by Kasim [26].
¶ Rule of five violations Molinpiration (http://molinspiration.com).
‖ NA specific program did not calculate a value.

28 Å resolution [76,83] and these preliminary studies were refined to address the mechanism of action of calcium pump inhibitors.

One key concept involves drug partitioning into a membrane prior to interaction with a membrane-localized target. The interaction kinetics of the calcium channel blocker 1,4-dihydropyridine with cardiac sarcolemmal membranes was examined [78] and calculated for two distinct approaches: an aqueous approach wherein the drug reaches the receptor by diffusion through the bulk solvent, and the membrane approach wherein the drug partitions into the membrane bilayer and laterally diffuses to a specific receptor site [78,80]. The membrane approach was considered the most likely based on calculated data and the high partition coefficients and specific positions of the drug in the membrane bilayer. This suggests that small, hydrophobic compounds are capable of inserting into the phospholipid bilayer wherein they diffuse laterally through the membrane and inhibit metabolic function by interacting with a specific membrane protein, or by perturbing membrane lipids such as boundary lipids (see below). Diffusion in membranes [79] was directly examined using an active rhodamine-labeled dihydropyridine analog and a fluorescent phospholipid probe. The drug probe was found to diffuse over macroscopic distances within the bilayer at a rate of similar to the phospholipid probe (3.8×10^{-8} cm^2/s), a rate compatible with the membrane diffusion model for drug action. Data were also presented showing that lipid bilayer partitioning occurs before drug binding to specific receptors [80], which may explain why AU1235 analogs devoid of growth inhibition still accumulate into bacteria.

Gaining access to the lipid area of the membrane may also influence drug pharmacokinetics [84,85] and passage into other cellular compartments, e.g. nimodipine transport across the blood–brain barrier [86]. Propranolol and nimodipine (membrane partition coefficients of approximately 1200 and 5000, respectively) are located approximately 6 Å from the phosphate head-group region of the membrane bilayer near the hydrocarbon core/water interface and are readily washed out of membranes [87]. In contrast amiodarone has a much higher partition coefficient (~1,000,000), is located deeper in the membrane, approximately 12 Å from the phosphate head-groups and closer to the terminal methyl groups of the lipid acyl chains, thus resisting washout from the membrane bilayer, leading to prolonged action. Hydrophobic compounds targeting the bacterial membrane could possibly prolong the post antibiotic effect by a similar mechanism. Interestingly, amiodarone (Table 4) has an MIC on Mtb of about 2–6 μg/ml (tested from the Prestwick Chemical Library screening at the NIH, see http://pubchem.ncbi.nlm.nih.gov) and is hydrophobic (logP = 8.5–9.9). The Prestwick Chemical Library (1200 small molecules) consists of drugs that were approved for human use (FDA, EMEA and other agencies). Other hydrophobic calcium channel blockers were identified as active on Mtb from the NIH sponsored Prestwick Chemical Library screen (see Table 4). Fendiline, lidoflazine, and bepridil had MICs of about 6–10 μg/ml, with logP values of 4.8–6.2. Nimodipine, a dihydropyridine, gave weak activity (partial inhibition at 20 μ/ml with a logP of 3.0–4.1. Other hydrophobic compounds in the Prestwick Chemical Library (Table 4) also had activity on Mtb: suloctidil (logP 5.4 – 6.0, MIC 2–3 μg/ml), perhexiline maleate logP 6.2 – 7.5, MIC 2–4 μg/ml), and pimozide (logP 5.3 – 6.4, MIC 2–3 μg/ml).

The above work was summarized and reviewed [82] in the context of the complex physical and chemical interactions of drugs with model and biological membranes and the potential role of nonspecific drug interactions [88–90]. For a description of how hydrophobic compounds access the membrane bilayer and perturb membrane function see Figure 2.

4.2. Boundary lipids

Several functional types of membrane proteins are embedded in the lipid matrix wherein the lipids can interact with proteins and modulate function, stability, folding, and evolution [91–93]. These interacting lipids are sometimes referred to as boundary lipids, which are mobile and capable of interacting with membrane proteins and affecting activity. Membrane proteins are also susceptible to physical stresses (e.g. membrane curvature, lateral pressure) that can alter function. Specific shells of boundary lipids [94] were also reported.

