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Novel Inhibitors of an Emerging Target in *Mycobacterium tuberculosis*; Substituted Thiazolidinones as Inhibitors of dTDP-rhamnose Synthesis

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Abstract—The emergence of multi-drug resistant tuberculosis, coupled with the increasing overlap of the AIDS and tuberculosis pandemics has brought tuberculosis to the forefront as a major worldwide health concern. In an attempt to find new inhibitors of the enzymes in the essential rhamnose biosynthetic pathway, a virtual library of 2,3,5 trisubstituted-4-thiazolidinones was created. These compounds were then docked into the active site cavity of 6′hydroxyl; dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase (RmlC) from *Mycobacterium tuberculosis*. The resulting docked conformations were consensus scored and the top 5% were slated for synthesis. Thus far, 94 compounds have been successfully synthesized and initially tested. Of those, 30 (32%) have $\geq$50% inhibitory activity (at 20 μM) in the coupled rhamnose synthetic assay with seven of the 30 also having modest activity against whole-cell *M. tuberculosis*.

The global burden of tuberculosis is immense.[1,2] In 1997 there were an estimated 7.96 million new and 16.2 million existing cases with the worldwide mortality rate at 23%. A major concern is the rise of multi-drug resistant tuberculosis (MDRTB); fatality rates are much higher with MDRTB and they are up to 100-fold more expensive to treat.[1,3] There is an urgent need to develop new, potent, fast-acting, anti-tuberculosis drugs with low toxicity profiles. One validated target for anti-mycobacterials is the cell wall, as many of the current drugs used to treat TB target the cell wall.[4–6] The mycobacterial cell wall is unique in that it contains an mycolylarabinogalactan layer that is tethered to the peptidoglycan layer via a rhamnose–GlcNAc sugar linker.[7] Recently, the synthesis of rhamnose has been shown to be essential for mycobacterial cell growth, validating the role of the rhamnose synthetic enzymes as potential drug targets.[8] Since rhamnose is not found in humans, enzymes involved in its biosynthesis may prove to be a very fertile ground for the development of anti-tuberculosis drugs.

L-Rhamnose is incorporated into bacterial polysaccharides from a common precursor, dTDP-L-rhamnose. This precursor is synthesized from glucose-1-phosphate and dTTP (deoxythymidine triphosphate) via a pathway (Fig. 1) that consists of four distinct enzymes, whose three dimensional structures have all been determined.[9] Briefly, glucose-1-phosphate thymidyl transferase (RmlA), couples the glucose-1-phosphate moiety to deoxythymidine triphosphate. dTDP-D-glucose 4,6-dehydratase (RmlB), then oxidizes the 4′hydroxyl and dehydrates the 6′hydroxyl. dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase (RmlC), inverts the 3′ and 5′ hydroxyls, creating an unstable ring structure which flips. Finally, dTDP-6-deoxy-D-xylo-4-hexulose reductase (RmlD) reduces the 4′ketone to form the end product, dTDP-rhamnose. We currently believe that RmlC is the best drug target in the cascade, as it is highly specific, structurally unique and does not require cofactor binding. This eliminates hits that may potentially bind to non-specific co-factor binding sites.

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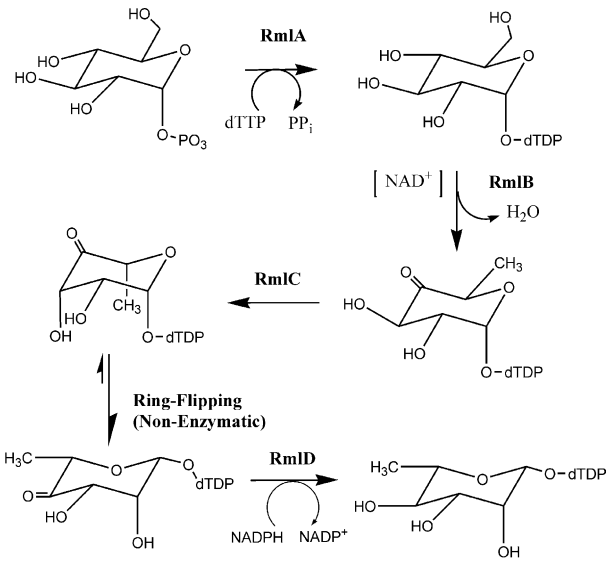

Figure 1. dTDP rhamnose biosynthetic pathway in *M. tuberculosis*. See text for details.

