Original Article

Structural Modification of a Novel Inhibitor for Mycobacterium Enoyl-Acyl Carrier Protein Reductase Assisted by *In silico* Structure-based Drug Screening

Junichi Taira[1], Toshiki Nagano[1], Mitsuru Kitamura[2], Miho Yamaguchi[1], Hiroshi Sakamoto[1], Shunsuke Aoki[1]

[1]Department of Bioscience and Bioinformatics, Graduate School of Computer Science and Systems Engineering, Kyushu Institute of Technology, Iizuka, [2]Department of Applied Chemistry, Kyushu Institute of Technology, Kitakyushu, Kyushu, Japan

Abstract

Background: *Mycobacterium tuberculosis* enoyl-acyl carrier protein reductase (mtInhA) is involved in the biosynthesis of mycolic acids, a major component of mycobacterial cell walls, and has been targeted in the development of anti-tuberculosis (TB) drugs. In our previous *in silico* structure-based drug screening study, we identified KES4, a novel class of mtInhA inhibitor. KES4 is composed of four ring structures (A–D-rings) and molecular dynamic simulation predicted that the D-ring is essential for the interaction with mtInhA. **Methods:** The structure–activity relationship study of the D-ring was attempted and aided by *in silico* docking simulations to improve the mtInhA inhibitory activity of KES4. A virtual chemical library of the D-ring-modified KES4 was then constructed and subjected to *in silico* docking simulation against mtInhA using the GOLD program. The candidate compound showing the highest GOLD score, referred to as KEN1, was synthesized, and its biological properties were compared with those of the lead compound KES4. **Results:** We achieved the synthesis of KEN1 and evaluated its effects on InhA activity, mycobacterial growth, and cytotoxicity. The antimycobacterial activity of KEN1 was comparable to that of the lead compound (KES4), although it exhibited superior activity in mtInhA inhibition. **Conclusions:** We obtained a KES4 derivative with high mtInhA inhibitory activity by *in silico* docking simulation with a chemical library consisting of a series of D-ring-modified KES4.

Keywords: Enoyl-acyl carrier protein reductase, GOLD program, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, structure-based drug screening

Submitted: 28-Nov-2019 **Accepted:** 12-Jul-2019 **Published:** 06-Mar-2020

Introduction

Tuberculosis (TB), a severe respiratory infectious disease, is initiated by the infection of *Mycobacterium tuberculosis*.[1-4] Mycobacterial enzymes of the type II fatty acid synthase (FAS-II) system are attractive targets for the rational design of anti-TB agents.[5-8] The FAS-II system elongates acyl fatty acid precursors of mycolic acids, which are known as an essential component of the mycobacterial specific cell wall structure (tetramycolypentaarabinosyl clusters).[9] These structures have been linked to virulence, to the ability of the bacillus to survive and replicate inside macrophages, and shown to act as a barrier to the intracellular entry of a number of common antibiotics. The enoyl-acyl carrier protein reductase (InhA, EC 1.3.1.9) from *M. tuberculosis* (mtInhA), which is a member of the mycobacterial FAS-II system, is among the most widely studied enzymes given its role as a target of antitubercular drugs such as isoniazid (INH), ethionamide, and triclosan (TCS).[10-13] This enzyme catalyzes the NADH-dependent reduction of the double bond of 2-trans-enoyl-(acyl-carrier protein), an essential step in the fatty acid elongation cycle of the FAS-II system. An accumulation of high-resolution structures of mtInhA has

Access this article online

Quick Response Code:

Website: www.ijmyco.org

DOI: 10.4103/ijmy.ijmy_184_19

Address for correspondence: Dr. Shunsuke Aoki
Department of Bioscience and Bioinformatics, Graduate School of Computer Science and Systems Engineering, Kyushu Institute of Technology, Iizuka 820-8502, Kyushu, Japan.
E-mail: aokis@bio.kyutech.ac.jp

ORCID:
https://orcid.org/0000-0001-8222-6713

How to cite this article: Taira J, Nagano T, Kitamura M, Yamaguchi M, Sakamoto H, Aoki S. Structural modification of a novel inhibitor for mycobacterium enoyl-acyl carrier protein reductase assisted by *in silico* structure-based drug screening. Int J Mycobacteriol 2020;9:12-7.

enabled the rapid and precise screening of mtInhA-targeted inhibitor with *in silico* structure-based drug screening (SBDS). Currently, this technique is one of the most powerful tools to find lead compounds with novel pharmacophores and is a promising tool rather than random high-throughput screening in terms of cost and labor intensiveness.

