Tetrahydro-2-furanyl-2,4(1H,3H)-pyrimidinedione Derivatives as Novel Antibacterial Compounds against *Mycobacterium*

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**Abstract**

**Objective/Background:** *Mycobacterium tuberculosis* thymidine monophosphate kinase (mtTMPK) is a potential enzymatic target for the treatment of tuberculosis (TB). **Materials and Methods:** In this study, we performed pharmacophore-based *in silico* screening, targeting mtTMPK with a compound library of 461,383 chemicals. We evaluated the candidate compounds for inhibitory effects on the growth of the model mycobacteria, *Mycobacterium smegmatis*. **Results:** The compound KTP3 completely inhibited the growth of *M. smegmatis* at 100 µM. A similarity search and rescoring with the structure of compound KTP3 using a web-based database identified two similar compounds (KTPS1 and KTPS2) with improved potency. The KTP3 analogs, KTPS1 and KTPS2, exhibited strong growth inhibitory effects with half-maximal inhibitory concentration values of 8.04 µM and 17.1 µM, respectively, against *M. smegmatis*. Moreover, the most potent chemical compound, KTPS1, did not exhibit toxic effects on the model enterobacteria and several mammalian cells. Two active chemicals, KTPS1 and KTPS2, inhibited mtTMPK activity by 18% and 36%, respectively, suggesting that these compounds have off-target activities against *Mycobacterium*. **Conclusion:** Structural and biological information on these chemicals is likely to be useful for the development of novel antibiotics for the treatment of TB.

**Keywords:** *In silico* drug screening, *Mycobacterium*, pharmacophore model, thymidine monophosphate kinase

**INTRODUCTION**

*Mycobacterium tuberculosis*, an infecting agent of human tuberculosis (TB), remains a serious public menace. The World Health Organization (WHO) estimated that there are 8.6 million new cases of TB and 1.3 million deaths arising from TB annually. These deaths also include 0.3 million human immunodeficiency virus coinfected deaths. Currently, the directly observed treatment short-course drug therapy program developed by the WHO is a widely used control strategy for TB. However, drug compliance is often incomplete, contributing to an increasing prevalence of drug-resistant *M. tuberculosis* strains such as the multidrug-resistant and extensively drug-resistant strains. In addition, the total drug-resistant strain of *M. tuberculosis* has been identified. Therefore, the development of novel anti-TB drugs is important for the treatment of patients with increasingly drug-resistant *M. tuberculosis* strains.

Advances in the identification of novel anti-TB drugs have progressed using a large panel of biological pathways including mycobacterial cell wall synthesis, energy production (adenosine 5′-triphosphate [ATP] synthase), DNA/RNA synthesis, and protein synthesis. Currently, the drug development pipeline of anti-TB agents contains several compounds in clinical trials or preclinical trials. These novel compounds are based on the following chemical scaffolds: Fluoroquinolone (gatifloxacin, moxifloxacin), nitroimidazoles (PA-824, OPC-67683), oxazolidinone (PNU-100480), 1,2-ethylenediamine (SQ109), and diarylquinoline (TMC207). In particular, TMC207 is the first new drug for the treatment of TB in more than 40 years. However, the discovery of novel compounds with potential anti-TB activity is still needed because of the high dropout rates.