To develop inhibitors of these enzymes, we are using a structure guided library approach: A scaffold is selected and a large virtual library is generated based on commercially available starting materials; the library is then filtered in silico by docking experiments to provide a manageable and prioritized list of compounds for synthesis. The scaffold we have chosen is derived from a recent study of inhibitors for an analogous sugar nucleotide-utilizing enzyme MurB.[10] In this study, a series of substituted 4-thiazolidinones were developed as diphosphate surrogates and inhibitors of MurB that bind at the nucleotide sugar site. Therefore, it was our hope that we could use the 4-thiazolidinone scaffold to mimic the diphosphate and generate specificity through the different R-groups placed around the ring (Fig. 2).

A virtual library of 3888 compounds was created by CombiLibmaker®[11] in the reactant based mode using 24 free amino acids, 27 aldehydes and two thioacids (Fig. 3). The library included all of the stereoisomers at the aldehyde position and the thioacid position. 3D coordinates

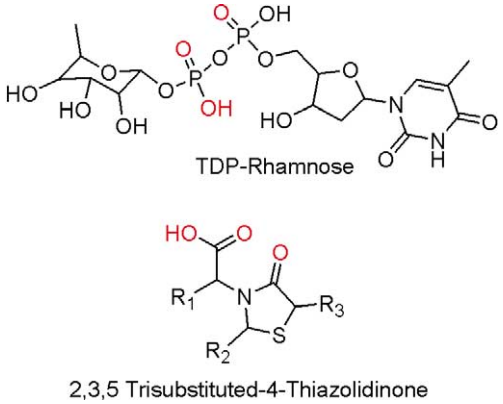

Figure 2. The thiazolidinone scaffold. The scaffold shown in this figure was used to build a virtual combinatorial library. The atoms highlighted in red show how this scaffold functions as a diphosphate mimietic.

were generated for the product database using Concord® stand-alone module[11] with default settings. The acid groups were retyped as O.co2 atoms to reflect their charged deprotonated state. All molecules were then minimized using the TRIPOS forcefield[11] to a max energy change of 0.5 kcal/mol. Using a crystal structure of the *Mycobacterium tuberculosis* RmlC with the bound substrate analogue, TDP-Rhamnose,[12,13] to define the active-site cavity, the virtual library was docked into the active site using FlexX.[14,15] The active site was defined as all momomers with at least one atom 6.5 Å around the bound TDP-rhamnose. A total of 3519 compounds were successfully docked. Consensus scoring was conducted using the Cscore module associated with the Sybyl package.[11] A CScore extraction was performed using the following criteria. The best docked orientation of each individual molecule was chosen with a multifunction criterion using all five scoring functions, with G score and D score given 2× the weight of the other scores. The Overall consensus was generated using only F score, PMF score and Chem score. The top 5% by overall consensus (144 compounds) were extracted and slated for synthesis.

The synthesis of the thiazolidinone library was performed in parallel on a Radley's Carousel Synthesizer by the three-component condensation of amino acid esters, carboxy acid thiols and aryl aldehydes, according to the procedure of Homes et al. (Fig. 4).[16] The chemistry was

