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The Search For Novel Inhibitors Of The Mycobacterial Enoyl Reductase InhA

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The Search For Novel Inhibitors Of The Mycobacterial Enoyl Reductase InhA Through Structure Based Drug Design

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Abstract

Isoniazid (INH), one of two first-line drugs used to treat tuberculosis (TB), has been shown to be a potent inhibitor of InhA, the mycobacterial enoyl reductase. However, the increasing resistance to INH makes it imperative to find alternative drugs that are as effective as the first-line drugs, yet active against INH-resistant strains. Since InhA has been validated as an excellent target of TB, there have been attempts to find novel inhibitors of InhA. Through rational drug design, a variety of high affinity InhA inhibitors were synthesized. Triclosan itself was observed to be a suboptimal inhibitor of InhA with a K of .22 μM, but with modifications to the 5-position of the A ring, the new diphenyl ether compounds demonstrated higher affinities with nanomolar constants. A particularly potent triclosan derivative, compound PT70, showed slow, tight-binding inhibition with a K value of 22 pM. In addition, arylamides showed moderate to high inhibition of InhA. They did not show sufficient anti-tubercular activity, and therefore future modifications are needed before they can be considered seriously for use against TB. Lastly, pyridomycin, a natural anti-tubercular drug was rediscovered and confirmed to have high activity against TB. It was found to be the first compound ever published to bind to both binding sites of InhA simultaneously. Current research is ongoing in the synthesis and testing of pyridomycin analogs to further increase their anti-tubercular properties.

Introduction

Drug resistance in Mycobacterium tuberculosis (TB) has become a severe global health threat. The fight against TB faces major challenges due to the appearance of Multi-Drug Resistant Tuberculosis (MDR-TB) and more recently, the virtually untreatable Extensively Drug Resistant Tuberculosis (XDR-TB). MDR-TB are strains that are resistant to both top first-line drugs, Isoniazid and Rifampin, while XDR-TB are MDR-TB strains that are also resistant to any fluoroquinolone and one or more of 3 injectable drugs. With this new resistance there emerges a need to find new drugs that are as effective yet bypass the problem of resistance. One method of research is to find new vulnerabilities of M. tuberculosis to use as new target sites of drugs. This method is highly expensive and requires intense and lengthy research just to implicate a new target site (Scheffler et al., 2013). An alternative is to develop new drugs that work on the same known targets as the first-line drugs but by different mechanisms thereby bypassing the resistance of TB to the drug.

Isoniazid (INH) is a powerful anti-TB drug that works by inhibiting the action of InhA, a crucial enzyme in the process of manufacturing the cell wall. Instead of searching for new targets on TB, there is a goal to expand on this target and develop drugs that inhibit InhA by different mechanisms and thus show anti-tubercular activity against MDR-TB strains.

The virulence of tuberculosis is due to its greatly complex cell envelope. Mycobacteria contain cell walls with unusually high lipid content due mainly to the presence of mycolic acids which are alpha-alkyl, beta-hydroxy fatty acids with atypically long alpha alkyl chains (up to C). The existence of mycolic acids in the cell wall makes the cell impervious to common antibiotics (Niederweis et al., 2010). Mycolic acids were shown to be essential for the survival of tuberculosis thereby making them a great target for drug therapy. InhA (NADH-dependent-2-trans-enoyl-ACP-reductase)'s role in mycolic acid biosynthesis is catalyzing the final step in a cycle of elongation by reducing 2-trans-enoyl chains to their saturated forms (Takayama et al., 2005).

Isoniazid (INH) has been used to treat tuberculosis since 1952. It is a prodrug that enters cells via passive diffusion where is it then converted from an inactive nontoxic form to its active toxic form. This conversion is mediated by KatG, a mycobacterial multi-functional catalase-peroxidase that activates INH by peroxidation to form a variety of intracellular INH-derived radical species. The key intermediate in this reaction is the isonicotinoyl radical (Figure 1, compound 3) which then covalently binds to coenzyme NAD+ to generate an isonicotinoyl-NAD adduct (INH-NAD) (Timmins, Deretic, 2006). The addition of the isonicotinoyl radical to NAD+ creates a stereocenter in the INH-NAD adduct, and it is the acyclic 4S isomer that selectively binds to InhA as a slow tight-binding competitive inhibitor (K = 0.75 nM) (Rozwarski et al., 1998; Rawat et al., 2003). The binding of the INH-NAD adduct inhibits InhA from taking part in mycolic acid biosynthesis leading to impaired cell wall integrity and eventual cell death.

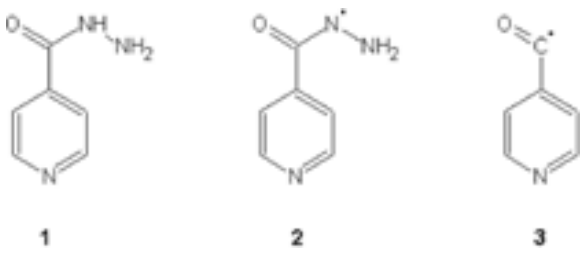


Figure 1
Structures of isoniazid (1), the isonicotinic hydrazyl radical (2) and the isonicotinoyl radical (3) Timmins, Deretic, 2006

Despite its powerful anti-TB potential, the foremost problem with INH is the increasing resistance to the drug. In fact, INH resistance is the most common type of drug-resistant TB. The predominant mechanism of such resistance involves a single point mutation in the gene that codes for the KatG enzyme which results in an amino acid change from serine to asparagine at position 315 in the heme binding catalytic domain of KatG (Zhang, Yew, 2009). This S315T mutant is observed to contain a narrower heme access channel on KatG (4.7 Å compared to the wild type 6 Å) suggesting that the loss of INH access to the oxidizing site of KatG might be the key to INH-resistance (Timmins, Deretic, 2006). While isolates with this mutation completely lose the ability to form the INH-NAD adducts, most retain good catalase activity and are therefore able to survive despite the mutation (Gumbo, 2011). Less commonly, resistance is also seen to occur by mutations in the promoter region of the InhA gene, leading to overexpression of InhA; or by mutations at the active site of InhA, resulting in lower InhA affinity to the INH-NAD adduct (Zhang, Yew, 2009).