The bacterial K^+ channel protein KcsA is a α-helix bundle non-voltage-gated channel of the two-transmembrane-helix inward-rectifier type subject to regulation by lipid interactions [95,96]. Modulation of KcsA action can occur via specific, cooperative binding of anionic nonannular lipids to the channel's selectivity filter [97], and simulation studies revealed the interactions of aromatic amphipathic side chains (e.g. W, Y) with lipid headgroups, and "snorkeling" interactions of basic side chains (e.g. K, R) with phosphate groups [98,99] with H-bonds fluctuations on the ~1- to 5-ns timescale. Similar results were obtained with OmpA, a bacterial membrane porin. Key protein segments with affinity for

Table 4
Properties of Prestwick Library compounds.

Compound and MIC (μg/ml)	MIC (μg/ml)*	clogP†	logP§	logP**	clogP‡	Rule of 5 violations¶	Comment
Amiodarone hydrochloride	2 to 6	9.9	7.9	9.7	8.5	1	Cardiac membrane Ca channel
Nimodipine	>20 with 50% inhibition at 20	4.0	3.0	3.1	4.1	0	Cardiac membrane Ca channel
Fendiline HCl	7 to 9	5.7	5.9	5.8	5.5	1	Cardiac membrane Ca channel
Lidoflazine	4 to 6	4.8	6.2	5.0	5.3	1	Cardiac membrane Ca channel
Bepridil	9 to 13	6.2	5.4	5.2	5.0	0	Cardiac membrane Ca channel
Perhexiline maleate	2 to 4	7.2	5.2	7.5	6.2	1	Vasodilator; increases glucose metabolism at the expense of free-fatty-acid metabolism, enhancing oxygen efficiency during myocardial ischaemia
Pimozide	2 to 3	6.4	5.3	6.2	5.6	1	Dopamine antagonist
Suloctidil	2 to 3	6.0	5.4	5.6	5.9	1	Peripheral vasodilator; nonselective cyclooxygenase inhibitor
Econazole	1 to 4	5.1	4.5	5.6	5.1	1	Inhibits 14-α demethylase, (cytochrome P-450) converts lanosterol to ergosterol; active in mouse model of TB infection (half-life is short)
Clotrimazole	3 to 7	5.3	5.2	6.3	5.5	1	Inhibits 14-α demethylase
Sulconazole	2 to 5	6.3	5.3	6.9	5.6	0	Inhibits 14-α demethylase
Miconazole	1 to 3	5.8	5.1	6.3	5.7	1	Inhibits 14-α demethylase
Isoconazole	2 to 5	5.8	5.1	6.3	5.7	1	Inhibits 14-α demethylase
Ketoconazole	2 to 5	2.6	3.5	4.5	3.8	1	Inhibits 14-α demethylase
Terconazole	6 to 10	4.2	5.3	5.1	4.6	1	Inhibits 14-α demethylase
Butoconazole	1 to 2	6.9	5.9	7.4	6.3	1	Inhibits 14-α demethylase
Enilconazole	1 to 5	3.7	3.0	4.1	3.5	0	Inhibits 14-α demethylase
Sertaconazole	3 to 5	6.1	5.5	6.6	6.0	1	Inhibits 14-α demethylase
Astimazole	5 to 8	6.1	5.3	6.4	5.7	1	Antihistamine
Cinnarizine	4 to 10	6.1	5.7	5.4	5.8	1	Antihistamine
Terfenadine	2 to 3	6.1	7.0	7.6	6.2	1	Antihistamine
Meclizine	6 to 10	6.7	6.2	5.9	3.9	1	Antihistamine
Flunarizine	5 to 8	6.3	6.0	5.8	6.1	1	Antihistamine

* MIC determined 3 times with range indicated (NIH data see http://pubchem.ncbi.nlm.nih.gov).
† logP estimated by Chemdraw (http://www.cambridgesoft.com/support/DesktopSupport/Documentation/ChemDrawPlugin/).
§ clogP calculated by Chemdraw (http://www.cambridgesoft.com/support/DesktopSupport/Documentation/ChemDrawPlugin/).
** clogP calculated by KowWin (http://esc.syrres.com/esc/est_kowdemo.htm).
‡ clogP calculated Molinspiration (http://www.molinspiration.com/cgi-bin/properties).
¶ Rule of five violations Molinspiation (http://www.molinspiration.com).

specific lipid chain lengths were discovered from the crystal structure of KcsA [100].

