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Lead selection and characterization of antitubercular compounds using the Nested Chemical Library

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SUMMARY

Discovering new drugs to treat tuberculosis more efficiently and to overcome multidrug resistance is a world health priority. To find novel antitubercular agents several approaches have been used in various institutions worldwide, including target-based approaches against several validated mycobacterial enzymes and phenotypic screens. We screened more than 17,000 compounds from Vichem's Nested Chemical Library™ using an integrated strategy involving whole cell-based assays with *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*, and target-based assays with protein kinases PknA, PknB and PknG as well as other targets such as PimA and bacterial topoisomerases simultaneously. With the help of the target-based approach we have found very potent hits inhibiting the selected target enzymes, but good minimal inhibitory concentrations (MIC) against *M. tuberculosis* were not achieved. Focussing on the whole cell-based approach several potent hits were found which displayed minimal inhibitory concentrations (MIC) against *M. tuberculosis* below 10 μM and were non-mutagenic, non-cytotoxic and the targets of some of the hits were also identified. The most active hits represented various scaffolds. Medicinal chemistry-based lead optimization was performed applying various strategies and, as a consequence, a series of novel potent compounds were synthesized. These efforts resulted in some effective potential antitubercular lead compounds which were confirmed in phenotypic assays.

1. Introduction

Tuberculosis (TB) is a very common and very dangerous disease. One third of the world's population is infected with TB, and based on statistics, around 15 million persons will die of TB over the next decade, although mortality rates appear to be falling in most countries. There were an estimated 8.7 million new cases of TB in 2011 and an estimated 1.4 million deaths, making this disease one of the world's biggest infectious killers [1]. About five hundred thousand people die annually due to HIV-TB co-infection1. Tuberculosis caused by *Mycobacterium tuberculosis (Mtb)* is considered to be one of the oldest infectious diseases affecting mankind.

The currently applied TB drugs are almost 50 years old and the length of the drug treatment varies from six to nine months in the case of drug-sensitive disease, while in the case of drug-resistant disease the treatment could last up to two years. Improper administration of the drugs can result in drug resistance, treatment failure or even death. Resistance is the consequence of the fact that *Mtb* has a great success by surviving within macrophages and thereby causing persistent infection [2–4].

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The highly expansive multidrug-resistant (MDR-TB) and the extensively drug-resistant (XDR-TB) tuberculosis strains - XDR-TB involves resistance to isoniazid and rifampicin, such as MDR-TB, as well as to any of the fluoroquinolones (such as ofloxacin or moxifloxacin) and to at least one of three injectable second-line drugs (amikacin, capreomycin or kanamycin) - and the TB–HIV co-infection, necessitate drugs with different modes of action. Several years ago the basic approach of researchers so-called target-based drug discovery was to identify specific enzymes essential for the replication and propagation of the bacteria, screen for inhibitors against pharmacologically validated target enzymes and identify new chemical entities that could serve as novel TB drug candidates. In the late 1990s a development in microbiology enabled scientists to identify and validate new classical and innovative targets in the TB genome. During the past two decades several chemical libraries were screened for specific enzyme targets. It is important to note that target-based drug discovery offers medicinal chemistry the possibility to improve the activity, selectivity and minimize the toxicity and side-effects of a drug-like compound. In spite of the results in target-based approach another strategy, the whole cell-based [5] approach seems to be more efficient in the field of TB drug discovery.

There are ongoing efforts in several research institutions with the aim of more efficient TB drug development. The More Medicines for Tuberculosis (MM4TB) [6] consortium evolved from the highly successful FP6 project, New Medicines for Tuberculosis (NM4TB) [7], which delivered a candidate drug for clinical development. MM4TB continues to aim to discover and develop new drugs for TB treatment.

Here we would like to summarize the work that has been performed and the results we have got until now under the framework of the NM4TB and MM4TB projects including both the target-based and the whole cell-based strategies using the Nested Chemical Library™ of Vichem Research Ltd. in this field.

First we turned our attention to kinases present in *Mtb* as kinases have been considered to be attractive targets in drug research for cancer therapy where they play important roles in cell signalling pathways. Now several kinase inhibitors are on the market as successful drugs. The *Mtb* genome encodes 11 eukaryotic-like Ser/Thr kinases (PknA, PknB, PknC, PknD, PknE, PknF, PknG, PknH, PknJ, PknK, PknL) [8,9] and three of them (PknA, PknB, PknG) have an essential role in bacterial growth/survival [10]. PknA proved to be responsible for morphological changes occurring during cell division. PknB was reported as an essential enzyme for the bacteria *via* regulation of cell growth and cell division [11]. PknG is responsible for the survival of the bacteria within the macrophage of the host by preventing the fusion of phagosome and lysosome [12,13].