There are many methods of drug development. Older methods rely on trial and error testing of chemical substances on cells or whole organisms. This method is extremely lengthy and expensive as it requires the screening of hundreds of thousands of compounds for activity. A newer method called rational drug design employs prior detailed knowledge of a vulnerable target and its three-dimensional structure to synthesize biologically active compounds that specifically interact with the target. One manner of rational drug design is structure-based drug design, which employs the principle of SAR (Structure-Activity Relationship) that theorizes that similarly structured molecules have similar activities. Thus, if a compound is known to possess a desired activity, the essential functional groups and moieties responsible for this activity can be identified and a variety of new compounds containing those groups can be synthesized. The new compounds will then be tested for efficacy and functional similarity to the parent compound. SAR commonly employs the use of molecular mechanics/dynamics software to analyze and predict the conformations of known molecules and to subsequently model the conformational changes that occur in a bound target molecule. This information is then used to predict how similar synthesized compounds may interact with the target. Additionally, the 3D structure of a target molecule both alone and in complex with the synthesized compounds is frequently obtained through X-ray crystallography and NMR spectroscopy to determine the underlying basis for the efficacy (or lack thereof) of the newly synthesized compounds.

In the following literature review, a variety of SAR studies that attempted to find novel InhA inhibitors are summarized. The majority of the compounds discussed used a backbone of the biocide triclosan to create triclosan derivatives and substituted diphenyl ethers that exhibit InhA inhibition as well as efficacy against a variety of tuberculosis strains. Additionally, arylamide moieties have been confirmed as good InhA inhibitors although additional research is still needed to improve their anti-tubercular activity. Lastly, pyridomycin, a natural anti-Mycobacterial drug was recently rediscovered and shown to be effective against multiple strains of TB, as well as showing inhibition of InhA in a unique manner.

Methods

Literature searches were performed using the Touro College Online Library. In particular, the Health Sciences related databases (Medline, PubMed, Proquest Medical Library (Health and Medical Complete), and EBSCO Multisearch were employed. Additionally, Google Scholar and the Touro Quicksearch option proved to be very helpful in finding the necessary relevant articles. The following keywords were searched: Mycobacteria, tuberculosis, InhA inhibitors, triclosan, triclosan derivatives, high affinity InhA inhibitors, SAR, rational drug design, arylamides, pyridomycin. As a final point, only articles published in scholarly peer-reviewed journals after the year 2005 were included in the search.

Results and Discussion

Triclosan derivatives and diphenyl ethers

The biocide triclosan (Figure 2), originally thought to have non-specific antimicrobial activity, has been shown to target and uncompetitively inhibit enoyl reductases, the family of enzymes that includes InhA (Heath et al., 2001). Consistent with uncompetitive inhibition, triclosan binds to the enoyl reductases in the presence of NAD+. Triclosan is an excellent inhibitor of the E. coli enoyl reductase FabI with a picomolar inhibition constant (K = 7 pM) but is shown to be only a submicromolar inhibitor (K = 0.2 μM) of InhA (Ward et al., 1999). To understand the 30,000 fold difference in triclosan's affinity for FabI versus InhA, crystal structures of FabI in complex with NAD+ and triclosan were compared to structures of InhA in complex with NAD+ and triclosan, so as to compare the ligand bindings and use the information to develop new inhibitors with high affinity for InhA (Sullivan et al., 2006).

The structures of triclosan bound to FabI and to InhA were generally very similar yet an important difference was seen with respect to the ordering of the amino acid loop that covers the substrate

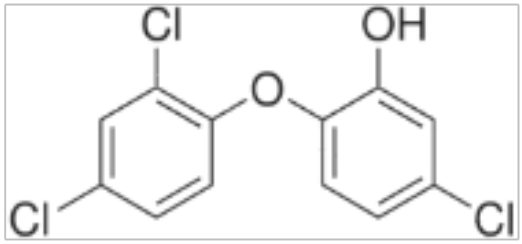


Figure 2:
Molecular structure of triclosan Sullivan et al., 2006

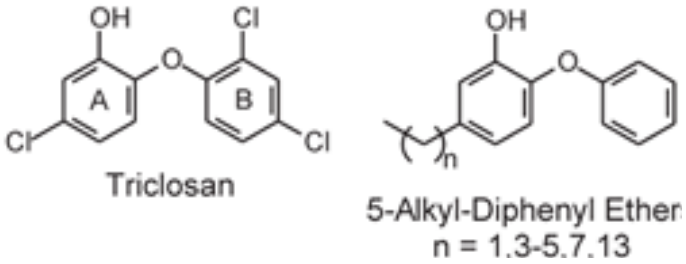


Figure 3
Modified diphenyl ether inhibitors Sullivan et al., 2006

binding site of each enzyme. In the FabI:NAD:triclosan molecule, the substrate binding loop (residues 195-200) is ordered when triclosan is bound to it. In contrast, the substrate binding loop of InhA (residues 197-210) is disordered when triclosan binds to it. It has been suggested that ordering of the loop is indicative of slow, tight binding inhibition (Heath et al., 2001). When the substrate binding loop becomes structured, additional Van der Waals interactions strengthen the binding. A key feature of slow-binding inhibitors is a conformational change in the enzyme upon drug binding. This is likely the slow step in the reaction, a hypothesis that is supported by the fact that triclosan has been identified as a slow, tight binding inhibitor of FabI but not InhA. Since slow-order kinetics has not been determined in the inhibition of InhA by triclosan, the loop is disordered in InhA (Sullivan et al., 2006). Ordering of the loop has been observed in the inhibition of InhA by the INH-NAD adduct, which has also been identified as a tight-binding inhibitor of InhA (Rawat et al., 2003), further supporting the hypothesis that connects slow, tight-binding inhibition to ordering of the active site loop.

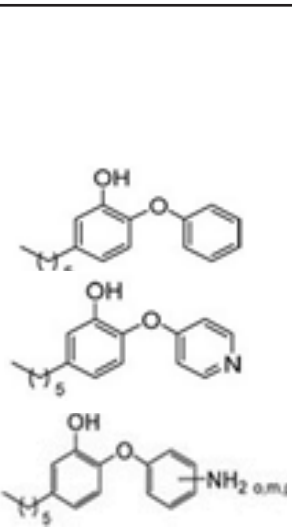
Comparison of FabI and InhA crystal structures showed that InhA contains a larger substrate binding loop than FabI (14 residues vs. 6 residues), making it harder to order the loop in InhA. Sullivan et al. hypothesized that longer substituents on the diphenyl ether A ring of triclosan might result in additional hydrophobic contacts between triclosan and the substrate binding pocket residues, and thereby increase the affinity for InhA. They removed the chlorine atoms from the B ring and substituted the chlorine atom on the A ring with alkyl groups of varying length (Figure 3).