The structure of the mechanosensitive channel MscL from Mtb is known and was evaluated in the context of interaction with membrane lipids [101,102]. Lipid binding was heterogeneous, with a hot-spot for binding anionic lipid on the cytoplasmic side associated with a cluster of three positively charged residues R98, K99, and K100, where they might interact with the headgroup of an

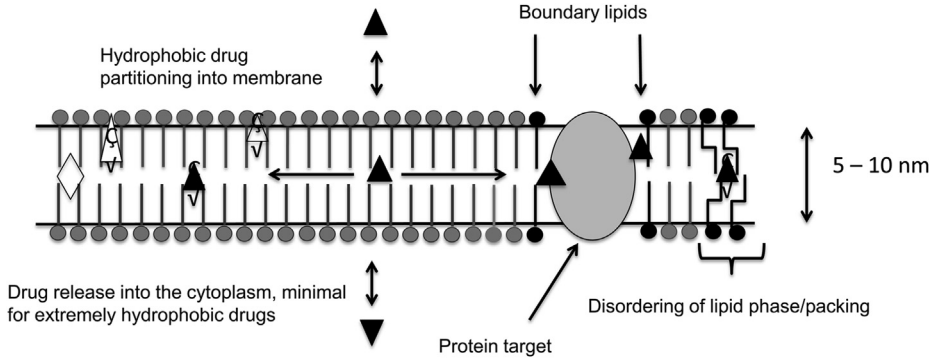


Figure 2. Pathways for access and inhibition of membrane functions. Hydrophobic drugs can partition into the lipid bilayer and orient based on physiochemical properties, and then diffuse laterally within the membrane. Once in the membrane, diffusion is in two dimensions with unique kinetics for the drug-target interaction. Initial membrane interaction can be 1000 times the forward rate constant of binding to receptors. Drugs can position in the membrane near the polar lipid head groups, in the nonpolar region or extend through both regions depending on structure and physiochemical properties. Drugs can also interfere with the interaction of boundary lipids with proteins and mechano-sensing. Depending on the logP, some degree of drug would also enter the cytoplasm but the higher the logP (e.g. 5.0 = 5 orders of magnitude) the greater the concentration in the membrane.

anionic phospholipid. Furthermore, differing sizes of cavities exist between the transmembrane alpha-helices of MscL that are effectively solvated by lipid fatty acyl chains [103].

The antibiotic gramicidin S will localize in the glycerol backbone region of the bilayer below polar headgroups, and above the hydrocarbon chains, where it perturbs lipid packing and induces formation of inverted cubic phases at lower temperatures [104]. Effects on boundary lipids were also reported [105]. Other antimicrobial peptides have specificity based on membrane lipid profiles, and act via formation of specific lipid-peptide domains, lateral phase segregation of zwitterionic from anionic phospholipids and even the formation of non-lamellar lipid phases.

5. Uptake of hydrophobic compounds and access to membrane targets

Compounds (including drugs) enter and cross the bacterial cytoplasmic membrane primarily by one of three mechanisms: passive diffusion, active transport, and facilitated uptake. Hydrophobic compounds are capable of diffusing across membrane bilayers and will equilibrate with the outside concentration unless there is a binding sink within the cell, for examples antibacterial macrolides [106]. Given the hydrophobic nature of the compounds and target identification studies (i.e. no transport mutants identified as resistance factors), the new TB leads are likely taken up by passive diffusion of unionized drug (for example see erythromycin [106]). Passive diffusion is defined by the equation $v = PA(C_{out} - C_{in})$, where v = net flux, P = permeability coefficient, A = cross sectional membrane area, and C_{out} and C_{in} are the molar concentrations of compound on the outside and inside of the barrier, respectively. P is in cm/sec, v in moles/sec, and C in molarity. Hydrophobic drugs will also partition between the aqueous space and the membrane (see below). Thus, the uptake rate is proportional to P and the net difference between the drug concentrations on the two sides of the membrane barrier. The term P bears a complex relationship to the structure and hydrophobicity of the drug and the structure of the particular lipid bilayer [106]. Size, shape, hydrophobicity, and charge are parameters that influence diffusion rate. Size is inversely related to the diffusion rate, whereas increasing hydrophobicity is positively correlated to diffusion rate.

While the mode of uptake is not usually studied as part the development process it is a critical event, which deserves more attention given that the potency of analogs of lead compounds can change for various reason, including transport properties. Also the example of the antifungal cispentacin is quite noteworthy. Cispentacin is accumulated by active transport mainly via the proline amino acid permeases but is not metabolized or pumped out of cells and thus attains an intracellular concentration of up to 36 mM [107] where it likely affects multiple metabolic processes.