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M. tuberculosis thymidine monophosphate kinase (mtTMPK; EC 2.7.4.9), a member of the large superfamily of nucleoside monophosphate kinases, catalyzes the reversible phosphorylation of deoxythymidine 5'-monophosphate (dTMP) to deoxythymidine 5'-diphosphate in the presence of Mg²⁺ and ATP. This catalysis step plays a crucial role in both the de novo and salvage pathways of DNA synthesis for mycobacterial growth.⁴⁻⁵ Significant progress has been made in the development of mtTMPK inhibitors using several in silico drug screening methods (e.g., docking studies, pharmacophore models, and quantitative structure–activity relationship methods).²⁰⁻²³ In fact, several research groups have reported on the successful design and synthesis of nucleotide analog mtTMPK inhibitors (i.e., α-thymidine, acyclic nucleoside, and bicyclic nucleoside derivatives).²⁴⁻²⁶ Therefore, mtTMPK is considered to be an attractive target for the development of effective antibiotics against TB. In this study, we performed pharmacophore-based in silico screening, targeting mtTMPK with a compound library of 461,383 chemicals, which yielded one hit (KTP3) that was able to inhibit the growth of Mycobacterium smegmatis. Subsequently, we rescreened compounds similar to KTP3 and obtained potentially active compounds from a web-based database. We identified two KTP3 analogs (KTPS1 and KTPS2) that showed antibacterial effects against M. smegmatis. Moreover, the most potent compound, KTPS1, did not exhibit any significant toxic effects on model intestinal bacteria or several mammalian cells. In addition, we experimentally demonstrated that two chemical compounds (KTPS1 and KTPS2) directly inhibited mtTMPK enzymatic activity to some extent, suggesting that these compounds have off-target activities against Mycobacterium. We anticipate that structural and experimental information on these active chemicals will contribute to the development of anti-TB medical drugs.

Materials and Methods

Chemical compound library

For the pharmacophore-based in silico screening, we generated a virtual chemical compound library (ChemBridge; 461,383 chemicals) using the wash and energy minimize modules of Molecular Operating Environment (MOE) version 2011.10.²⁷ First, the two-dimensional structures were converted into three-dimensional structures. After generating the three-dimensional structures, the protonation state was treated using the partial charge module in MOE. The multiconformation structural data of the compounds were generated using the energy minimize module of MOE 2011.10.²⁷

Pharmacophore-based in silico screening

The crystal data for mtTMPK (PDB ID: 1MRN)²² were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank.²³ Before using the mtTMPK crystal structure in the in silico drug screening, we removed all water molecule atoms and the P₁-(adenosine-5')-P₁-(thymidine-5')-pentaphosphate (Ap₂T) atoms of the inhibitor from the original crystal structure data. In addition, hydrogen atoms were added to mtTMPK using the Protonate 3D module in MOE 2011.10 at pH 7.0 with the MMFF94x force field. The energy was minimized using the energy minimize module of MOE 2011.10.²⁷ As part of this process, all of the heavy atoms, except the hydrogen atoms, were tethered to relieve any short contacts.

The pharmacophore-based in silico screening was performed using MOE 2011.10²⁷ and CCDC GOLD suite version 5.3.³⁴ The first pharmacophore-based screening was performed using the pharmacophore search module in MOE 2011.10 with default parameters.²⁷ The candidate compounds obtained from the first screening were then docked into the active site cleft of mtTMPK, and putative protein–chemical compound binding affinities were predicted using the GOLD program, which uses a genetic algorithm to search for flexible ligand conformations and allows for partial receptor flexibility.³⁴ The docking simulations were performed with the default settings in GOLD (operations = 100,000, island = 5, migration = 15, mutation = 95, and crossover = 95).²⁹⁻³¹ The pharmacophore-based in silico screening results were analyzed using the protein–ligand interaction fingerprint and ligand interaction modules in MOE 2011.10.²⁷

Screening of similar chemical compounds

A total of 13 compounds similar to the initial active compound KTP3 were chosen from the Hit2Lead web-based database after two- and three-dimensional similarity searches.³⁵ The docking studies were performed with a GOLD screening using the multiconformation structural data of the 13 analog compounds.

Docking metric

We generated a decoy set of chemical compounds of mtTMPK based on the active ligands of mtTMPK (Kₐ values = 7.2–116 µM)²⁴⁻²⁵ using the directory of useful decoys enhanced test set generator.³⁶ Three-dimensional structures of the ligand and decoy set chemical compounds were generated using the energy minimize and LowMode MD modules in MOE 2011.10. The docking studies were performed using the same methods and parameters as the single- and multi-conformational GOLD docking processes. The receiver operating characteristic (ROC) curve and area under the curve (AUC) values were calculated using Epi package in R version 3.0.2 (Gentofte, Denmark).