As the length of the alkyl substituents increased from 2 carbons to 8 carbons, the IC value (the concentration of inhibitor needed to inhibit 50% of the enzymes) decreased significantly from 2 μM to 5 nM, indicating considerably higher affinity for InhA. In each case, the compounds were rapid, reversible uncompetitive inhibitors like the parent molecule triclosan. The most potent diphenyl ether, which was identified as 5-octyl-2-phenoxyphenol (8PP), displayed an IC of 5 nM (K =1.1 nM). The octyl chain in this molecule adopted a linear confirmation and burrowed into the pocket, forming numerous hydrophobic interactions with residues in the substrate binding loop which led to the observed significant increase in inhibitory activity. In addition to lower IC values, the alkyl diphenyl ethers showed significantly lower MIC (minimum inhibitory concentration) values than triclosan (MIC 6.5μM vs. 43.1μM) against drug sensitive TB (H37Rv strain) as well as 5 INH resistant strains, thus demonstrating their potential for use against MDR-TB and validating the hypothesis that compounds that do not require activation by KatG will be active against most INH resistant strains of TB. The results of this study revealed that the reason for the differences in triclosan's inhibition of FabI and InhA is that InhA has a large substrate binding loop that triclosan cannot fill as entirely as it can the smaller active site of FabI. Substituting alkyl chains on the triclosan molecule thus allowed for more hydrophobic interactions deeper into the binding site and consequently higher affinity was demonstrated for InhA. Testing of the alkyl diphenyl ethers on H37Rv and 5 INH resistant strains confirmed that this higher affinity also led to greater anti-tubercular activity (Sullivan et al., 2006).

Treatment of TB is lengthy (6-24 months), so toxicity is a significant concern in the development of potential anti-tubercular drugs. The two most potent inhibitors identified by Sullivan et al., 6PP and 8PP, were tested using a Vero cell line and shown to be five times less toxic than triclosan with a therapeutic index of 5-6 compared to <1 for triclosan. Treatment with triclosan at 1xMIC for one day proved lethal to the cells. Furthermore, TB-infected macrophages treated with 2xMIC of 6PP and 8PP demonstrated high level growth inhibition, indicating an ability of these drugs to enter macrophages and remain effective against intracellular tuberculosis growth. Oral administration of 6PP and 8PP in mice also showed no adverse effects, substantiating a lower toxicity for these molecules. These studies demonstrated that alkyl substituted diphenyl ethers exhibited lower toxicity towards cells and TB-infected macrophages than did triclosan (Boyne et al., 2007).

While 6PP and 8PP showed high potency against InhA (nM inhibition constants) and improved efficacy against both drug-sensitive and drug-resistant strains, they were observed to have low bioavailability with significantly high cLogP (partition coefficient) values (6PP= 6.47). The clogP value is important for predicting oral

Table 1

Inhibition and solubility data for modified B ring heterocycles					
Compound	**Structure**	**$IC_{5(}$(nM)**	**MIC (µg/ml)**	**ClogP**	**LogP**
19 (6PP)		11 ± 1	2.1 ± 0.9	6.47	5.76
3c		160 ± 16	3.13	4.97	4.93
14a		62 ± 5	3.13	5.24	
14b		1090 ± 90	100 ± 0	5.24	5.27
14c		55 ± 6	12.50	5.24	4.93

amEnde et al., 2008

absorption as it relates to a compound's solubility and ability to permeate through cell membranes. Compounds that are too hydrophilic (low cLogP) are not able to pass through the hydrophobic membranes, while compounds that are too hydrophobic (high cLogP) tend to be insoluble and have trouble permeating through membranes because they can get stuck in the hydrophobic bilayer. In general, a cLogP greater than 5 predicts poor absorption or permeation (Lipinski et al., 2001).

To lower the cLogP values, modifications were made to the B ring of the diphenyl ether backbone of 6PP. A series of analogs that incorporated different functional groups on the ring, designed to increase the polarity of the alkyl diphenyl ethers, were synthesized. Two series were made: one series replaced the B ring with isosteric heterocycles that incorporated nitrogen atoms into the ring to increase polarity without added steric strain while the other series incorporated polar bulky substituents at different positions on the B ring. The results were primarily stereoselective. Overall, the addition of bulky substituents at either ortho, meta, or para positions of the B ring and the incorporation of most nitrogen heterocycles caused significantly higher MIC values and reduced inhibitory activity. However, the addition of nitro or amino substituents at ortho/para positions only slightly reduced inhibitory activity. While none of the newly synthesized compounds showed IC or MIC values lower than the parent compound 6PP, compounds 3c and 14a (Table 1) had inhibitory values similar to 6PP but also had lower cLogP values (4.97 and 5.24 respectively). The results demonstrate that the addition of nitrogen containing compounds at certain positions on the B ring can lower the hydrophobicity of the compounds. Additional studies are needed to test whether the modifications result in increased bioavailability and improved in vivo antibacterial activity (amEnde et al., 2008).

InhA has two hydrophobic cavities that are capable of being filled: the hydrophobic substrate binding pocket in which the substrate of InhA (2-trans-dodecenoyl-CoA) binds, and the NADH binding

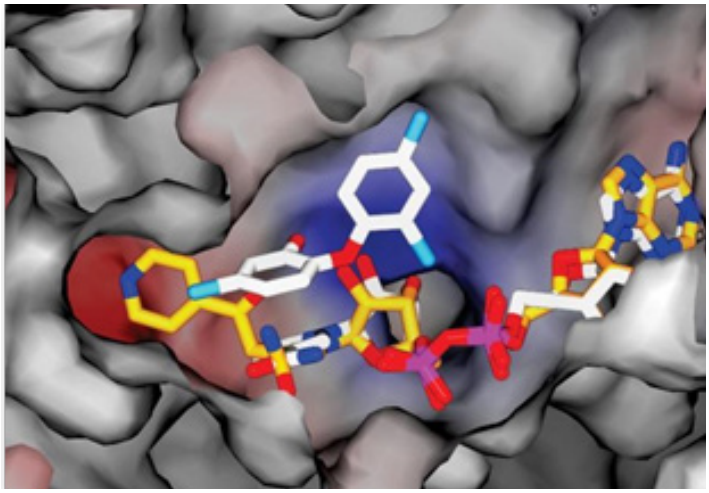


Figure 4:
Cross-section of the InhA active site with superimposed structures of InhA in complex with the INH-NAD adduct (colored in gold) and triclosan (white). The A ring chlorine of triclosan (cyan) is observed to be very close to the isonicotinoyl binding site. Freundlich et al., 2009

active site. The NADH active site sits underneath the substrate binding pocket and is lined primarily with hydrophobic residues. Most InhA inhibitors, including triclosan and its derivatives, inhibit InhA through binding to the substrate binding pocket. The competitive inhibitor INH-NAD adduct, is one of the few inhibitors that binds directly to the NADH active site. Freundlich et al., (2009) observed the structure of InhA:NAD+:triclosan superimposed on InhA:INH:NAD and saw that the chlorine atom at position 5 of the triclosan A ring was only 2 Å from the binding pocket of the isonicotinoyl moiety of the INH-NAD adduct (Figure 4). They hypothesized that triclosan derivatives can be synthesized that can extend into the hydrophobic isonicotinoyl binding pocket and increase inhibition of InhA by occupying both the substrate binding pocket and the NADH binding site. This hypothesis was different than Sullivan et al. (2006) who created analogs to increase interactions with the substrate binding loop, mimicking the structure of the substrate.

