Antimycobacterially active salicylanilide diethyl phosphates were evaluated to identify their potential drug target(s) for the inhibition of several mycobacterial enzymes, including isocitrate lyase, L-alanine dehydrogenase (MtAlaDH), lysine ε-aminotransferase, chorismate mutase, and pantothenate synthetase. The enzymes are related to the nongrowing state of Mycobacterium tuberculosis. Salicylanilide diethyl phosphates represent new candidates with significant inhibitory activity especially against L-alanine dehydrogenase. The most active MtAlaDH inhibitor, 5-chloro-2-[(3-chlorophenyl)carbamoyl]phenyl diethyl phosphate, has an IC₅₀ of 4.96 μM and the best docking results. Other mycobacterial enzymes were mostly inhibited by some derivatives but at higher concentrations; isocitrate lyase showed the highest resistance to salicylanilide diethyl phosphates.

1. Introduction

The increased number of drug-resistant tuberculosis (TB) cases worldwide and the evidence of recently reported totally drug-resistant strains demonstrate the urgent need for novel therapeutic interventions [1] including innovative antimycobacterial drugs with no cross-resistance to clinically used drugs.

Recently, salicylanilide diethyl phosphates (diethyl [(2-phenylcarbamoyl)phenyl] phosphates I; Figure 1) have been synthesized as potential antimycobacterial agents with activity in the micromolar range (minimum inhibitory concentrations (MICs) from 1 μM). They inhibit nontuberculous mycobacteria and both drug-susceptible and drug-resistant Mycobacterium tuberculosis (Mtbt) strains [2]. Previously, some salicylanilide-based derivatives were reported as mild inhibitors of mycobacterial isocitrate lyase (ICL) and methionine aminopeptidase [3]. The exact mechanism(s) of their action as antimicrobial agents has still not been fully elucidated. Therefore, we screened the presented derivatives for new enzymatic targets of Mtbt, especially those related to the nongrowing state. No inhibitor of the selected enzymes has been established for clinical practice to date.

Mycobacterium tuberculosis exhibits a tendency to remain latent or persistent for decades before its activation into symptomatic disease. The bacterium has developed ingenious mechanisms to survive inside a hostile environment and to acquire essential nutrients. These metabolic processes appear to provide potential targets for novel anti-TB agents [4]. Genetic analysis has revealed a set of new potential drug targets in Mtbt.

Isocitrate lyase (ICL; EC 4.1.3.1) is one of two enzymes comprising the glyoxylate shunt and splits isocitrate into succinate and glyoxylate; this metabolic pathway is absent in vertebrates. ICL is responsible for the persistence of Mtbt and, additionally, disruption of theicl gene attenuated...
bacterial virulence and adaptation to hypoxia [3]. Based on the fact that salicylanilides and their esters with various acids have been reported as isocitrate lyase inhibitors [3, 5], we evaluated salicylanilide diethyl phosphates 1 against this enzyme (Table I).

The mycobacterial L-alanine dehydrogenase (MtAlaDH; EC 1.4.1.11) catalyzes the NADH-dependent reversive oxidative deamination of L-alanine to pyruvate and ammonia [6]. Both L-alanine and D-alanine are important components of peptidoglycan. MtAlaDH plays a key role in the utilisation of carbon and nitrogen sources. It was observed that in the persistent state of the microorganism the gene coding MtAlaDH is upregulated [7, 8].

Lysine ε-aminotransferase (MtLAT; EC 2.6.1.36) has been implicated in the mycobacterial stress response and is upregulated by approximately 40-fold in nutrient-starved models designed to mimic the persistent/latent state of TB [9]. MtLAT also plays an important role in adaptation to long-term persistence in Mtb [10]. In addition, this enzyme has been shown to be upregulated during adaptation to the stationary phase and low-oxygen dormancy [11].

Chorismate mutase (MtCM; EC 5.4.99.5) is another promising selective drug target [12]. This enzyme catalyzes the Claisen rearrangement of chorismate to prephenate in the shikimate pathway, which is the first committed step in the biosynthesis of the aromatic amino acids phenylalanine and tyrosine.

Pantothenate synthetase (MtPS; EC 6.3.2.1) catalyzes the essential adenosine triphosphate-dependent condensation of D-pantoate and β-alanine to form pantothenate in bacteria, yeast, and plants. Pantothenate is a key precursor for the biosynthesis of coenzyme A and acyl carrier protein, which are essential cofactors for bacterial growth [13, 14].

We evaluated salicylanilide diethyl phosphates 1 also against these four mycobacterial persistence-related enzymes as a pilot screening.