Sequence alignment and homology modeling

The amino acid sequence of mtTMPK (accession number: WP_003904223) retrieved from the Basic Local Alignment Search Tool (BLAST) database was aligned with that of msTMPK (accession number: WP_01727970) using MOE 2011.10.²⁷ The three-dimensional structure of msTMPK was generated using the homology modeling module in MOE 2011.10 with default parameters.³¹

Chemical compounds

All chemical compounds (KTP1–KTP5 and KTPS1–KTPS4) were purchased from ChemBridge Corporation and...
dissolved in dimethyl sulfoxide (DMSO) (Sigma) for the in vitro assays. The chemical compounds (supplier IDs are given in parentheses) used include the following:

KTP1: 2-chloro-N-[(3-(5-ethyl-1,3-benzoazol-2-yl)-4-hydroxy-5-methylphenyl) amino] carbonothioyl)-4-methylbenzenesulfonate (6578689);

KTP2: 4-[[2-butyl-5-imino-7-oxo-5H-[1,3,4]thiadiazolo[3,2-a] pyrimidin-6 (7H)-ylidene] methyl]-2-methoxyphenyl benzenesulfonate (7912813);

KTP3: 1-[(3,4-dihydroxy-5-[[4-(methoxyphenyl)(diphenyl) methoxy] methyl] tetrahydro-2-furanyl]-2,4 (1H,3H)-pyrimidinedione (5135608);

KTP4: N-6-[[5-methyl-1,3,4-thiadiazol-2-yl]thio]methyl]-4-oxo-1,4-dihydro-2-pyrimidinyl)-N’-[2-(phenylthio) phenyl] guanidine (7722897);

KTP5: 3-[[4,6-dihydroxy-5-propyl-2-pyrimidinyl]thio]-1-(4-phenoxoynyl)-2,5-pyrrolidinedione (7733852);

KTPS1: 2-[[5-[[bis(4-methoxyphenyl)(phenyl) methoxy]methyl]4-hydroxy-3-(tetrahydro-2H-pyran-2-yl)oxy] tetrahydro-2-furanyl]-1,2,4-triazine-3,5 (2H,4H)-dione (5141276);

KTPS2: 1-[[5-[[bis(4-methoxyphenyl)(phenyl) methoxy]methyl]4-hydroxy-3-(tetrahydro-2H-pyran-2-yl)oxy] tetrahydro-2-furanyl]-2,4 (1H,3H)-pyrimidinedione (5141274);

KTPS3: [3,4-dihydroxy-5-(5-methyl-2,4-dioxo-3,4-dihydro-1 (2H)-pyrimidinyl) tetrahydro-2-furanyl] methyl benzoate (5141196);

KTPS4: [5-(2,4-dioxo-3,4-dihydro-1 (2H)-pyrimidinyl)-3,4-dihydroxytetrahydro-2-furanyl] methyl benzoate (5141262).

The nuclear magnetic resonance or liquid chromatography-mass spectrometry data related to these compounds are available on the ChemBridge Corporation’s website at http://www.chembridge.com.

Bacterial strains and bacterial growth assays

The model bacterial strain *M. smegmatis* was obtained from the RIKEN BioResource Center (Saitama, Japan). The Escherichia coli JM109 and BL21 strains were kindly provided by Dr. S. Sueda (Kyushu Institute of Technology).

The bacterial growth assays were performed using previously described methods.[19] Purified recombinant mtTMPK was used in the enzymatic assay.[18] The enzymatic reactions were performed at 30°C and with a final volume of 500 µL containing 50 mM Tris–HCl buffer (pH 7.4), 50 mM KCl, 200 µM NADH, 1 mM phosphoenol pyruvate, and 2 units each of LDH, pyruvate kinase, and nucleoside diphosphate kinase. The concentrations of dTMP and ATP were kept constant at 50 µM and 500 µM, respectively. The oxidation of NADH (at 340 nm) was measured with an Eppendorf ECOM 6122 photometer.