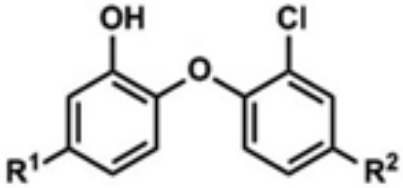
The analogs of Freundlich et al., were similar to those of Sullivan et al., but differed in that a variety of both hydrophobic and hydrophilic substituents were added to position 5 on the A ring. Additionally, contrary to the previous studies of Sullivan et al., it was suggested that the two chlorine atoms on the B ring do in fact participate in hydrophobic interactions with Phe97 and Met103 residues and so they remained in the compounds. The results showed that the compounds with alkyl (hydrophobic) substituents were the more potent inhibitors. As seen with Sullivan et al., inhibition of InhA increased with increasing alkyl chain length. The highest inhibitory potencies for the alkyl chain additions were observed in the 4 carbon chain compounds (Table 2), namely compound 10 (Freundlich et al., 2009).

Surprisingly, while most hydrophobic substituents were observed to show lower IC values, the addition of a phenyl group at the 5 position (Compound 6) showed a much greater IC (IC >10,000 nM) than even triclosan (IC =1,100 nM). The same values were obtained for a pyridyl group at the 5 position. Modeling the inhibitors in the active site showed that the aryl group was too far from the isonicotinoyl binding pocket by 2.5 Å and was also positioned too close to Phe149 which led to steric clashes. Therefore, to better fit the phenyl group into the hydrophobic pocket, a carbon linker chain consisting of 1-3 carbons was added to the 5 position (Compounds 24-26) between the phenyl group and the A ring. This resulted in significantly increased inhibitory potency of the phenyl substituted compounds (IC of 21-51 nM). Compound 25, with a 2 carbon linker chain showed the lowest IC of all the compounds tested with a measured potency 50 times that of triclosan. Adding linkers to the pyridyl substituents (Compunds 17-19) also proved effective in producing powerful inhibitors (IC of 29-75 nM).

Through analysis of crystal structures, it became apparent that the goal of mimicking the isonicotinoyl moiety and reaching the isonicotinoyl binding site was not possible. The triclosan derivatives did reach the isonicotinoyl binding site but they did not occupy the same volume as the INH-NAD adduct. Instead, their actions mimicked the substrate analogs like Sullivan et al.'s compounds did. Attempts to increase the volume of the triclosan analogs in order to mimic the isonicotinoyl moiety would cause a rotation of Phe149, which in turn would displace Tyr158 and result in the loss of a crucial hydrogen bond, leading to the complete loss of inhibitor binding. Therefore, it was concluded that triclosan derivatives can only attempt to place their substituents near the binding pocket and increase hydrophobic interactions in a way that mimics the substrate rather than the isonicotinoyl-NAD adduct (Freundlich et al., 2009).

Six of Freundlich et al.'s compounds (three alkyl and three aryl with IC < 110 nM) were tested for anti-tubercular activity. All of the compounds were more active than triclosan against H37Rv strain (wild type). Additionally, all showed high inhibition against five INH resistant strains demonstrating their potential for use in treatment against MDR-TB. These results revealed that while triclosan analogs cannot be used to mimic the INH-NAD adduct in the isonicotinoyl binding pocket they can act as substrate analogs. The aryl and pyridyl substituents with carbon chain linkers as well as many straight chain substituents showed considerably lower IC values than triclosan, displaying higher affinity for InhA. They also showed greater anti-tubercular activity than triclosan when tested against both drug-sensitive and drug-resistant tuberculosis (Freundlich et al., 2009).

Table 2

In vitro activities of select triclosan derivatives against InhA			
			
Compound	R^1	R^2	InhA IC_5(nM)
Triclosan	Cl	Cl	1100 ± 180
6	Ph	Cl	>10000
10	$(CH_2)_3$ CH_3	Cl	55 ± 20
14	2-pyridyl	CN	>10000
15	3-pyridyl	Cl	>10000
16	4-pyridyl	CN	>10000
17	CH_2 (2-pyridyl)	Cl	29 ± 11
18	CH_2 (3-pyridyl)	Cl	42 ± 10
19	CH_2 (4-pyridyl)	CN	75 ± 16
24	CH_2 Ph	Cl	51 ± 6
25	$(CH_2)_2$ Ph	Cl	21 ± 8
26	(CH_2)3 Ph	Cl	50 ± 14

Feundlich et al., 2009

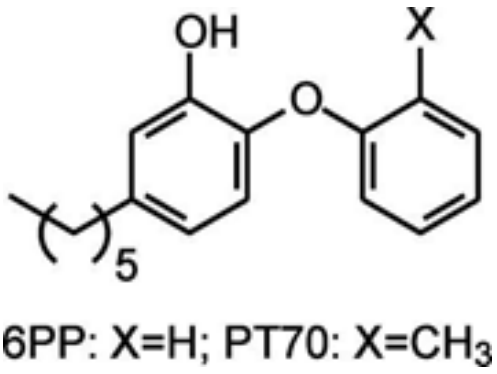


Figure 5
Addition of a methyl group on the ortho position of the B ring of 6PP led to PT70, a slow onset, tight binding diphenyl ether inhibitor. Luckner et al., 2010

One problem with the triclosan derivatives and diphenyl ethers thus far is that they are shown to be rapid reversible inhibitors of InhA. An important and vital factor of in vivo drug activity is long residence times on the targets. Furthermore, structures of the previous compounds had indicated a disordered substrate binding loop. It had earlier been theorized that slow onset inhibition is coupled with ordering of an active site loop, which leads to the closure of the substrate binding pocket. Luckner et al., (2010) attempted to create a slow onset triclosan-derived inhibitor of InhA using the hypothesis that compounds with the ability to order the loop will be slow tight-binding inhibitors of InhA. They used Sullivan et al.'s 6PP analog as a backbone and introduced a variety of groups onto the B ring in the hopes of promoting increased hydrophobic interactions between the inhibitor and the loop residues.