2. Materials and Methods

2.1. Chemistry. The synthesis and characterization of the salicylanilide diethyl phosphates were published previously [2]. Yield of esters synthesized via reaction of parent salicylanilides with diethyl chlorophosphate in the presence of triethylamine ranged from 11% up to 78%.

2.2. Enzyme Inhibition Measurement

2.2.1. Isocitrate Lyase Assay (ICL). Isocitrate lyase activity was assayed according to the protocol reported by Dixon and Kornberg (glyoxylate phenyl hydrazone formation) [15] at 10 μM of the investigated compounds. Isoniazid was employed as a negative control (inhibition of 0%), and 3-nitropropionic acid (3-NP) served as a positive control. A description of the method can be found in the literature [5].

2.2.2. Mycobacterial L-Alanine Dehydrogenase (MtAlaDH) [16] Assay. A reaction mixture consisting of 125 mM glycine/KOH (pH 10.2), 100 mM L-alanine, 1.25 mM NAD+, and 6.026 pM of MtAlaDH in a final volume of 200 μL diluted in 125 mM glycine/KOH (pH 10.2) was added to each well of a 96-well plate. The compounds were then added to the plates. The reaction was initiated by the addition of 10 μL enzyme diluted in buffer. Enzymatic activity was measured by the rate of production of NADH that accompanies the conversion of alanine to pyruvate by oxidative deamination [17]. The reaction components, except for MtAlaDH, were mixed in the well and the background reaction was measured; MtAlaDH was then added and the reaction kinetics were monitored. All measurements were performed at 340 nm with a heat-controlled Perkin Elmer Victor V3 spectrophotometer.

2.2.3. Mycobacterial Lysine ε-Aminotransferase (MtLAT) [18] Assay. The reaction mixture consisting of 1 mM L-lysine HCl, 1 mM α-ketoglutarate, 15 μM pyridoxal-5’-phosphate, and 1.25 pM MtLAT in a final volume of 200 μL diluted in 200 mM phosphate buffer (pH 7.2) was added to each well of a 96-well plate. Compounds were then added to the plates. The reaction was initiated by the addition of 10 μL of MtLAT, diluted in buffer. The mixture was incubated at 37°C for 1 h. The reaction was terminated by the addition of 10% trichloroacetic acid in ethanol. Piperidine 6-carboxylate (P6C) was detected by measuring the colour intensity of its adduct with 2-aminobenzaldehyde spectroscopically at 465 nm. The reaction components except for MtLAT were

![Figure 1: General structure of salicylanilide diethyl phosphates 1 (diethyl [2-(phenylcarbamoyl)phenyl] phosphates; R^1 = 4-Cl, 5-Cl, 4-Br; R^2 = 3-Cl, 4-Cl, 3,4-diCl, 3-Br, 4-Br, 3-F, 4-F, 3-CF_3, 4-CF_3).](attachment:image_url)
mixed in the well and the background reaction was measured; *Mt*LAT was then added and the reaction kinetics were monitored. Reactions were carried out at 37°C in a heat-controlled Perkin Elmer Victor V3 spectrophotometer.

One LAT unit (1 U) is the activity that produces 1 μM of P6C per min under these conditions.

2.3. Molecular Docking Studies. The crystal structure of *Mt*AlaDH was then obtained from the Protein Data Bank (http://www.pdb.org/, pdb code 2VHW). Water molecules and NAD+ were removed, polar hydrogens were added, partial charges were assigned, and the energy of the molecule was minimised using UCSF Chimera software 1.6.2. [22] The ligand structure was created using CS ChemOffice version 10.0 (CambridgeSoft), and its conformation optimised with the aid of UCSF Chimera 1.6.2 using the Amber force field.

Docking calculations were carried out using Autodock Vina [23]. The three-dimensional affinity grid box was designed to include the full active site of *Mt*AlaDH (box centre: x = –58, y = 57, and z = 8; size of the box 20 points in each direction). The enzyme structure was kept rigid during the docking procedure. The visualisation of enzyme-ligand interactions was prepared using PyMol 1.1r1. [24].

3. Results and Discussion

With respect to isocitrate inhibition, most of the evaluated compounds 1 were inactive at the concentration of 10 μM; some of these molecules displayed the ability to activate the tested enzyme. Only seven derivatives showed very weak inhibition, within the range from 4 to 7%, but without any significant activity. It seems that only halogen monosubstitution of the aniline ring and the presence of 4-bromo or 5-chloro substitution on the salicylic ring retain some inhibitory activity. Salicylanilide diethyl phosphates I failed with respect to finding more efficient ICL inhibitors and when compared with previously described esters [3, 5]. The low inhibition rates observed for these phosphate esters I indicate the importance of the acid used for the esterification of parent salicylanilides in the inhibition results.