We also previously reported that 1-(2-furoyl)-4-(3-[phenoxy] benzyl) piperazine, designated as KES4, was identified as an mtInhA inhibitor, aided by *in silico* SBDS.[14-17] KES4 exhibited inhibitory effects on mycobacterial growth as well as InhA enzymatic activity.[14] As shown in Figure 1a, KES4 is composed of two-phenyl, piperazinyl, and furoyl groups (the ring structures are referred to as the A–D-rings), and our molecular dynamic simulations predicted that the hydrogen atom on methylene, between the piperazinyl group (C-ring) and the 3-phenoxybenzyl group (B-ring), interacts with Phe149 by CH-π interaction. Notably, the 2-furoyl group (D-ring) and the proximal carbonyl group were also shown to be involved in hydrophobic interactions with Leu218 and hydrogen bonding with Tyr158, respectively. Since Tyr158 and Leu218 are located in the active site of mtInhA, the D-rings could play an important role in terms of binding with mtInhA. Thus, in the present study, to obtain a potent mtInhA inhibitor, the D-ring structure in KES4 was modified with the assistance of *in silico* SBDS. A chemical library of D-ring-modified KES4 derivatives was prepared and subjected to *in silico* docking simulations. The biological properties of the selected compounds were also compared with those of the lead compound.

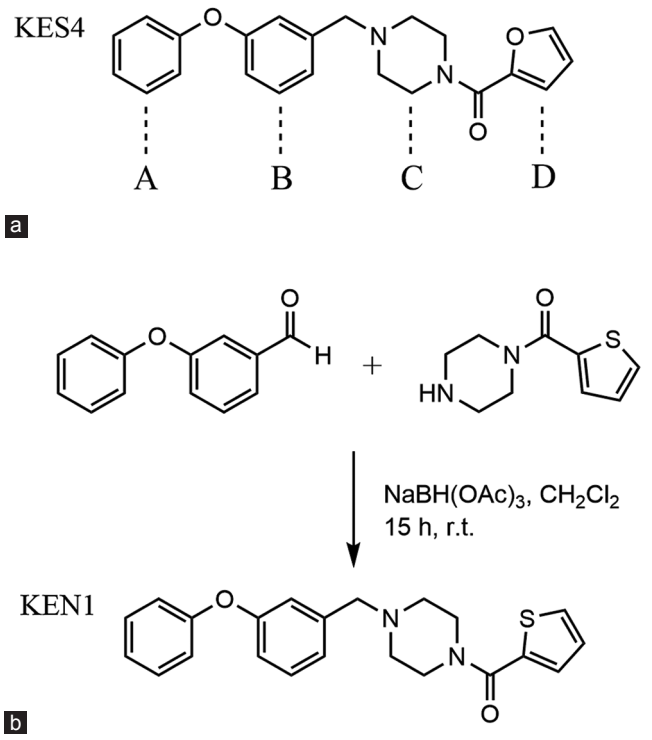

Figure 1: Chemical structures and synthesis of mtInhA inhibitors. (a) Chemical structure of KES4. The phenyl and heterocyclic rings are referred to as A–D-rings. (b) Synthetic scheme of KEN1

Methods

Preparation of compound library and protein structural data

A virtual chemical library comprising 579 compounds of the D-ring-modified KES4 analog was prepared. In brief, the 579 sets of structural data similar to the D-ring segment were extracted from the PubChem database (https://pubchem.ncbi.nlm.nih.gov), based on Fingerprint Tanimoto-based two-dimensional similarity search. Then, the chemical library of D-ring-modified KES4 derivatives was generated using the canonical Simplified Molecular Input Line Entry System (SMILES) notation. Conversion to MOL2-formatted three-dimensional chemical structures, the addition of hydrogen atoms and electric charges, and structural optimization were performed with the Energy Minimize, Protonate 3D, and Energy Minimize modules, respectively, which were implemented in Molecular Operating Environment (MOE) version 2011.10 (Chemical Computing Group, Montreal, Canada). The PDB structural data of mtInhA in complex with pyrrolidine carboxamide derivative (PDB-id 4U0J)[18] were employed in the docking simulation with the following structural adjustments. The hydrogen atoms and electric charges were added using the Protonate 3D module in the MOE software after the removal of water oxygen atoms and the pyrrolidine carboxamide-derived inhibitor from the structure. Then, hydrogen atoms were optimized with the Energy Minimize module in MOE software.