One resulting compound, PT70 (Figure 5), contained a methyl group ortho to the diphenyl ether linkage and showed a substantially higher affinity for InhA, with a K of 22 pM, a value 430-fold lower than that of 6PP (K =9.4 nM). Additionally, PT70 displayed slow-onset inhibition which is essential for in vivo antibacterial activity. Crystal structures of InhA:NAD+:PT70 showed that PT70 binds to InhA in almost the same way that triclosan and its derivatives do. The main difference in the binding is that the methyl group on PT70 generates additional hydrophobic interactions with Ala198, Met199, Ile202 and Val203 residues on the substrate binding loop, leading to a defined loop structure (Figure 6). The

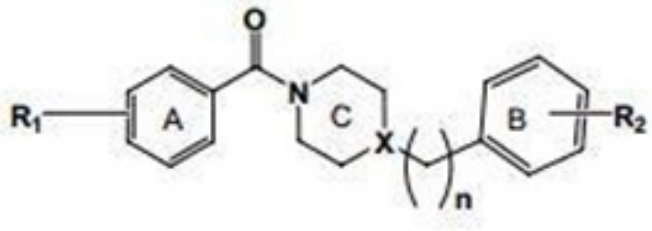


Figure 7
Scaffold of He et al.'s arylamides He et al., 2007

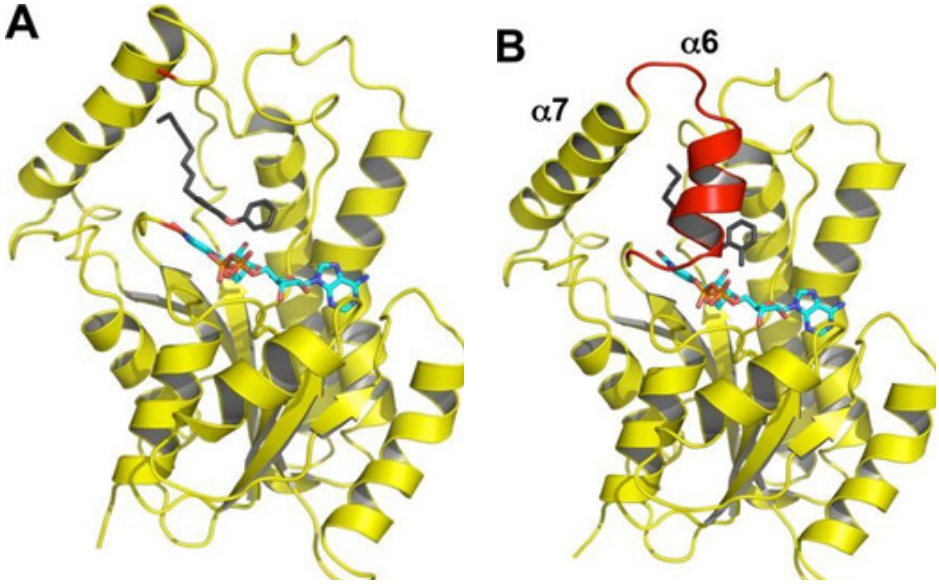


Figure 6
Loop ordering is observed upon slow binding inhibition. A) InhA•NAD+•8PP complex in the binding of 8PP (Sullivan et al.'s most potent inhibitor) to InhA. NAD+ is displayed in cyan and the 8PP molecule is in black. The substrate-binding loop is disordered in the 8PP structure, and the loop ends are depicted in red. B) InhA•NAD+•PT70 complex. The PT70 compound is depicted in black. The substrate-binding loop (shown in red) is ordered in this structure and covers the binding pocket. Luckner et al., 2010

ordered binding loop covers the entrance to the binding pocket locking PT70 inside and increases the residence time on the target. Kinetic data show that PT70 has a residence time of 24 minutes on the target, a 14,000 fold increase over the residence time of its parent compound 6PP (0.1 sec). This study also confirmed the hypothesis that ordering of the active site loop is coupled to slow-onset inhibition. Further research is needed to test PT70 on tuberculosis strains to confirm that the increase in affinity of PT70 for InhA does indeed correlate with enhanced anti-bacterial activity (Luckner et al., 2010).

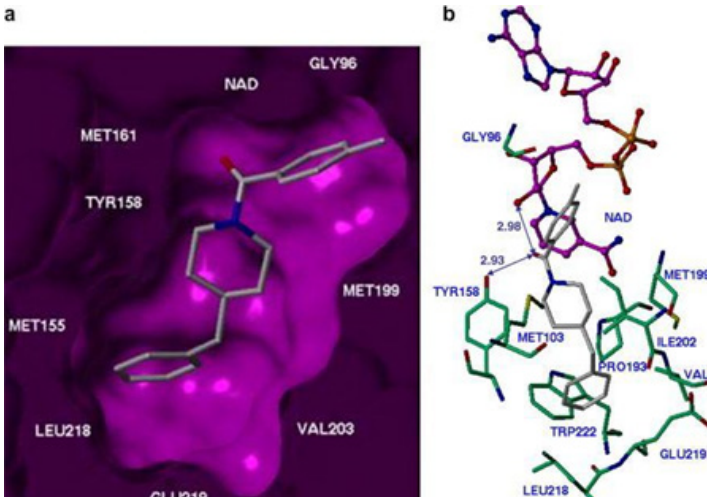


Figure 8

a) Inhibitor b3 bound to the active site of InhA. As illustrated, extra space is observed in the area below the B ring of the inhibitor b) Details of the InhA-b3 interactions. Relevant residues of the binding pocket are shown. The amide oxygen hydrogen-bonds with the 2`-hydroxyl moiety of the nicotinamide ribose and the hydroxyl group of Tyr158 (blue arrows). He et al., 2007

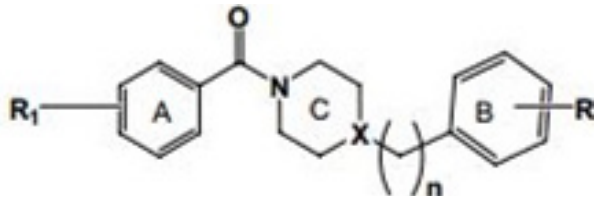
Triclosan has been recognized as a great molecular scaffold for InhA inhibition. The previous studies all employed the diphenyl ether backbone of triclosan. Modifications to both the A and B ring demonstrated increased affinity for InhA which was also coupled to greater anti-tubercular activity. The compounds show tremendous promise for rational drug design and SAR use in the treatment of TB. Significantly, it was confirmed that these compounds do not require KatG activation affording them great potential against the most common strains of drug-resistant TB. Further research is still needed to test the compounds in vivo to confirm their anti-tubercular capacity.