Statistical analysis

All statistical analyses were performed using R version 3.0.2 (The R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism version 4 (GraphPad Software, Inc. (San Diego, CA)).

Supporting information

Five additional figures and two additional tables of supporting information are available: An evaluation of the docking accuracy of the ROC analysis [Figure S1]; a sequence alignment of mtTMPK and msTMPK [Figure S2]; the inhibitory effects of the KTP3 analogs (KTPS1–KTPS4) on the growth of *M. smegmatis* after 24 h [Figure S3]; the dose-dependent effects of INH on the growth of *M. smegmatis* [Figure S4]; the effects of the active compounds (KTP3, KTPS1, and KTPS2) on the JM 109 and BL 21 *E. coli* strains [Figure S5]; the cytotoxic effects of the active compounds (KTP3, KTPS1, and KTPS2) on MDCK, SH-SY5Y, THP-1, HL-60, and
K562 cells [Figure S6]; the inhibitory effects of the active compounds (KTP3, KTPS1, and KTPS2) on the enzymatic activity of mtTMPK [Table S1]; and the physicochemical properties of the active compounds [Table S2]. These materials are available free of charge through the Internet at http://www.sciencedirect.com.

RESULTS
Pharmacophore-based in silico screening
We performed a three-step pharmacophore-based in silico screening using the pharmacophore search module in MOE and docking simulations with the GOLD program to identify active compounds against mtTMPK [Figure 1]. The dTMP binding site comprises the following amino acid residues: Asp 9, Tyr 39, Phe 70, Arg 74, Tyr 103, Arg 153, and Glu 166 [Figure 2].[32,39] First, the constructed structural library of 461,383 chemical compounds was screened using the pharmacophore search module in MOE,[9,27] and 2645 hit compounds were identified. After the pharmacophore screening, we performed a GOLD screening using a single conformation per compound (AUC values of the ROC analysis = 0.713; Figure S1a) and identified the top 500 compounds with GOLD scores greater than 75. Subsequently, we performed a second GOLD screening using five conformations per compound (AUC values of the ROC analysis = 0.733; Figure S1b). After the second GOLD screening, the top 239 ranked compounds showed average GOLD scores >80. We then eliminated compounds based on the following criteria: (1) The compound was evaluated through past high-throughput screening (HTS) using the PubChem database[40] and (2) the compound has no direct interactions (i.e. vDW and/or hydrogen bonds) with the dTMP binding site. Finally, we selected five chemical compounds (KTP1–KTP5) with average GOLD scores >80 (80.92–94.11). The binding potencies of these five compounds to the homology-modeled M. smegmatis TMPK (msTMPK) were verified by GOLD simulations. The GOLD scores of these compounds (KTP1–KTP5) ranged from 80.4 to 100.17 [Table 1].

In vitro antimycobacterial assay of the candidate compounds KTP1–KTP5
We performed an antimycobacterial assay to confirm that the five candidate compounds (KTP1–KTP5) actually exhibited inhibitory effects on the growth of the model mycobacteria M. smegmatis (biosafety level 1). A BLAST analysis[37] showed that the amino acid sequence of mtTMPK was similar to that of msTMPK (identity rate = 66%, positive rate = 72%). All amino acid residues in the active site of msTMPK are completely conserved in mtTMPK [Figure S2]. One of the five compounds, KTP3 (100 µM), significantly inhibited the growth of M. smegmatis after 24 h compared with the negative control (DMSO). In contrast, KTP1, KTP2, KTP4, and KTP5 had no significant or weak inhibitory effects on M. smegmatis growth when compared with the negative and positive controls [Figure 3]. Notably, KTP3 showed inhibitory effects on the growth of M. smegmatis similar to those of INH.