In silico structure-based drug screening

Binding affinity between the 579 compounds in the chemical library and mtInhA substrate-binding cavity was predicted by tandem (two-step) GOLD screening with Genetic Optimization for Ligand Docking (GOLD) suite version 5.3 (Cambridge Crystallographic Data Center, Cambridge, United Kingdom). The primary screening of the compounds with a single conformation identified 49 compounds with a GOLD score above that of KES4 (81.0 as a threshold).[14] Then, ten conformations of each selected compound were generated by the Conformation Search module of MOE, followed by them being subjected to secondary screening. As a result of the secondary screening, seven compounds showed higher scores than the lead compound. Among them, 1-(2-thienyl)-4-(3-phenoxybenzyl) piperazine showed the highest score (82.5) and is hereafter referred to as KEN1. The manner of binding between the compounds and amino acids constituting the mtInhA substrate-binding cavity was estimated using the ligand interaction (LI) and Protein LI Fingerprint (PLIF) modules in MOE software.

Synthesis of 1-(2-thienyl)-4-(3-phenoxybenzyl) piperazine (KEN1)

To a solution of 3-phenoxybenzaldehyde (1 equivalent) in dichloromethane, piperazin-1-yl (thiophen-2-yl) methanone hydrochloride (1.1 equivalent) and sodium acetoxyborohydride (1.2 equivalent) were added. The reaction mixture was stirred at room temperature. After stirring

overnight, the reaction was quenched by the addition of water and was then extracted with chloromethane. The organic layer was washed with brine and was dried with anhydrous Na_2SO_4. The organic solvent was evaporated under reduced pressure to obtain the crude product. The reaction was carried out under an argon atmosphere. The compound was purified by silica gel column chromatography (Kanto Chemical Silica Gel 60N). NMR spectra were recorded on Bruker Avance 500 (Bruker) in $CDCl_3$ (Tetramethylsilane for 1H, $\delta = 0$) or $CDCl_3$ (for 13C, $\delta = 77.0$) was used as an internal standard. Mass spectra were obtained with a JEOL JMS-SX102A mass spectrometer (JEOL). Yield: 46%. ^{1}H NMR ($CDCl_3$, 500 MHz): 7.51–6.91 (m, 12H, Ar-H), 3.75 (s, 4H, $CH_2 \times 2$), 3.52 (s, 2H, CH_2), 2.48 (t, 4H, $CH_2 \times 2$). ^{13}C NMR ($CDCl_3$, 126 MHz): δ (ppm) 163.50, 157.36, 140.20, 139.86, 137.12, 131.92, 130.44, 129.76, 129.61, 128.76, 128.51, 127.60, 126.64, 123.85, 123.26, 122.58, 119.41, 118.84, 117.66; mass: exact mass $C_{22}H_{23}N_2O_2S$ 379.1 (M + H), found 379.

Assay of mtInhA activity

The mtInhA activity was determined by the method reported by Delaine *et al*. with minor modifications.[19] Briefly, the pre-incubation of enzyme and inhibitors was performed in 50 µl (total volume) of 30 mM PIPES (pH 6.8), 150 mM NaCl, 200 µM NADH, 700 nM mtInhA, and compounds (50 µM). dimethyl sulfoxide (DMSO) was used as co-solvent, with a final concentration of <0.5%. After 1 min of pre-incubation at 25.5°C, the addition of 200 µM substrate (trans-2-decenoyl-CoA) initiated the reaction, which was followed at 340 nm (oxidation of NADH) and at 25°C using Infinite M200 PRO microplate reader (TECAN, Männedorf, Switzerland). The inhibitory activity of each compound tested was determined based on the absorbance 5 min after the addition of trans-2-decenoyl-CoA and is expressed as the percentage relative to the mtInhA activity recorded in the absence of inhibitor.

Cytotoxic assay

Madin–Darby canine kidney (MDCK) and SH-SY5Y human neuroblastoma cell lines were maintained in high-glucose Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin and were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The cell lines (MDCK cells, 5.0×10^2 cells/well; SH-SY5Y cells, 1.5×10^4 cells/well) were seeded into 96-well plates 24 h before serum starvation, with 0.25% FBS containing DMEM. After the serum starvation, the candidate compounds (50 µM) were added. DMSO (0.3%) was used as a negative control. The cultures were incubated for an additional 24 or 48 h, followed by the analysis of the cytotoxicity of the compounds with Cell Counting Kit-8 (Dojin, Kumamoto, Japan), as previously described.[14]