Arylamides

Arylamides (Figure 7) have also been identified as a potential scaffold of InhA inhibitors. Using High Throughput Screening (HTS), He et al., (2007) identified a variety of arylamides all of which contained either a piperazine or piperidine compound as the core structure. Compounds with the piperazine scaffold exhibited the best inhibitory activity among the initial compounds tested, with compound a4 being the most potent inhibitor (IC =3.07 μM) (Table 3). In order to enhance inhibition, He et al. crystallized Compound b3 (IC = 5.16 μM), containing a piperidine with InhA and NADH and that structure was used as a reference for future compounds. The key hydrogen bond seen in all enoyl reductase inhibition reactions, involving the catalytic residue Tyr158 was observed in the structure. The carbonyl oxygen of the arylamide was hydrogen bonded to both the 2'-hydroxyl moiety of the nicotinamide ribose and to the hydroxyl group of Tyr158. The unsubstituted B ring fit into the substrate binding pocket and interacted with many hydrophobic residues there. However, underneath the B ring, extra space was observed (Figure 8). He et al. hypothesized that addition of hydrophobic substituents to the B ring could form more interactions and lead to higher affinity (He et al., 2007).

Table 3

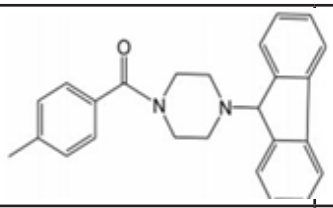
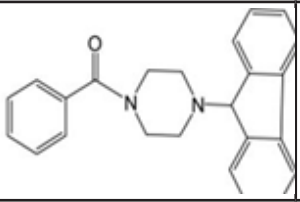
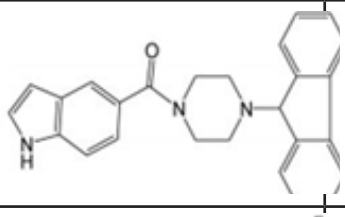
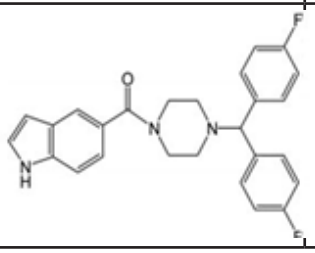
The most potent inhibitors found by He et al. using High Throughput Screening



Compound	*X*	*N*	*R^1*	*R^2*	*IC_{50}(μM)*
a4	*N*	*0*	*4-CH3*	*3-Cl*	*3.07 ± 0.48*
b3	*C*	*1*	*4-CH3*	*H*	*5.16 ± 0.45*

He et al., 2007

Table 4

Inhibition activity of newly synthesized arylamides			
	p1	99	0.4±0.02
	p2	97	0.09±0.00
	p3	94	0.2±0.01
	p4	84	1.04±0.04

He et al., 2007

A variety of arylamides containing the piperazine scaffold were synthesized. 12 of the 60 compounds synthesized showed high InhA inhibitory activity. All 12 contained large polyaromatic moieties on the B ring, substantiating the hypothesis that larger substituents on the B ring form more interactions and lead to higher affinity. Compound p2 (Table 4) of the newly synthesized molecules showed the highest inhibitory activity (IC = 90 nM), a 34 fold decrease in IC when compared to compound a4, the most potent of the HTS compounds (He et al., 2007).

Unfortunately, when the new compounds were tested against the H37Rv strain for anti-tubercular activity, most had MIC values >125 μM (He et al., 2007). This data suggests that the inhibitors do not possess optimal membrane permeability, likely due to their amphiphilicity, caused by the hydrophobicity of the B ring substituents and simultaneous hydrophilicity of the potentially protonated C ring piperazine.

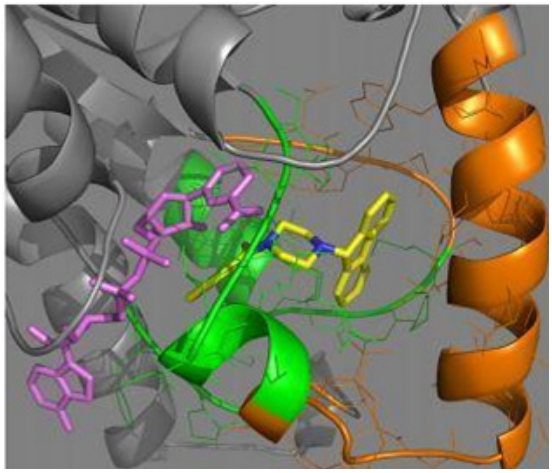


Figure 9

Compound p2, the most potent arylamide inhibitor synthesized by He et al. (yellow) with NADH (purple) in the InhA substrate binding site. The rigid residues around the binding site are displayed in green, while the flexible residues are displayed in orange. As discussed, the A ring of the arylamide is situated near the rigid residues, while the B ring is near the flexible residues. Punkvang et al., 2010

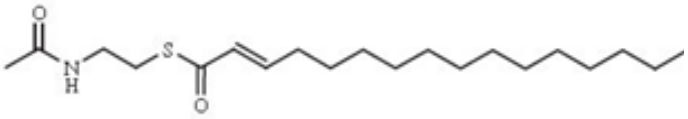


Figure 10a
Structure of the substrate analog, trans-2-hexadecenoyl-(N-acetylcysteamine)-thioester Punkvang et al., 2010

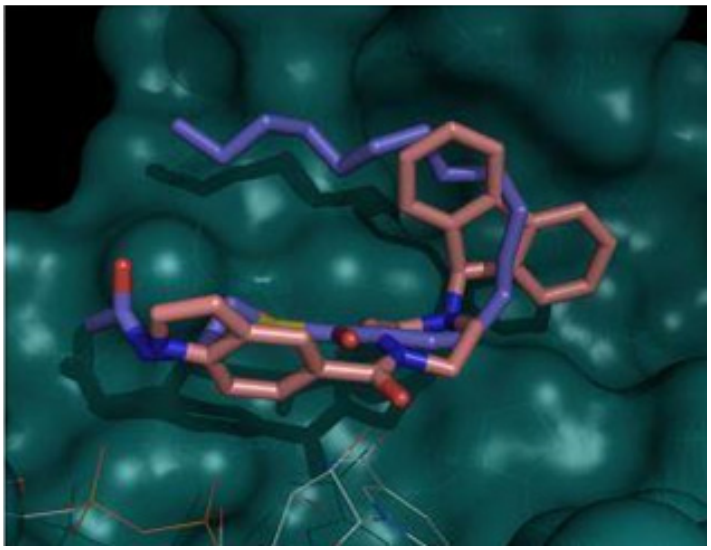


Figure 10b
Superimposition of x-ray structures of compound p3, an arylamide inhibitor synthesized by He et al. (pink) and fatty acid substrate (purple) in the InhA substrate binding site. As discussed, the A ring binds to the hydrophilic part of the binding site where the acetylcysteamine moiety of the substrate binds. The B ring is shown to bind to the hydrophobic part of the binding site where the fatty acid chain of the substrate binds. Punkvang et al., 2010

Punkvang et al. (2010) attempted to identify the reasons behind the suboptimal anti-tubercular activity of the arylamides and to propose modifications to the compounds to increase both their affinity for InhA and anti-tubercular activity. Using molecular dynamics, a computer simulation that mathematically predicts the physical movements of atoms and molecules, they investigated the structural features and dynamic behavior of the InhA-inhibitor interactions providing detailed information about the molecule's flexibility and conformation. RMSF (root mean square fluctuation) of residues around the substrate binding site of InhA was calculated to reveal the mobile flexibility of the residues. Residues 96-99, 155-165, and 192-200 (located around the binding pocket of the aryl A ring of the arylamide) were shown to be too rigid to bind the arylamide inhibitors (Figure 9). In contrast, most of the residues around the aryl B ring binding pocket (100-104, 149-154, 201-223) indicated greater flexibility consistent with He et al.'s proposal that additional large substituents can be added to the B ring to increase the number of hydrophobic interactions (Punkvang et al., 2010).