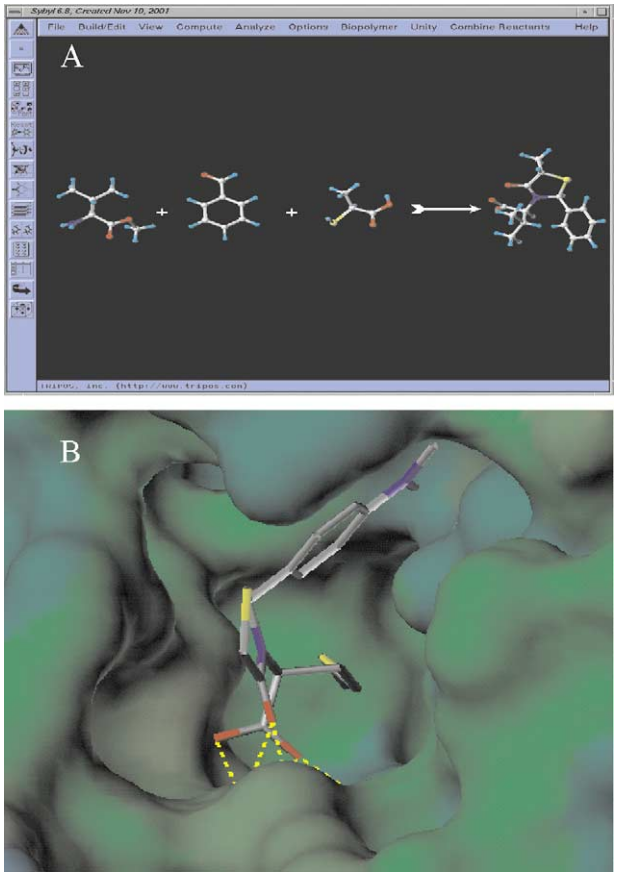

Figure 3. Virtual screening of in silico library; (a) schematic of first reaction product in the library made with Combilibmaker; (b) docked solution of compound **8** in the active site cavity of RmlC.

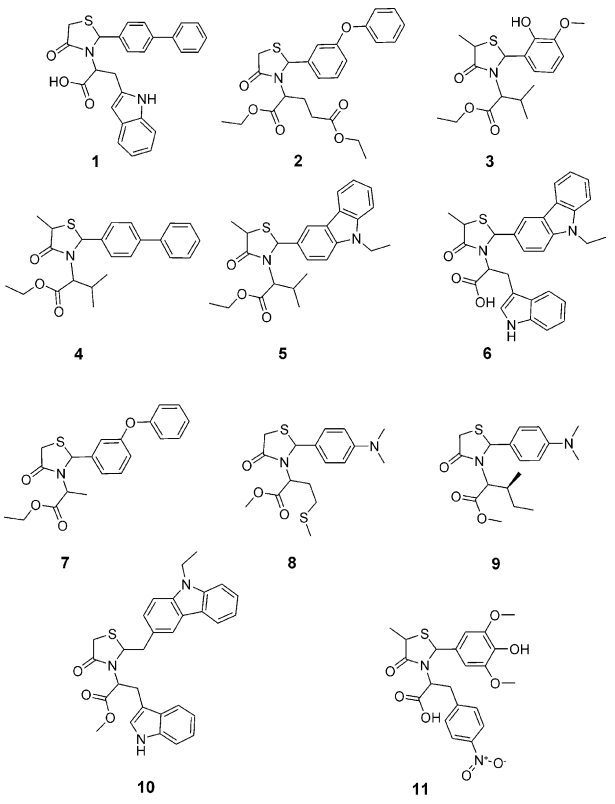

Figure 4. Structures of active compounds.

optimized to a smaller scale on the Carousel using activated molecular sieves to scavenge the water byproduct. On a 2-mmol scale, the building blocks were added in a 1:2:2:1.5 ratio (amino acid esters HCl/carboxy acid thiols/aryl aldehydes/diisopropyl ethylamine) to toluene (10 mL). The tube, with reaction mixture, stirrer and freshly activated molecular sieves was then heated to 80 °C overnight. Reaction mixtures were allowed to cool and taken directly for flash silica gel chromatography using a petroleum ether/ethyl acetate solvent gradient. Product fractions were identified by thin layer chromatography and confirmed by ^{1}H NMR and LC–MS analysis.

Of the 144 compounds slated for synthesis, 47 have been successfully synthesized in both the esterified and free acid forms and also tested. For initial testing, a 'coupled' assay was used where dTDP-Glc (100 μM) and NADPH (200 μM) are converted to dTDP-Rha and NADP using RmlB, RmlC, and RmlD.[8] The enzymes concentrations are balanced so that inhibition of any of them will inhibit the overall reaction. 30 of 94 (32%) have ≥50% inhibitory activity (at 20 μM) in this assay.[17]

Seven of the 30 also have modest MIC values against whole cell *M. tuberculosis* of ≤50 μg/mL (Table 1). Unfortunately, the most potent Rml inhibitors showed no activity versus whole cells. The reason for this is unclear but may be due to poor *M. tuberculosis* cell permeability or inactivation by the cellular enzymes.