Screening of KTP3 analogs based on chemical structure similarity
We rescreened the KTP3 analogs expected to have higher efficacy than KTP3. We obtained 13 KTP3 analogs from a web-based database (Hit2Lead).[35] Subsequently, we performed GOLD docking simulations targeting mtTMPK with multiconformations of the 13 KTP3 analogs. As a result, we identified four structurally similar compounds (KTPS1–KTPS4) with average GOLD scores >70. In addition, the GOLD scores of KTPS1–KTPS4 were calculated from the msTMPK simulations, which showed similar values (75.04–95.82) to the calculated values from the mtTMPK simulations (74.70–97.03). The compounds KTP3 and KTPS1–KTPS4 have a tetrahydro-2-furanyl ring as a common scaffold [Table 2].

![Figure 1: General flowchart of the pharmacophore-based in silico screening applied to identify novel antimycobacterial agents targeting mtTMPK.](image1)

![Figure 2: The three-dimensional structure of mtTMPK. The secondary structures are indicated by ribbon representations (α-helix: Orange, β-sheet: Green). The amino acid residues of the active site are shown as a stick model. mtTMPK: Mycobacterium tuberculosis thymidine monophosphate kinase, PLIF: Protein–ligand interaction fingerprint.)](image2)
In vitro antimycobacterial assay of the KTP3 analogs

We examined whether the four KTP3 analogs (KTPS1–KTPS4) truly exhibited an inhibitory effect on the growth of *M. smegmatis*. Compounds KTPS1 and KTPS2 (100 µM) exhibited significant inhibitory effects (more than 50%) on the growth of *M. smegmatis* after 24 h, whereas KTPS3 and KTPS4 had weak inhibitory effects on the growth of *M. smegmatis* [Figure S3].

Determination of the half-maximal inhibitory concentration and minimum inhibitory concentration 50% values of the active compounds

We investigated the dose-dependent effects of the active compounds (KTP3, KTPS1, and KTPS2) on the growth of *M. smegmatis*. The experimentally determined half-maximal inhibitory concentration (IC\textsubscript{50}) values of KTP3, KTPS1, and KTPS2 for *M. smegmatis* were 23.9, 8.04, and 17.1 µM, respectively [Figure 4]. It should be noted that the IC\textsubscript{50} value of KTPS1 is at the same level as that of INH (experimentally determined IC\textsubscript{50} = 10.3 µM; Figure S4).

Furthermore, we determined the minimum inhibitory concentration 50% (MIC\textsubscript{50}) values of INH, KTP3, and KTPS1 for *M. smegmatis* [Table 3]. We did not determine the MIC\textsubscript{50} value of the active compound KTPS2 because KTPS2 did not completely inhibit the growth of *M. smegmatis* at 100 µM [Figure S3].

Enterobacterial and mammalian cell toxicity assays of the active compounds

We performed enterobacterial toxicity assays using *E. coli* as the model bacteria. For the treatment of TB, it is necessary for patients to take the first-line anti-TB drugs for a long period (at least 6 months). Drugs with enterobacterial toxicity are not suitable for TB treatment. Therefore, we assessed the enterobacterial toxicity of the compounds KTP3, KTPS1, and KTPS2 against the JM109 and BL21 *E. coli* strains. None of the compounds had any toxic effects on the growth of either strain after 8 h [Figure S5].

Furthermore, we assessed the cytotoxicity of the KTP3, KTPS1, and KTPS2 compounds against cultured mammalian cells (Madin–Darby canine kidney cells: MDCK, human neuroblastoma cells: SH-SY5Y, human acute monocyclic leukemia cells: THP-1, human promyelocytic leukemia cells: HL-60, and human acute myelocytic leukemia cells: K562). KTPS1 did not show any significant toxic effects on cultured MDCK, SH-SY5Y, THP-1, HL-60, and K562 cells, whereas KTP3 and KTPS2 showed significant toxic effects on these mammalian cells [Figures 5 and S6].