Beside kinases we were also interested in other targets such as phosphatidyl-*myo*-inositol mannosyltransferase (PimA) and DNA topoisomerases (topoisomerase I, DNA gyrase). PimA is involved in the biosynthesis of phosphatidyl-*myo*-inositol-mannosides (PIM) and their highly glycosylated counterparts lipomannans (LM) and lipoarabinomannans (LAM). The enzyme transfers mannose from the GDP-mannose donor to position 2C of the inositol moiety in phosphatidyl-*myo*-inositol forming phosphatidyl inositol monomannoside (PIM_1). This molecule serves as the basis for the build-up of PIM, LM and LAM which have been shown to play important roles not only in maintaining the cell envelope integrity [14] but also in establishment of host–microbe interactions in the case of pathogenic mycobacteria [15].

DNA topoisomerase enzymes control the topology of the DNA in the cell by passing one strand of the DNA through a break in the opposing strand, or by passing a region of a duplex from the same or a different molecule through a double-stranded gap in the DNA molecule. Topoisomerases that cleave only one strand of the DNA are classified as the type I, and enzymes that cleave both strands to generate staggered double-stranded breaks are classified as the type II subfamily of topoisomerases [16].

Although several promising compounds with different structures have been selected as hit molecules from the biochemical assays performed on the targets of our interest, none of them proved to be effective inhibitors of *Mtb* itself. As a consequence, we decided to change our strategy and screen the NCL library directly on living bacteria and at the same time we made an effort to find an explanation as to why the target-based approach did not result in good antitubercular compounds. We assumed that the main reason lies in the thick, impermeable cell wall of the pathogen and the activity of efflux pumps.

In order to find new drugs to treat both active and latent TB, whole cell-based assays seemed to be a better approach to apply, for which *Mtb* strains modelling replicating and non-replicating bacteria were used. Although phenotypic screening proved to be more straightforward and several hits came into sight, target identification is a more complicated issue. Taking into account that both the target-based and the whole cell-based strategies have their own benefits, an integrated approach could facilitate drug discovery efforts.

2. Materials and methods

2.1. Compounds

All the compounds screened in this study belong to the Nested Chemical Library™ (NCL) of Vichem Chemie Research Ltd., which contains more than 19,000 molecules organized around 110 core structures and more than 600 scaffolds, including most of the published clinically and pre-clinically relevant kinase inhibitors and new, patentable compounds as well [17,18].

2.2. Protein kinase assays

The eukaryotic-like PknA, PknB and PknG radiometric and fluorescence based assays are described in several publications [19–21].

2.3. PimA assay

The effects of the selected compounds on the activity of PimA were tested in a radioactive assay. The reaction mixtures consisted of 0.5 μg of purified recombinant PimA from *Mycobacterium smegmatis*, 5 μg of PI from soybean of 98% purity (Sigma), 0.8 μM GDP-[^{14}C]-mannose (s. a. 262 mCi/mmol, Amersham Biosciences), 0.1% (w/v) CHAPS (Sigma), 3% (v/v) DMSO (Sigma), 50 mM Tris–HCl pH 7.8 in a total volume of 25 μl. The tested compounds were added in DMSO to 30 μM final concentration in the reaction mixture. Reactions were incubated for 30 min at 30 °C, then stopped by adding 150 μl of chloroform:methanol (2:1, v/v) and vigorously vortexed. Centrifugation (1000 × g, 5 min, RT) of the mixture resulted in formation of two phases. The bottom, organic phase, was removed, dried under stream of nitrogen gas and resuspended in 40 μl of chloroform:methanol (2:1, v/v). The activity of PimA was determined as the amount of radioactivity in the organic phase (corresponding to PIM_1) quantified by scintillation counting.

2.4. DNA gyrase supercoiling assays

For the DNA supercoiling assay, in a 30 μl reaction mixture 55 nM of *M. tuberculosis* DNA gyrase was incubated with 0.5 μg of relaxed plasmid pBR322 and 40 μM of the compounds in basic assay buffer 50 mM HEPES, KOH (pH 7.9), 6 mM magnesium