Table 1. Bioassay of selected compounds

Compd	% Inhibition (20 μM)	MIC versus *M. tuberculosis* H37Ra (μg/mL)
1	66.5	50
2	64.0	50
3	60.0	25
4	52.5	50
5	52	25
6	70.5	50
7	51	50
8	100	>200
9	110	>200

Shown below is the FlexX docking solution for the binding of the most potent RmlC inhibitor **8** (Fig. 5). The binding mode is similar to the one predicted based on the MurB inhibitors.[10] The substrate diphosphate moiety is being mimicked by the acid or ester and the carbonyl of the thiazolidinone ring.

This scaffold may serve as a general diphosphate mimetic. Thus, it was not surprising to discover that many of the compounds showed activity versus both RmlC and RmlD when tested using the same Rml assay[8] with the enzyme concentrations adjusted so that RmlC or RmlD is limiting. However, we have discovered compounds (Table 2) that do show specificity for one enzyme over the other. This demonstrates that specificity is possible, even among enzymes in the same pathway with similarly structured substrates.

Interestingly, most of the active compounds, with the exception of compound **2**, have a lipophilic group at the R_1 position that points towards the carbohydrate binding site. At first site, this seems counter-intuitive for a sugar binding site. However, an examination of the RmlC active site shows that there is a lipophilic pocket directly under the sugar binding site resulting from its location at the center of a lipophilic β-barrel.

Thus far, 30 compounds with ≥50% inhibitory activity (at 20 μM) of the rhamnose biosynthetic pathway have been pulled out of the virtual library. Of the active compounds, seven have modest inhibitory action against *M. tuberculosis* in vitro. Unfortunately, the best

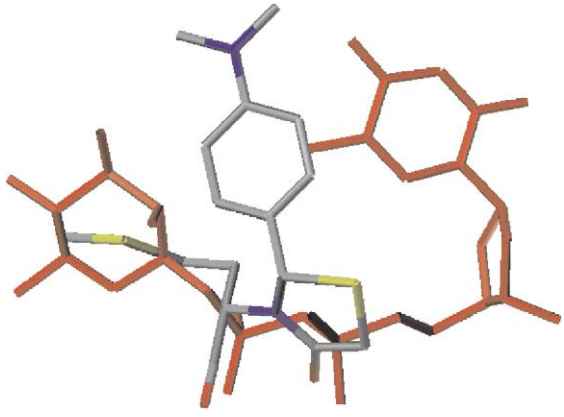

Figure 5. Overlay of compound **8** docking solution and the crystal structure of dTDP-rhamnose in the active site of RmlC.

Table 2. Activity of compounds **10** and **11** against individual enzymes

Compd	Activity versus RmlBCD inhibition at 20 μM (%)	Activity versus RmlC inhibition at 20 μM (%)	Activity versus RmlD inhibition at 20 μM (%)	MIC versus *M. tuberculosis* H37Ra (μg/mL)
10	45	48	Not active	>200
11	42	Not active	71	25

performing compounds in the enzymatic assay, and the rmlC specific compound **10**, had relatively little effect on bacterial growth. This result is most probably due to poor penetration, or bacterial metabolism of this class of inhibitors.

This work supports a previously proposed hypothesis that the thiazolidinone scaffold can act as a diphosphate mimietic, but importantly, we have also shown that it is possible to design in specificity for this class of inhibitor against enzymes in the same pathway. This study also demonstrates the successful use of structural and in silico techniques to guide and prioritize combinatorial library design. Interesting hit compounds have been discovered and further studies, including attempts at obtaining a co-crystal structure, further biochemical characterization of the hits, SAR studies, as well as the design of new libraries based on the initial hits, are currently underway.

Acknowledgements

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