Crystal structures and subsequent kinetic studies identify arylamides as competitive inhibitors of the substrate binding site of InhA. Consequently, arylamides should mimic the binding of the substrate. Superimposition of arylamide inhibitors bound to InhA and a substrate analog (trans-2-hexadecenoyl-(N-acetylcysteamine)-thioester) (Figure 10a) bound to InhA shows the conformational change that occurs in the enzyme when the arylamide or the substrate bind. It was observed that the A ring of the arylamide binds to the hydrophilic part of the pocket where the hydrophilic acetylcysteamine moiety of the substrate binds. In contrast, the B ring lies in the same site as the fatty acyl chain of the substrate surrounded by flexible hydrophobic residues (Figure 10b). The hydrophilic A ring is favored in the hydrophilic site and the hydrophobic B ring substituent is favored in the area with the hydrophobic residues (Punkvang et al., 2010).

Based on this data, Punkvang et al. proposed a series of modifications to the arylamides to mimic substrate binding and increase inhibitory activity. Introduction of small hydrophilic substituents such as an NH moiety or an acetyl oxygen on the A ring can be used to mimic the analogous part of the substrate and increase the hydrophilicity of the ring. Care must be taken, however, to ensure that the substituents are not too large and do not lead to steric interactions with the inflexible A ring residues and preclude proper binding as discussed. Additionally, binding affinity of arylamides can be increased with the addition of bulky hydrophobic substituents to the B ring (Punkvang et al., 2010).

These results explained the findings of He et al. and led to suggested future modifications of arylamides that may increase their inhibitory potency. Molecular dynamic simulations showed that only the hydrophobic part of the substrate binding pocket is flexible enough to bind arylamide inhibitors. Therefore, the addition

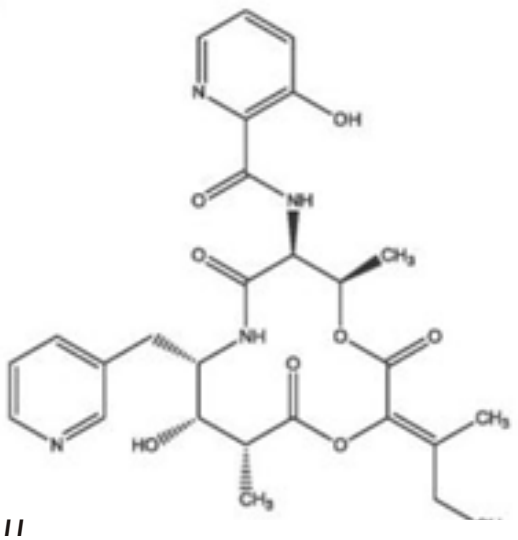


Figure 11
Pyridomycin structure Hartkoorn et al., 2012

of bulky hydrophobic substituents on the B ring can increase the binding affinity for the arylamides and also potentially increase membrane permeability. More research is needed to synthesize these proposed compounds and to measure their InhA inhibitory properties as well as their anti-tubercular properties and to see if membrane permeability indeed increased.

Pyridomycin

Discovered in 1953, pyridomycin (Figure 11) is a natural antibiotic produced by the bacteria Streptomyces pyridomyceticus and Dactylosporangium fulvum that exhibits specific activity against Mycobacteria (Maeda et al., 1953). Nevertheless, since it was only discovered a short time after the introduction of isoniazid as an exceedingly effective treatment for tuberculosis, pyridomycin was neglected and research into the drug ceased. Presently, with the rise of INH-resistant strains of TB, it is imperative to reexamine effective alternatives to INH. Despite earlier reports of pyridomycin's effectiveness against Mycobacteria as a genus, its efficacy against M. tuberculosis specifically had not yet been completely demonstrated. Additionally, pyridomycin's target and mechanism of action had also not yet been elucidated. Hartkoorn et al. (2012) ascertained the drug's target and specificity, and assessed its efficacy against TB.

Pyridomycin was tested to confirm earlier reports about its specificity, efficacy, and low toxicity. The new research showed pyridomycin to be a competent inhibitor of all species of Mycobacteria including tuberculosis (MIC= 0.62-1.25 µg/ml). It remained ineffective against other genera of both gram positive and gram negative classification (MIC > 100 µg/ml), confirming its specificity to a feature unique to Mycobacteria. It effectiveness against TB-infected THP-1 derived macrophages was verified which indicated its ability to physically enter macrophages and inhibit growth. Toxicity testing demonstrated pyridomycin's higher selectivity for tuberculosis than for human cells with a selectivity index of >100-fold. (Hartkoorn et al., 2012)

The identity of the target of pyridomycin was obtained by selecting for mutant pyridomycin-resistant strains (PYR7) on solid medium containing pyridomycin at 10xMIC (3µg/ml). The genome of mutant PYR7 was referenced to H37Rv (wild type strain) and an a443g point mutation in InhA was isolated in PYR7 which resulted in replacement of the aspartic acid at position 148 by a glycine (D148G). Asp148 was previously identified in the binding pocket of InhA thus validating this as pyridomycins's target. Subsequent experiments involving overexpression of InhA and the resulting increase in pyridomycin resistance further confirmed these findings (Hartkoorn et al., 2012).