The uptake mechanisms of several of the currently used antitubercular drugs are known (see Table 3 for properties of these drugs, note footnote e is data from Kasim [26]). Isoniazid [108], rifampicin [109,110], quinolones [111,112] and ethambutol [113] enter by passive diffusion while cycloserine transport is active via glycine/D-alanine transporters [114]. Cycloserine is a competitive inhibitor of two cytoplasmic enzymes, L-alanine racemase, which forms D-alanine from L-alanine, and D-alanylalanine synthase, which incorporates D-alanine into the pentapeptide necessary for peptidoglycan formation. The targets for rifampicin, isoniazid, and quinolones are all cytoplasmic enzymes and compounds like aminoglycosides and capreomycins (which inhibit protein synthesis) also affect a cytoplasmic target, the ribosome. Aminoglycoside uptake is complex and may involve a facilitator for initial entry followed by membrane damage caused by errors in protein synthesis producing altered membrane proteins that disrupt the barrier function allowing more drug entry [115,116]. EmbA-C (proteins believed to reside in the cytoplasmic membrane) may be involved in resistance to ethambutol (logP = −0.3) [117–119]. The mode of action of pyrazinamide (logP = −0.6) is likely analogous to cispentacin in that uptake is active (requires energy) via the nicotinamide uptake system [120] and in the absence of efflux pyrazinamide likely accumulates to high levels wherein various metabolic pathways may be targeted [121–127]. PAS appears to be taken up by an active process independent of the salicylate uptake system, possibly the same route as for p-aminobenzoate [128] .The mode of action was recently updated (previously assumed to inhibit dihydropteroate synthase) but it is now believed to serve as alternative substrate of folate metabolism [129].

Mtb possess two other unique components that play a role in compound uptake, the outer membrane, and porins. The outer membrane consists of outer lipids, mycolic acids, polysaccharides (arabinogalactan), peptidoglycan, and lipoarabinomannan forming a barrier, and specific outer membrane protein such a porins, through which specific compounds can pass [130]. The Mtb outer membrane may contain over 100 proteins [131] most of which have not yet been identified. Porins, proteins forming selective channels through which molecules can pass [132–136], appear to exist in greater numbers than previously anticipated [137].

None of the resistance factors identified to date for the new TB inhibitors (see above) were in transport systems for drug uptake, suggesting that these hydrophobic drugs are taken up by passive diffusion. Definitive studies on transport should be conducted to confirm this hypothesis. Also at least one drug that enters mycobacteria by passive diffusion (macrolides) [138] can accumulate (12%) in the cell integument, and defined a complex entry pathway with at least two barriers to diffusion.

6. Additional hydrophobic compounds from the Prestwick chemical library with activity on Mtb

Another series of compounds from the Prestwick Chemical Library of compounds active on Mtb are the antifungal azoles. Eleven azoles were active on Mtb (Table 4) with MIC values 1–20 μg/ml and average logP of 5.7. Antifungal azoles appear to target Mtb CYP121 and CYP130 [139–141] and some medicinal chemistry had ensued [142–144]. While the half-life of econazole is short in mice appropriate dosing does show efficacy [145]. Antihistamines (astimazole, cinnarizine, terfenadine, meclizine, and flunarizine) are another set of hydrophobic compounds with activity on Mtb (Table 4). Mefloquine (logP 4.1, see http://www.drugbank.ca/drugs/DB00358) is also is active on Mtb (MIC ∼ 3–5 μg/ml) and is active in mouse models of TB infection at high doses near the toxic levels for mice (C. Lambros, and R. Goldman, NIH unpublished data). Mefloquine is also active on *Mycobacterium avium in vivo* [146], and may act by affecting membrane transport and/or regulation of cellular replication [147]. Overall these actives from the Prestwick Chemical Library in Table 4 have an average logP of 5.7 and all are believed to target a membrane protein.

7. Role and philosophy of screening library design

Stanley and colleagues [22] observed that inhibitors of DprE1 and MmpL3 were somewhat divergent in structure and that whole cell screening appears to be selecting for membrane targets. They hypothesized that MmpL3 and DprE1 might be highly druggable and thus easily inhibited by a wide variety of chemical structures or that target identification using resistant mutants might be biasing towards targets that easily accommodate mutations in their active site while preserving target function (see Qcrb above where this is known to occur in *R. capsulatus*). However, inhibition of membrane