Antimicrobial assay

Mycobacterium smegmatis (IAM 12065 strain; RIKEN BioResource Center, Saitama, Japan) was grown at 37ºC for 24 h in 3.7% brain heart infusion broth (Sigma, St. Louis, MO, USA). Cultures were then diluted 30-fold with broth that contained the candidate compounds in a 96-well flat-bottomed clear plate. Each well was inoculated with 200 µL of culture. INH (LKT Laboratories, St. Paul, MN, USA) and 0.3% DMSO were used as positive and negative controls, respectively. The plates were incubated at 37ºC for 24 h, after which the cell cultures were subjected to growth inhibition assays. The inhibition of bacterial growth was determined by measuring OD_{595} using a Bio-Rad Model 680 microplate reader (Bio-Rad, Hercules, CA, USA).

Results

Identification and chemical synthesis of the candidate compound (KEN1)

A chemical library composed of the 579 compounds of the D-ring-modified KES4 derivatives was prepared and subjected to *in silico* SBDS. The GOLD program predicts the binding affinity of compounds to mtInhA active sites (PDBid: 4U0J),[18] represented by the GOLD score, by calculating protein–ligand hydrogen-bonding energy, van der Waals contact energy, and molecule torsion energy based on a genetic algorithm.[20,21] The screening resulted in the identification of eight compounds with GOLD scores (81.0–82.5) higher than that of the lead compound (KES4). The compound with the highest GOLD score [82.5, Table 1], referred to as KEN1, was successfully synthesized at sufficient quantity and purity [Figure 1b].

Cytotoxicity of the compounds

To probe any potential damaging effects of the compounds on mammalian cells, cytotoxicity was measured with the MDCK and SH-SY5Y cell lines [Figure 2]. TCS, a representative InhA inhibitor with a diphenyl ether pharmacophore, was used as a positive control. TCS showed dose-dependent cytotoxicity, while KEN1 did not show significant cytotoxicity on either cell line. Since the FAS-II system is absent in mammals, low toxicity of KEN1 may be preferable for specific antimycobacterial action.

Inhibitory effect of KEN1 on the enzymatic activity of mtInhA

The effect of KEN1 on the enzymatic activity of recombinant mtInhA was examined. As shown in Figure 3 and Table 1, the enzymatic activity of mtInhA was suppressed to 68.0%

Table 1: Effects on *Mycobacterium tuberculosis* enoyl-acyl carrier protein reductase activity and global initiative for chronic obstructive lung disease score of the compounds

Compounds	mtInhA activity (%)*	GOLD score
DMSO	100.0±3.1	-
KES4	68.0±0.5	80.1±0.6
KEN1	42.6±0.8	82.5±0.6

*mtInhA (0.7 µM) was preincubated with each compound (50 µM) at room temperature for 1 min and then its activity was measured. Vehicle (0.3% DMSO) was used as a negative control. All experiments were performed in triplicate and data are expressed as mean±SEM. mtInhA: *Mycobacterium tuberculosis* enoyl-acyl carrier protein reductase, GOLD: Global initiative for chronic obstructive lung disease, SEM: Standard error of mean

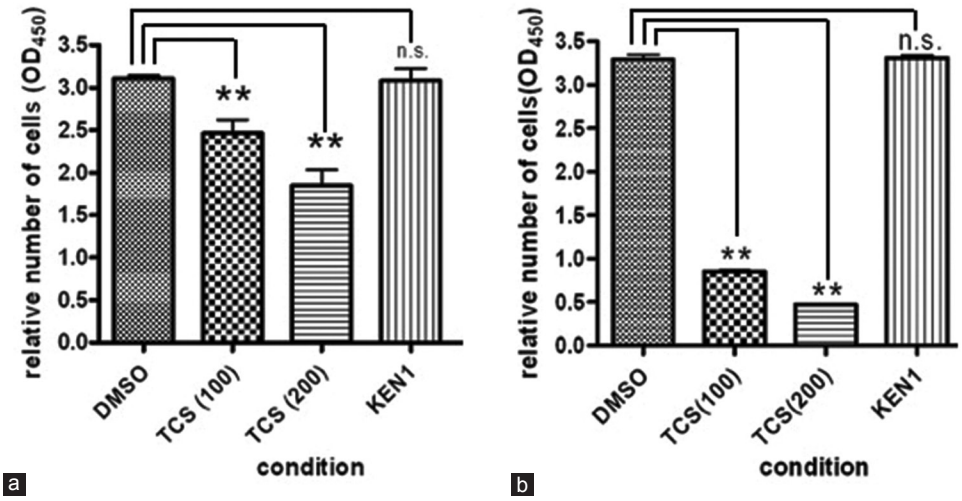

Figure 2: Cytotoxicity of KEN1 on (a) Madin–Darby canine kidney and (b) SH-SY5Y human neuroblastoma cell lines. The concentration of the compounds was 50 μM. Here, 0.3% DMSO and TCS (100 and 200 μM) were used as negative and positive controls, respectively. All experiments were performed in quadruplicate, and cell survival rates were compared using Dunnett's test for significance: $^{**}P < 0.001$