To explore other possible target(s) of derivatives I, the inhibitory activity at 50 μM was screened against four other mycobacterial enzymes, *Mt*AlaDH, *Mt*LAT, *Mt*CM, and *Mt*PS (Table 2), which play very important roles in *Mt*M persistence. The investigated compounds showed weak activity against *Mt*CM (the highest activity was approximately 26% for 1r) and *Mt*LAT, where only compound 1s showed inhibition higher than 50%. More than 50% inhibitory activity against *Mt*CM was found for thirteen compounds, with the highest in vitro efficacy for compounds 1o and 1s (above 60%). The highest percentage activity was found for inhibition of *Mt*AlaDH; compounds 1s, 1q, and 1n had more than 70% inhibition. Therefore, their IC50 values were determined and are reported in Table 2. We plotted the graphs of the inhibition rates [%] of 50, 25, 12.5, 6.25, 3.13, and 1.56 μM and calculated IC50 values. 5-Chloro-2-[(3-chlorophenyl)carbamoyl]phenyl diethyl phosphate, 1s, was found to be the most potent compound against *Mt*AlaDH with an IC50 of 4.96 μM. Interestingly, this molecule also exhibited superior inhibition of *Mt*LAT and *Mt*CM, as pointed previously. Its MICs against actively growing *Mt*H37Rv strain were 4–8 μM [2].

Based on the inhibition results, the whole series of salicylanilide diethyl phosphates 1 was investigated in a molecular docking study to identify possible interactions with amino acid residues in the active site of *Mt*AlaDH. The three-dimensional structure of native *Mt*AlaDH is described as a hexamer formed by three associated dimers of protein subunits. Each subunit consists of two distinct domains, the substrate-binding domain (residues 1–129 and 311–370) and the NAD+/NADH binding domain (residues 130–310). These domains are separated from each other by a cleft in which most of the active site amino acid residues are located [16].
### Table 2: Mycobacterial enzyme activity inhibition results.

| Comp. code | R¹ | R² | % inhibition at 50 μM against L-MtAlaDH | L-MtAlaDH IC₅₀ (μM) | % inhibition at 50 μM against MtLAT | % inhibition at 50 μM against MtCM | % inhibition at 50 μM against MtPS |
|------------|----|----|----------------------------------------|---------------------|-------------------------------------|---------------------------------|---------------------------------|
| 1a         | 4-Br | 3-F | 69.94                                  | 39.75               | 7.80                                | 40.50                          | 7.47                            |
| 1b         | 4-Br | 3-Cl | 59.46                                  | 41.12               | 18.73                               | 43.50                          | 3.89                            |
| 1c         | 4-Br | 4-Cl | 39.35                                  | >50                 | 29.10                               | 48.31                          | 13.25                           |
| 1d         | 4-Br | 3,4-di-Cl | 59.02                                  | 31.92               | 30.27                               | 41.30                          | 20.54                           |
| 1e         | 4-Br | 3-CF₃ | 42.08                                  | >50                 | 40.20                               | 53.20                          | 17.76                           |
| 1f         | 4-Br | 4-CF₃ | 69.28                                  | 34.73               | 26.15                               | 52.09                          | 11.33                           |
| 1g         | 4-Br | 3-Br | 22.62                                  | >50                 | 12.42                               | 46.07                          | 18.76                           |
| 1h         | 4-Br | 4-Br | 34.35                                  | >50                 | 18.25                               | 38.19                          | 18.33                           |
| 1i         | 4-Br | 4-F  | 54.58                                  | 15.58               | 2.90                                | 46.67                          | 5.41                            |
| 1j         | 4-Cl | 3-Cl | 12.30                                  | >50                 | 29.30                               | 54.56                          | 3.64                            |
| 1k         | 4-Cl | 4-Cl | 18.73                                  | >50                 | 38.00                               | 43.78                          | 14.74                           |
| 1l         | 4-Cl | 3-F  | 61.87                                  | 23.11               | 8.06                                | 50.75                          | 17.93                           |
| 1m         | 4-Cl | 4-F  | 57.70                                  | 42.26               | 20.12                               | 49.33                          | 7.76                            |
| 1n         | 4-Cl | 4-Br | 70.64                                  | 29.17               | 17.29                               | 50.19                          | 6.57                            |
| 1o         | 4-Cl | 3,4-di-Cl | 44.46                                  | >50                 | 18.24                               | 60.96                          | 23.01                           |
| 1p         | 4-Cl | 3-Br | 54.64                                  | 36.11               | 19.13                               | 37.41                          | 20.59                           |
| 1q         | 4-Cl | 3-CF₃ | 73.88                                  | 36.32               | 19.05                               | 50.02                          | 11.53                           |
| 1r         | 4-Cl | 4-CF₃ | 47.28                                  | >50                 | 23.18                               | 50.05                          | 26.25                           |
| 1s         | 5-Cl | 3-Cl | 73.94                                  | 4.96                | 53.64                               | 60.12                          | 11.18                           |
| 1t         | 5-Cl | 3-Br | 41.51                                  | >50                 | 12.86                               | 57.58                          | 5.04                            |
| 1u         | 5-Cl | 3-F  | 59.85                                  | 39.20               | 23.42                               | 52.36                          | 22.22                           |
| 1v         | 5-Cl | 4-F  | 69.07                                  | 34.47               | 37.29                               | 52.05                          | 17.28                           |
| 1w         | 5-Cl | 4-Br | 14.97                                  | >50                 | 40.28                               | 52.84                          | 14.51                           |
| 1x         | 5-Cl | 4-Cl | 58.62                                  | 46.51               | 20.18                               | 53.79                          | 11.17                           |
| 1y         | 5-Cl | 3,4-di-Cl | 26.30                                  | >50                 | 28.17                               | 56.65                          | 12.38                           |
| 1z         | 5-Cl | 3-CF₃ | 58.32                                  | 17.82               | 10.01                               | 43.49                          | 4.51                            |
| 1zz        | 5-Cl | 4-CF₃ | 64.82                                  | 37.49               | 19.83                               | 54.09                          | 8.26                            |

