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Discovery of a new Mycobacterium tuberculosis thymidylate synthase X inhibitor with a unique inhibition profile

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ABSTRACT

Tuberculosis (TB), mainly caused by Mycobacterium tuberculosis (Mtb), is an infection that is responsible for roughly 1.5 million deaths per year. The situation is further complicated by the wide-spread resistance to the existing first- and second-line drugs. As a result of this, it is urgent to develop new drugs to combat the resistant bacteria as well as have lower side effects, which can promote adherence to the treatment regimens. Targeting the de novo synthesis of thymidylate (dTMP) is an important pathway to develop drugs for TB. Although Mtb carries genes for two families of thymidylate synthases (TS), ThyA and ThyX, only ThyX is essential for its normal growth. Both enzymes catalyze the conversion of uridylate (dUMP) to dTMP but employ a different catalytic approach and have different structures. Also, ThyA is the only TS found in humans. This is the rationale for identifying selective inhibitors against ThyX. We exploited the NADPH oxidation to $NADP^+$ step, catalyzed by ThyX, to develop a spectrophotometric biochemical assay. Success of the assay was demonstrated by its effectiveness (average $Z' = 0.77$) and identification of selective ThyX inhibitors. The most potent compound is a tight-binding inhibitor with an IC_{50} of 710 nM. Its mechanism of inhibition is analyzed in relation to the latest findings of ThyX mechanism and substrate and cofactor binding order.

1. Introduction

Antibiotics are essential drugs to combat bacterial infections. However, drug resistance leaves more and more bacterial infections untreatable, which increases the mortality due to infectious diseases [1,2]. One striking example is that of Mycobacterium tuberculosis (Mtb): the causative agent of tuberculosis (TB), remains to be a massive burden on public health. Reports by the World Health organization (WHO) give estimates of 9 million new cases and death tolls of about 1.5 million per year, making it one of the deadliest infectious diseases (http://www.who.int/tb/publications/global_report/en/). The standard quadruple therapy (isoniazid, rifampicin, pyrazinamide and ethambutol) is highly effective for drug-susceptible TB [3]. However, emergence of resistance occurs due to incorrect regimens, lack of patient adherence and insufficient drug supplies, especially in developing countries [4]. Patients with multi-drug resistant (MDR) and extensively-drug resistant (XDR) TB will have to endure therapies that are generally less effective, more toxic, more expensive and need to be administered for prolonged durations of at least 18 months, all making treatment success rates very low. Yet, despite these compelling factors, it was only recently that interest in finding more TB drugs revived. So far, 6 new compounds (belonging to 4 chemical classes) were introduced into clinical testing, two of which (bedaquiline and delanamid) have been approved for treatment of resistant TB [5,6]. However, of these compounds, only two inhibit novel targets [7–9]. To be able to combat the rapid emergence of resistance, new chemical entities as well as the functionality and essentiality of new targets needs to be characterized [10,11]. Thus, more work is needed to maintain a continuum in drug discovery to develop a pipeline of new drug candidates, in the different development and clinical assessment stages.

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In this work, we focus on the inhibition of de novo synthesis of dTMP from dUMP. Inhibition is associated with alterations in the dUTP and dTTP balance, within the cell, causing the misincorporation of dUTP into the DNA, followed by futile cycles of excision, repair and reinsertion. This leads to DNA strand breakage and ultimately 'thymine-less cell death' [12]. While in humans this reaction is catalyzed by the 'classic' thymidylate synthase (ThyA), an alternative enzyme (ThyX) was identified in a series of pathogenic organisms, including Mtb [13]. Thymidylate synthases (TS), of both classes, are evolutionarily unrelated and have distinct structures and mechanisms. The exact mechanism of ThyX is still not fully understood, but recent results indicate a unique transfer of the one-carbon unit, from the methylenetetrahydrofolate (mTHF) cofactor to dUMP, via a covalent intermediate formed with the reduced $FADH_2$ [14]. Mtb is one of the exceptional organisms, that carry both thyA and thyX genes, whereas in most organisms both TS families are mutually exclusive. Despite the presence of ThyA, ThyX is an essential enzyme in Mtb [15] and ThyX was associated with resistance to tuberculosis drugs, that target the folate pathway [16]. So far, TS was reserved as a target for development of anti-cancer agents; one example is 5-fluororuracil (5-FU), that has been successfully used in the clinic for decades [17]. Since the discovery of ThyX, the differences in structure and mechanism, between ThyX and ThyA, are paving the way to develop selective ThyX inhibitors, as a new class of antibiotics.

In this work, a high-throughput screening (HTS) campaign was initiated to screen a library of 40,000 compounds for ThyX inhibitors. The compound library is composed of small molecules and the selection was based on (i) chemical diversity, (ii) drug-like properties (Lipinski rule of five compliant), (iii) exclusion of unstable chemical groups and known toxicophores. HTS technology is a rapid and robust method to identify novel chemical scaffolds against target proteins. Large compound sets can be made available by combinatorial chemistry and can be selected to satisfy the properties needed for successful drug development [18]. A radioactive tritium assay is well-established and has been successfully used for identifying other ThyX inhibitors [19]. However, due to safety issues, stability of the reagents, cost and lack of proper containment facilities, the wide-spread use of radiometric screening has been hampered [20,21]. Here, we describe the optimization of an absorbance-based biochemical assay and its adaption into a HTS campaign. The assay monitors the ThyX NADPH oxidase activity in aerobic conditions, which can be followed spectrophotometrically at 340 nm. Optimization of the assay proved to be successful in identifying compounds selectively active against ThyX. One class of compounds, with a 1,4-benzoxazine moiety, was further studied to understand its biochemical mechanism of inhibition.