Table 1: Five selected compounds identified by pharmacophore-based in silico screening

| Chemical name | Chemical structure | cLogP\textsuperscript{a} | GOLD scores\textsuperscript{b} | mtTMPK | msTMPK |
|---------------|-------------------|-----------------|-----------------|-------|-------|
| KTP1          | ![Chemical structure](image1) | 6.16            | 94.11±0.37      | 88.44±0.45 |
| KTP2          | ![Chemical structure](image2) | 4.27            | 87.77±0.30      | 100.17±0.17 |
| KTP3          | ![Chemical structure](image3) | 2.83            | 87.34±1.60      | 86.82±0.95 |
| KTP4          | ![Chemical structure](image4) | 4.10            | 84.92±0.91      | 80.40±1.05 |
| KTP5          | ![Chemical structure](image5) | 4.06            | 80.92±0.52      | 85.35±0.44 |

\textsuperscript{a}The cLogP value was predicted using ligand properties module in MOE, \textsuperscript{b}Each value represents the mean±SEM. cLogP: Calculated LogP, MOE: Molecular operating environment, msTMPK: *Mycobacterium smegmatis* thymidine monophosphate kinase, mtTMPK: *Mycobacterium tuberculosis* thymidine monophosphate kinase, SEM: Standard error of the mean.
**Mycobacterium tuberculosis thymidine monophosphate kinase enzymatic assays**

Enzymatic assays were performed to assess whether the three active chemical compounds (KTP3, KTPS1, and KTPS2) directly inhibit the enzymatic activity of mtTMPK. Two of the three compounds, KTPS1 (100 µM) and KTPS2 (116 µM), inhibited mtTMPK activity by 18% and 36%, respectively, compared with the negative control, DMSO (0.3%). Compound KTP3 had no significant inhibitory effects on mtTMPK activity at 20 µM [Table S1]. It is noteworthy that these compounds formed precipitate at concentrations higher than 100 µM (approximately 20 µM for KTP3) when the compounds were added to the enzymatic reaction buffer. Therefore, we could not complete our analysis of the effects of these compounds on mtTMPK activity (e.g., dose-dependent effects).

**Discussion**

Our pharmacophore-based *in silico* screening targeting mtTMPK revealed three compound hits (KTP3, KTPS1, and KTPS2) exhibiting inhibitory effects on the growth of *M. smegmatis*. Among these compound hits, two compounds (KTPS1 and KTPS2) also exhibited inhibitory effects on mtTMPK enzymatic activity. Moreover, compound KTPS1 did not have statistically significant toxic effects on model intestinal bacteria (*E. coli*) and several mammalian cells (MDCK, SH-SY5Y, THP-1 HL-60, and K562 cells). Although compound KTP3 (calculated LogP [cLogP] =2.83) completely inhibited the growth of *M. smegmatis*, inhibitory effects on mtTMPK activity were not observed at a concentration of 20 µM. In contrast, the compounds KTP1 (cLogP =6.16), KTP2 (cLogP =4.27), KTP4 (cLogP =4.10), and KTP5 (cLogP =4.06) had no significant or weak inhibitory effects on *M. smegmatis* growth although these compounds had higher cLogP values compared with KTP3. It could be hypothesized that these compounds may modulate several biological targets, and these off-target activities may be essential for effectiveness. Indeed, previous research has suggested that effective drugs bind to multiple proteins (although the drugs are designed to be highly selective), and this polypharmacology may be essential for efficacy.\[41-43\] For example, d-cycloserine, associated with the second-line drugs for TB, inhibits mycobacterial cell wall biosynthesis through the inhibition of both d-alanine ligase and d-alanine racemase.\[44\] Combined with weak enzymatic inhibition, these results may suggest that the antibacterial activity was driven by polypharmacology rather than lipophilicity. Thus, the biological assay data for KTP3, KTPS1, and KTPS2 and the prediction of possible off-target activities with a computational approach (i.e., network analysis) are likely to be useful for the development of novel anti-TB drugs.