The best results for each enzyme are shown in bold.

![Figure 2: Molecular docking of the 1s derivative with H-bonds and hydrophobic interactions with amino acid residues of MtAlaDH.](image)

The top-scoring orientations of the compounds were located in the cavity normally occupied by NAD⁺ (Figure 2). All of the compounds in the series displayed a similar conformation in the active site. Since structural differences of the compounds 1 are very small, the docking studies enabled us only to predict possible orientation in the active site of the enzyme, but the influence of different position of substitution on the activity is not clear. In general, the orientation of all of the compounds suggests possible H-bond interactions, predominantly with two amino acid residues, Thr178 and Ala179. In addition, some of the compounds showed another H-bond interaction with Ala238, Leu240, Ser134, and Lys203. The most active compound in the series, 5-chloro-2-[(3-chlorophenyl)carbamoyl]phenyl diethyl phosphate 1s (IC₅₀ 4.96 μM), exhibited H-bond interactions with residues Ser134, Thr178, Ala179, Lys203, and Leu240 and additional hydrophobic interactions with amino acid residues Ile267 and Ala268 (Figure 2), which resulted in one of the highest affinities for the enzyme (docking score –7.8 kJ/mol). Good docking results were also observed for compounds with a trifluoromethyl substitution in the aniline part (1e, 1f, 1q, 1r, 1z, and 1zz). These compounds demonstrated the same H-bond interactions as 1s. Additionally, the trifluoromethyl group is positioned in a small cavity occupied by hydrophobic amino acid residues (Leu130, Ala137, and Ile267), and the potential to form hydrophobic interactions may cause a higher binding.
Salicylanilide derivatives share a complex mechanism of action with more structural similarity, related and analogous derivatives. Cocrystallisation of the enzyme with an inhibitor, etc. Based on structural similarity, related and analogous derivatives may be designed and evaluated as prospective inhibitors of enzymes, especially when the suppression of these enzymes should affect especially persistent mycobacterial subpopulation. The results confirm the fact that salicylanilide derivatives share a complex mechanism of action with more molecular/cellular targets.

4. Conclusions

To identify potential TB drug target(s) of salicylanilide diethyl phosphates, they were evaluated against five mycobacterial enzymes related to dormancy. Most of the compounds exhibited significant inhibition, especially against MtAlaDH.

We found that there is not a direct relationship of in vitro MICs of salicylanilide diethyl phosphates against actively growing \( \text{Mt} \) and the inhibition of five presented enzymes, especially when the suppression of these enzymes may be designed and evaluated as prospective inhibitors of enzymes. The results of enzyme inhibition screening. Further studies to verify that these compounds are true inhibitors of \( \text{Mt} \text{AlaDH} \) are required (e.g., inducing drug-resistant mutants and identification of possible mutations, cocrystallisation of the enzyme with an inhibitor, etc.). Based on structural similarity, related and analogous derivatives may be designed and evaluated as prospective inhibitors of this enzyme.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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