protein function need not occur at the active site as perturbation of lipid interactions can also cause inhibition. They also cautioned that focusing on leads to which resistance can occur, as opposed to those for which resistance is not selected, could lead to clinical resistance. However this concern is somewhat abrogated by the fact that for the foreseeable future TB therapy will continue to use multiple drugs, which if taken appropriately, suppresses the outgrowth of resistance even for isoniazid, which can occur at a frequency of 4.40×10^{-6} to 7.56×10^{-6} [148]. This frequency is also in the range of resistance to PA824 and OPC67683 (two compounds in clinical trials) due to loss of function of the bioreduction pathway [149]. The authors also comment that more attention should be paid to hits to which resistance cannot be selected. However target identification is still critical because if spontaneous resistance cannot be readily generated the compound could have a non-specific mode of action. The use of multiple, or parallel pathways for target identification was suggested. While not as focused, the past method of testing drug effects on macromolecular pathways (e.g. DNA, RNA, protein and cell wall) with follow up by *in vitro* dissection of the inhibition stage, was successful in identifying the targets of several historical antibiotics. Metabolic profiling of gene/protein express represents a modern version of these traditional techniques.

Properties of screening hits and libraries that led to identification of growth inhibitors of Mtb were recently analyzed [150,151] including the screens supported by the NIH, and compared to FDA approved drugs and know antitubercular drugs. For hits from the NIH primary screen for growth inhibitors (single dose), the mean number of hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), number of rotatable bonds (RBN) and polar surface area (PSA) was significantly lower compared with FDA approved drugs and pKa, Lipinski rule of five alerts, and number of aromatic rings while logP was significantly higher. The most active molecules from the MLSMR screen also revealed differences (molecular weight, logP, Lipinski score and pKa were higher, while the PSA was slightly lower). Examination of the most potent compounds (confirmed hits with reported follow up MIC determination) revealed that molecular weight, number of hydrogen bond donors, hydrogen bond acceptors, rule of five alerts, pKa, atom count, PSA and rotatable bond number *were all statistically higher*. Other reports address the specific rules used in library design vs. natural products [152], which tend to deviate from the basic rules used for small synthetic molecule library design [153–156], and how to improve on library design based on required elements for drug like behavior [157–163]. A new approach (Ligand Efficiency Indices) [164–166] was recently discussed, which considers binding energy/non-hydrogen atoms as a useful guide to optimize fragment and lead selection in the discovery process.

Some of the libraries used in the studies discussed above complied with the rule of five and some did not; new leads were discovered from both. Among the compounds listed in Table 1 most complied with the rule of five (20 of 24) while 4 did not (one with 2 violations, see Table 1). Three of the nine currently used TB drugs listed in Table 3 have rule of five violations (more than one violation). Among the actives from the Prestwick library (Table 4) three of the five cardiovascular drugs, eight of the 10 antifungal azoles, and all five active antihistamines possess rule of five violations.

The remaining three listed in Table 4 (perhexiline maleate, pimozide and suloctidil) each have one rule of five violation.

7.1. Strong inference

A very provocative analysis [167] discussed the complexities of modern drug discovery to those of a major historical event; Tycho Brahe's meticulous planetary readings that in the end required Kepler's insight to reveal the laws of planetary motion. One should bring to bear the full force of strong inference [168] (Platt, J.R. 'Strong Inference: Certain systematic methods of scientific thinking may produce much more rapid progress), which might in time lead to a better understanding of the vast amounts of preclinical data and the insights of a 'Kepler' for drug development. Several discussions on have insused [169], and the originator of the rule of five also addressed some of the issues surrounding its use in drug discovery [170,171]. Some, however, even questions whether or not properties/descriptors will ever be found that completely separates drug-like from non-drug-like space [159,167].

8. Summary

We do not know if the recent trend will continue and additional membrane protein targets will be linked to hydrophobic compounds discovered by phenotypic screening followed by genome sequencing. Perhaps it is just a statistical aberration and will not continue. One possibility is related to the relationship between potency and specificity previously discussed [172] wherein these hydrophobic compounds may be fairly weak inhibitors of the target but concentrate in the membrane driving inhibition. Regardless, given what is known about the mode of action of hydrophobic drugs targeting membrane protein function we must be careful that highly 'specific' drugs are selected for development in order to avoid toxicity that might be linked to inhibition of host membrane proteins or membrane integrity. The author has observed that the membrane bound demethylase involved in fungal ergosterol synthesis is particularly sensitive to perturbation of the membrane environment by hydrophobic compounds that were rather non-specific in action (i.e. also inhibited other membrane bound enzymes, e.g. fungal glucan synthase). While hydrophobic compounds per se are not necessarily promiscuous inhibitors, the use of hydrophobic drugs targeting membrane function is relatively new to the field of TB drugs and antibacterials in general, at least from the standpoint of detailed studies with approved drugs. Other surprises can occur when targeting specific pharmacophores to the membrane. For example lipophilic versions of the drugs vancomycin and eremomycin gain the ability to target the membrane bound transglycosylase involved in peptidoglycan synthesis [173–175] with subsequentl activation a novel cell death pathway [176].