2. Materials and methods

2.1. Chemicals

NADPH, dUMP and FAD were purchased from Chem Impex (Illinois, USA). Folinic acid (FA), 5-fluoro-deoxyuridine monophosphate (5-F-dUMP), HEPES, $MgCl_2$, BSA, Triton X-100, glycerol and DMSO were purchased from Sigma-Aldrich (Diegem, Belgium). mTHF was obtained from Merck & Cie (Schaffhausen, Switzerland).

2.2. Cloning, protein expression and purification

The preparation of Mtb ThyX and ThyA was done as previously described by Sampathkumar et al. [22] and Kögler et al. [19] respectively. The enzymes were stored at −20 °C in 50% glycerol. The enzyme concentrations were determined spectrophotometrically at 280 nm using NanoDrop 2000 (Thermo Fisher Scientific, Asse, Belgium) to give the mg of protein per ml of solution. The molecular weight of the monomers (28.7 and 30.7 kDa respectively) was used to calculate the molar monomer concentration. The total protein concentration is expressed as [monomer]. For the standard assay, the [monomer] is calculated as 1 μM (26 ng/μl) and 1.8 μM (51.3 ng/μl) for ThyX and ThyA respectively.

2.3. Active Mtb ThyX concentration

It is assumed that only active ThyX tetramers can bind to FAD [23]. The active Mtb ThyX concentration in applied experimental conditions was therefore determined by measuring the amount of FAD bound to Mtb ThyX by two collective methods. In the first step, the absorbance of FAD, bound to purified Mtb ThyX, was measured at 450 nm using the LVis plate with Clariostar plate reader (Isogen Life Science, Utrecht, The Netherlands). The results showed that 0.137 μM FAD are found in 1 μM ThyX (13.7%), indicating that only a small portion of ThyX is isolated bound to FAD. In the second, the FAD fluorescence quenched due to binding to ThyX was measured [24]. Different concentrations of FAD (0–50 μM) were added to total ThyX protein (1 μM). The different samples were excited at 450 nm and the fluorescence emission of FAD is measured at 520 nm. Measurements were taken after 5 min of mixing to allow for equilibration. A plot of relative fluorescence intensity (free FAD/total FAD) showed that 1 μM of purified ThyX is saturated by 0.65 μM FAD (65%) (data not shown). Since it is known that there is 1 FAD molecule per ThyX monomer in each active tetramer, the total active ThyX is 0.79 μM in 1 μM of total protein (13.7% + 65% = 78.7%).

Where mentioned, the ThyX concentration is expressed as the total protein concentration and not the active protein. Active ThyX concentration was only used in inhibition constant (k_i) calculation.

2.4. NADPH oxidation assay for ThyX activity

The ThyX activity is monitored using the NADPH oxidation assay, which measures the conversion of NADPH to $NADP^+$. Unless otherwise indicated, the following assay mixture (total volume = 100 or 200 μl) was used: 5 μM Mtb ThyX, 600 μM NADPH, 10 μM dUMP, 62.5 μM FAD, 30 μM mTHF, 1 mM $MgCl_2$, 50 mM HEPES (pH 7.5), 1 mg/ml BSA, 1% glycerol and 0.1% Triton X-100. Performed at room temperature (RT), the reaction was initiated by addition of NADPH. Absorbance measurements at 340 nm were taken in 96-well plates using the Envision microtiter plate reader (Perkin Elmer, Massachusetts, USA). ThyX activity (initial reaction rate) is calculated as the absorbance decrease over time.

2.5. NADPH oxidation assay for library screening

The above-mentioned assay was adapted to an end-point assay, compatible with a high throughput inhibitor screen. 40,000 compounds from the Centre of Drug Discovery and Design (CD3) were tested at a 20 μM concentration for a potential inhibitor of ThyX. Reagents were used in following concentrations: 1 μM Mtb ThyX, 600 μM NADPH, 3 μM dUMP, 12 μM FAD, 1 mM $MgCl_2$, 50 mM HEPES (pH 7.5), 0.5 mg/ml BSA, 0.5% glycerol and 0.05% Triton X-100. The compounds are dissolved in DMSO (5 mM concentration) and stored at −20 °C in 384-well master plates. Prior to screening, the compounds are thawed at RT and the screening plates are prepared using the Evo 200-based liquid handling robot system (Tecan Group Ltd., Männedorf, Switzerland). Initially, the compounds are diluted into 384-well daughter plates to give a concentration of 100 μM in 2% DMSO. 10 μl of the pre-diluted compounds are then transferred into empty 384 flat-bottom well plates. Each plate contained 32 positive (50 μM FA, 0.4% DMSO) and 32 negative (no compound, 0.4% DMSO) controls. Following plate preparation,