Since the primary goal of research into pyridomycin was to determine its potential as an alternative to isoniazid, pyridomycin was tested against INH-resistant strains of TB. KatG mutants

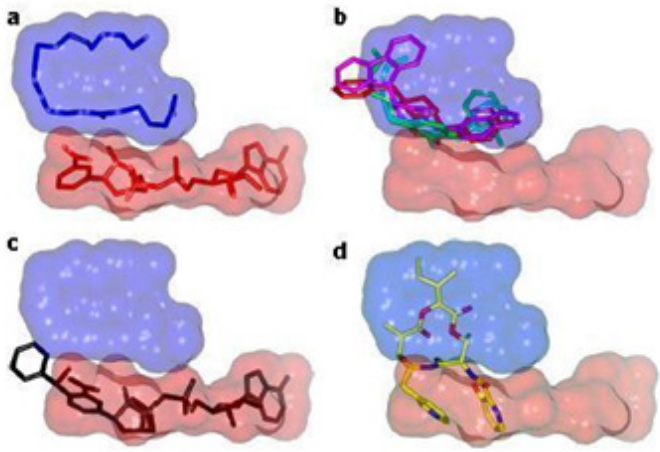


Figure 12

Binding comparison of various InhA inhibitors. The blue area represents the lipid substrate binding pocket, while the red area represents the NADH binding site. a) The lipid substrate is bound to the substrate binding site. b) Triclosan derivatives have all been shown to bind solely to the substrate binding site. c) The INH-NAD adduct binds solely to the NADH binding site. d) Pyridomycin binds to the NADH binding site and, because of its large size, extends into the lipid substrate binding site as well. Hartkoorn et al., 2014

(the majority of the resistant strains), despite being highly resistant to INH (MIC >10 µg/ml), showed no resistance to pyridomycin. This demonstrated that pyridomycin does not depend on catalase-peroxidase bioactivation and therefore holds increased potential for use against the more common KatG INH-resistant strain (Hartkoorn et al., 2012). The results of this research confirmed previous reports of pyridomycin's selectivity for and efficacy against tuberculosis including H37Rv and INH-resistant strains.

As discussed earlier, InhA contains two essential sites: the NADH binding site and the lipid substrate binding pocket. Crystal structures and kinetic experimentation show that pyridomycin is a direct competitive inhibitor of NADH binding in InhA and occupies the NADH binding site. Pyridomycin forms several interactions with residues in the NADH binding site including the crucial hydrogen bond between the side chain of Tyr158 and the C7 carbonyl oxygen of pyridomycin. Loss of this hydrogen bond and consequent lack of the crucial enoyl reductase-inhibitor interaction in D148G mutants (pyridomycin-resistant InhA) resulted in markedly decreased affinity for pyridomycin. Crystal structures show that, as a result of its unusually large size, pyridomycin not only occupies the NADH binding site, but its upper hemisphere also extends into the substrate binding pocket forming a hydrophobic interaction with Phe149. While previously identified InhA inhibitors exclusively inhibited only one of the sites, pyridomycin is the first published compound to bridge the gap between both sites and simultaneously inhibit the lipid substrate binding pocket as well as the NADH binding site (Figure 12) (Hartkoorn et al., 2014).

Pyridomycin is a naturally produced antibiotic and efforts to synthesize it have been hampered by the inability to form the double bond of the enol ester moiety between C1 and C2. The compound was instead synthesized with a saturation in that position (and a replacement of the sec-butyl group with a symmetrical isopropyl group) to identify if the double bond was indeed necessary for anti-tubercular activity. Saturation generated a new stereocenter, producing two stereoisomers: the R isomer (OH141) and the S isomer (OH139) (Figure 13). Both compounds, as well as natural pyridomycin were then tested for anti-tubercular activity. Pyridomycin was the most effective against TB (MIC = 0.39 µg/ml). OH139 had a MIC value 32-fold higher than pyridomycin while OH141 measured only 4-fold higher, indicating that the enol ester moiety is not actually a critical requirement for anti-TB activity (Horlacher et al., 2013).

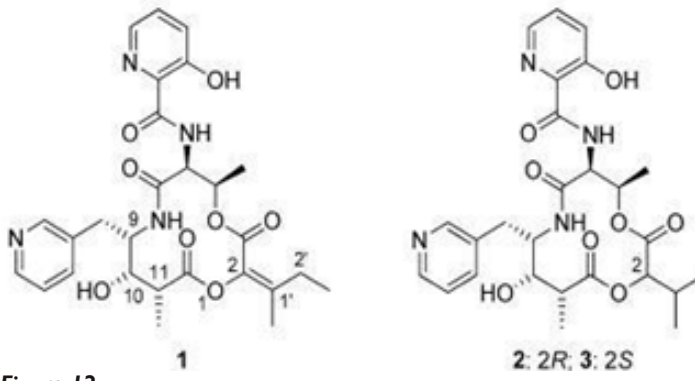


Figure 13

Pyridomycin (1) and the newly synthesized compounds (2,3) with a saturation in the C1-C2 bond and replacement of the sec-butyl group with an isopropyl group. A new stereocenter led to the production of two stereoisomers: the R isomer (OH141) and the S isomer (OH139). Horlacher et al., 2013

X-ray crystallography and in silico modeling highlight the reasoning behind the difference in efficacy between the two stereoisomers. Superposition of InhA:pyr on InhA:OH141 show pyridomycin and OH141 occupying the same position in the active site. The isopropyl group of OH141 was found in the equatorial position avoiding any steric clashes with residues in the active site. OH139 however, was shown with its isopropyl group in a steric interaction with the side chain of Met199 in the substrate binding loop, indicating why OH139 has a lower affinity for InhA than does OH141 (Horlacher et al., 2013). The slight decrease in the activity of OH141 compared to pyridomycin could be due to the lower hydrophobicity of the isopropyl group compared to the 2-butan-2-ylidene substituent. The results of this research demonstrate that pyridomycin can be synthesized with a saturation in the C1-C2 bond without significantly impacting its anti-tubercular activity. This establishes the potential for future SAR work in the development of pyridomycin-derived drug candidates for TB treatment. Current research is now focused on using OH141 as a scaffold for synthesis of new pyridomycin variants.

Conclusion

In this review, SAR has been shown to be an effective means to structurally design and modify drugs to increase their InhA inhibition abilities. The underlying assumption of the research was that compounds that do not require KatG activation would be effective against most INH-resistant strains. This hypothesis proved correct in the case of triclosan and pyridomycin derivatives. Additional modifications to arylamides are needed to optimize their activity. Through analysis of crystal structures, along with computer-assisted molecular dynamics and modeling, a variety of high affinity compounds were synthesized. Current efforts are now concentrated on optimizing the in vivo properties of these novel compounds.

Abbreviations

TB	Tuberculosis
MDR-TB	Multi Drug Resistant Tuberculosis
XDR-TB	Extensively Drug Resistant Tuberculosis
INH	Isoniazid
SAR	Structure Activity Relationship
IC_{50}	Half maximal inhibitory concentration (the concentration of inhibitor at which half of the enzymes are inhibited)
K_i	Inhibitory constant
MIC	Minimum Inhibitory Concentration
H37Rv	Wild type strain of TB
cLogP	Calculated partition coefficient

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