8.1. Path forward

Thus far several membrane protein targets were discovered by phenotypic screening followed by selection of resistant mutants and genome sequencing, and the future will us if tell if this trend continues. However at this stage, with the identification of compounds that are active in animal models of TB infection, one needs to consider whether any specific issues need to be addressed in their further development. First of all, detailed understanding of the target:ligand interactions will require the use of appropriate tools for working with membrane proteins from Mtb [177–185]. Secondly, it is important to verify specificity in the mode of action. While target identification is not required for clinical development, lack of information on target affinity makes it difficult to assess the relationship between affinity and specificity as pointedly address [172] wherein greater affinity likely equates with greater specificity and selectivity. The fact that so many hydrophobic Prestwick Chemical Library compounds inhibit the growth of Mtb at 10 μg/ml or less does signal an alert regarding specificity. However, the antifungal azoles do appear to specifically target P450 enzymes in Mtb [139–141].

It would be valuable to measure the degree of resistance in all resistant isolates by determining the actual MIC increase. The target

system should also be examined for degree of resistance, which should match the MIC resistance in as much as the mutational change is proposed to be in the target and not compound uptake (an example of doing an experiment to disprove one's hypothesis see [168]). One could also try to select higher-level resistance with the initial resistant mutants isolated, and if they arise repeat the genetic identification of the target. The use of mutagenesis with UV light (generating the most diverse types of mutations) could also be used. It is possible for a compound with more that one mode of action (e.g. potent activity on target A and moderate on target B) to select for resistance to a first target, then exposing a second target as the drug level increases. Two of the compounds discussed above (TCA1 and BM212) do appear to have more than one mode of action.

A few antiinfective drugs that target cell membranes have been approved for systemic treatment of infections (e.g. daptomycin, telavancin, amphotericin B, antifungal azoles, and antifungal lipopeptides). Daptomycin [186], telavancin [187], and amphotericin B [188] have complex modes of action, which includes disruption of pathogen membrane integrity. Telavancin (a lipopeptide) acts by inhibiting transpeptidation (as does vancomycin). However, being lipophilic it also disrupts bacterial membranes and it can associate with eukaryotic membranes [189], localizing in lysosomes and causing mild morphological alterations but no changes in lipid metabolism. A related lipopeptide oritavancin was being developed for human used but was not approved in the US and was withdrawn from the EU. It also accumulates in lysosomes of eukaryotic cells causing lipid/cholesterol alterations [190]. Daptomycin caused myopathies, manifested as muscle pain or weakness and is associated with elevations in creatine phosphokinase but the toxicity was ameliorated by appropriate dosing [191]. The antifungal azoles (small lipophilic compounds) specifically target the P450 demethylase involved in ergosterol synthesis. Approved antifungal lipopeptides such as caspofungin, micafungin, anidulafungin target the fungal membrane bound glucan synthase complex. A detailed study of a related preclinical lipopetamine [192] revealed that uptake was passive and unsaturable, and that 95% of the protoplast associated drug localized in fungal microsomes.

One also should be aware of the unique kinetics of interaction of hydrophobic compounds with cells and bio-membranes. Once a compound gains access to the membrane bilayer it will situate in the membrane bilayer based on physiochemical properties and diffuse laterally within the 2-dimensional membrane environment, as opposed to 3-dimensions in the cytosol, and thus the kinetics of inhibition will be unique. A decidedly hydrophobic drug (e.g. logP of 4.0) should effectively partition into the membrane by four orders of magnitude. If one were to determine cellular concentrations based on net uptake, one would need to correct these data because the bacterial membrane only represents a small portion of the total cellular volume (about 3–5%) requiring a 20–30-fold correction. Interactions with membrane proteins and lipids will likely be complex and appropriate tools should be used to characterize the interaction and specificity. While antibacterial peptides can show a preference for bacterial over mammalian membrane, this is not likely true for the types of small molecule reported herein, and thus toxicity will need to be addressed in a more non-traditional format (possibly by testing panels of membrane proteins), addressing possible perturbation of host membranes and thus function.

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