In general, unique physicochemical properties are required for antimycobacterial agents because of the distinctive architecture of mycobacterial cell walls (containing mycolic acids), which affect membrane and cell wall permeability.\[45,46\] Previously, Trias and Benz\[47\] reported that *M. smegmatis* is approximately

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**Figure 3:** Inhibitory effects of the candidate compounds (KTP1–KTP5) on the growth of *Mycobacterium smegmatis* after 24 h. The concentrations of the compounds were 100 µM. DMSO (0.3%) and INH (100 µM) were used as negative and positive controls, respectively. Each value represents the mean ± SEM of four independent experiments. Bonferroni’s all-paired comparison tests were performed (**P < 0.001, n.s.). DMSO: Dimethyl sulfoxide, INH: Isoniazid, n.s.: Not significant, OD: Optical density, SEM: Standard error of the mean.
20 times less permeable than *E. coli*. In addition, O’Shea and Moser\(^{(45)}\) reported that the physicochemical property values of chemical compounds with only Gram-positive activity (lipophilicity, molecular weight [MW], and polar surface area [PSA] values) were higher than those of chemical compounds with Gram-negative activity. Thus, taking physicochemical properties into account, LogP, MW, and topological PSA (tPSA) values are key factors that affect the efficacy of drugs. The physicochemical properties of each of the active compounds are shown in Table S2. The most active compound, KTPS1, had a higher lipophilicity (cLogP = 3.86) and higher MW and tPSA values (MW = 632, tPSA = 137.38) than KTP3.

Table 2: KTP3 analogs with different R substituents

| Chemical name | R<sub>1</sub> | R<sub>2</sub> | R<sub>3</sub> | cLogP<sup>a</sup> | GOLD scores<sup>b</sup> |
|---------------|--------------|------------|-------------|----------------|----------------------|
| KTPS1         | N            | –H         |             | 3.86          | 97.03±0.65, 95.82±1.07 |
| KTPS2         | C            | –H         |             | 4.39          | 92.30±0.90, 93.85±1.31 |
| KTPS3         | C            | –CH<sub>3</sub> | –OH         | -0.25         | 77.05±0.91, 76.44±0.25 |
| KTPS4         | C            | –H         | –OH         | -0.64         | 74.70±0.31, 75.04±0.09 |

<sup>a</sup>cLogP: Calculated LogP, msTMPK: *Mycobacterium smegmatis* thymidine monophosphate kinase, mtTMPK: *Mycobacterium tuberculosis* thymidine monophosphate kinase.

Figure 4: The dose-dependent effects of compounds KTP3, KTPS1, and KTPS2 on the growth of *Mycobacterium smegmatis*: (a) KTP3, (b) KTPS1, and (c) KTPS2. The bacterial growth (%) was determined after a 24-h cultivation of *Mycobacterium smegmatis*. Each plotted value represents the mean ± SD of four independent experiments. Each IC<sub>50</sub> value was determined by nonlinear regression analysis. IC<sub>50</sub>: Half-maximal inhibitory concentration, SD: Standard deviation.
...for the development of anti-TB chemical compounds with improved potency.

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**Conflicts of interest**

There are no conflicts of interest.

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Figure S2: Sequence alignments of mtTMPK and msTMPK. The identity and similarity amino acid residues are highlighted in black and gray, respectively. The sky blue upper triangle indicates the active site of TMPK. msTMPK: *Mycobacterium smegmatis* thymidine monophosphate kinase, mtTMPK: *Mycobacterium tuberculosis* thymidine monophosphate kinase.

Table S1: Effects of the compounds KTP3, KTPS1, and KTPS2 on the enzymatic activity of *Mycobacterium tuberculosis* thymidine monophosphate kinase

| Chemical name | mtTMPK activity (%) |
|---------------|---------------------|
| DMSO | 100±2.0 |
| KTP3 (20 µM) | 97.0±1.0 |
| KTPS1 (100 µM) | 82.0±5.0 |
| KTPS2 (116 µM) | 64.0±1.0 |

*Data in brackets indicate the final compound concentration, †Experiment was performed in duplicate, and each value represents the mean±SEM. DMSO: Dimethyl sulfoxide, mtTMPK: *Mycobacterium tuberculosis* thymidine monophosphate kinase, SEM: Standard error of the mean.*

Table S2: Physicochemical properties of the active compounds

| Chemical name | MW  | cLogP | tPSA  | HBA  | HBD  |
|---------------|-----|-------|-------|------|------|
| KTP3         | 517 | 2.83  | 117.56 | 7    | 3    |
| KTPS1        | 632 | 3.86  | 137.38 | 10   | 2    |

*The cLogP value was predicted using ligand properties module in MOE. HBA: H-bond acceptor, HBD: H-bond donor, MOE: Molecular operating environment, MW: Molecular weight, tPSA: The topological polar surface area
Figure S3: Inhibitory effects of the KTP3 analogs (KTPS1–KTPS4) on the growth of *Mycobacterium smegmatis* after 24 h. The concentrations of the compounds were 100 µM. DMSO (0.3%) and INH (100 µM) were used as negative and positive controls, respectively. Each value represents the mean ± SEM of four independent experiments. Bonferroni’s all-pair comparison tests were performed (**P < 0.001, n.s.). DMSO: Dimethyl sulfoxide, INH: Isoniazid, n.s.: Not significant, OD: Optical density, SEM: Standard error of the mean.

Figure S4: The dose-dependent effects of INH on the growth of *Mycobacterium smegmatis*. The bacterial growth (%) was determined after a 24-h cultivation of *Mycobacterium smegmatis*. Each plotted value represents the mean ± SD of four independent experiments. The IC$_{50}$ value was determined by nonlinear regression analysis. IC$_{50}$: Half-maximal inhibitory concentration, INH: Isoniazid, SD: Standard deviation.
Figure S5: Effects of the active compounds (KTP3, KTPS1, and KTPS2) on model enterobacteria strains: (a) *Escherichia coli* JM109 and (b) *Escherichia coli* BL21. The concentrations of the chemical compounds were 50 µM (close to IC$_{90}$ and MIC$_{90}$ value of the most potent compound KTPS1 against *Mycobacterium smegmatis* growth). DMSO (0.3%) and TCS (50 µM) were used as negative and positive controls, respectively. Each value represents the mean ± SEM of four independent experiments. DMSO: Dimethyl sulfoxide, IC$_{90}$: 90% inhibitory concentration, MIC$_{90}$: Minimum inhibitory concentration to inhibit growth of 90% of organisms, OD: Optical density, SEM: Standard error of the mean, TCS: Triclosan.

Figure S6: Cytotoxic effects of the active compounds (KTP3, KTPS1, and KTPS2) on cultured mammalian cells: (a) MDCK, (b) SH-SY5Y, (c) THP-1, (d) HL-60, and (e) K562 cells. The concentrations of the compounds were 50 µM (close to IC$_{90}$ and MIC$_{90}$ value of the most potent compound KTPS1 against *Mycobacterium smegmatis* growth). DMSO (0.3%) and TCS (50 µM) were used as negative and positive controls, respectively. Each value represents the mean ± SEM of four independent experiments. Dunnett’s multiple comparison tests were performed (**) $P < 0.01$, n.s.). DMSO: Dimethyl sulfoxide, IC$_{90}$: 90% inhibitory concentration, INH: Isoniazid, MIC$_{90}$: Minimum inhibitory concentration to inhibit growth of 90% of organisms, n.s.: Not significant, SEM: Standard error of the mean, TCS = Triclosan.