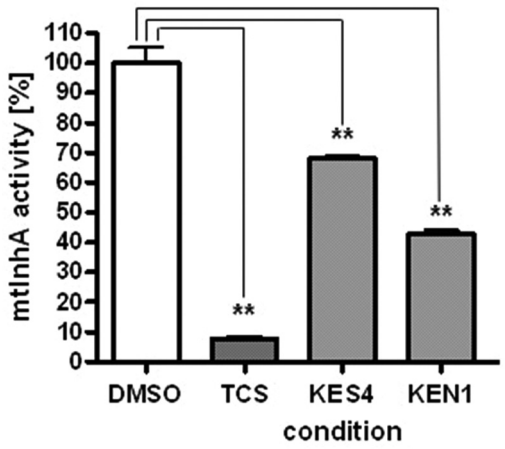

Figure 3: Effects of KES4 and KEN1 on enzymatic activity of mtInhA. mtInhA (0.7 μM) was preincubated with each compound (50 μM) at room temperature for 1 min before its activity was measured. TCS and 0.3% DMSO were used as positive and negative controls, respectively. All experiments were performed in quadruplicate, and data with error bars are expressed as mean ± standard deviation, $^{**}P < 0.05$

in the presence of 50 μM KES4. Interestingly, the mtInhA inhibitory effect of KEN1 was significantly stronger than that of KES4 (inhA activity was decreased to 42.6%). The affinity of the lead compound can be increased by the substitution of the 2-furoyl group of the D-ring by a 2-thienyl group. The results suggest that KEN1 has an advantage over the lead compound in terms of inhibitory activity against mtInhA.

Growth inhibition of *Mycobacterium smegmatis* by KEN1

As a primary evaluation of bioactivity, the effect of KES1 on mycobacterial growth inhibition was examined. *M. smegmatis* (biosafety level 1) was utilized as a model Mycobacterium. *M. smegmatis* InhA has 87% similarity to mtInhA. As shown in Figure 4a, mycobacterial growth was almost completely inhibited in the presence of 50 μM KES4 and KEN1 after 24 h of incubation. As shown in Figure 4b, the IC_{50} value (the concentration at which KEN1 achieves 50% inhibition of *M. smegmatis* growth) of KEN1 (11.8 μM) corresponded to that of KES4 (13.5 μM).

Discussion

In the present study, *in silico* docking simulation with a chemical library comprising a series of D-ring-modified KES4 was performed to obtain a KES4 derivative with high mtInhA inhibitory activity. KEN1, identified as the candidate with the highest GOLD score, showed improved activity regarding mtInhA inhibition [Figure 3 and Table 1]. Our previous simulations predicted that mtInhA forms a complex with KES4 through Phe149 via CH-π interaction and with Leu218 and Tyr158 via hydrogen bonds.[14] The complex-forming manner of KEN1–mtInhA, predicted by the LI and PLIF simulations, was indistinguishable from that of KES4 [Figure 5]. These results indicated that KEN1 and KES4 share similar mechanisms of action and that substitution of the 2-furoyl group (D-ring) by a 2-thienyl group promotes mtInhA inhibition. Despite the difference of mtInhA inhibitory activity, the IC_{50} value of KEN1 corresponded to that of KES4 [Figure 4b]. Since the cell wall of *Mycobacterium* is rich in mycolic acid,[6-9] cell wall permeability should also be considered to improve the antimycobacterial activity of KEN1 in future study.

An increase in TB patients has been attributed to the emergence of drug-resistant strains (multidrug-resistant TB and extensively drug-resistant TB) and the use of inappropriate drug regimens in chemotherapy (i.e., insufficient supply and quality of anti-infectives).[22] There has thus been demand for novel anti-infectives, and there is an urgent need to develop anti-TB drugs with new mechanisms of action. The *in silico*-assisted compound modification performed in this study is expected to be a time,

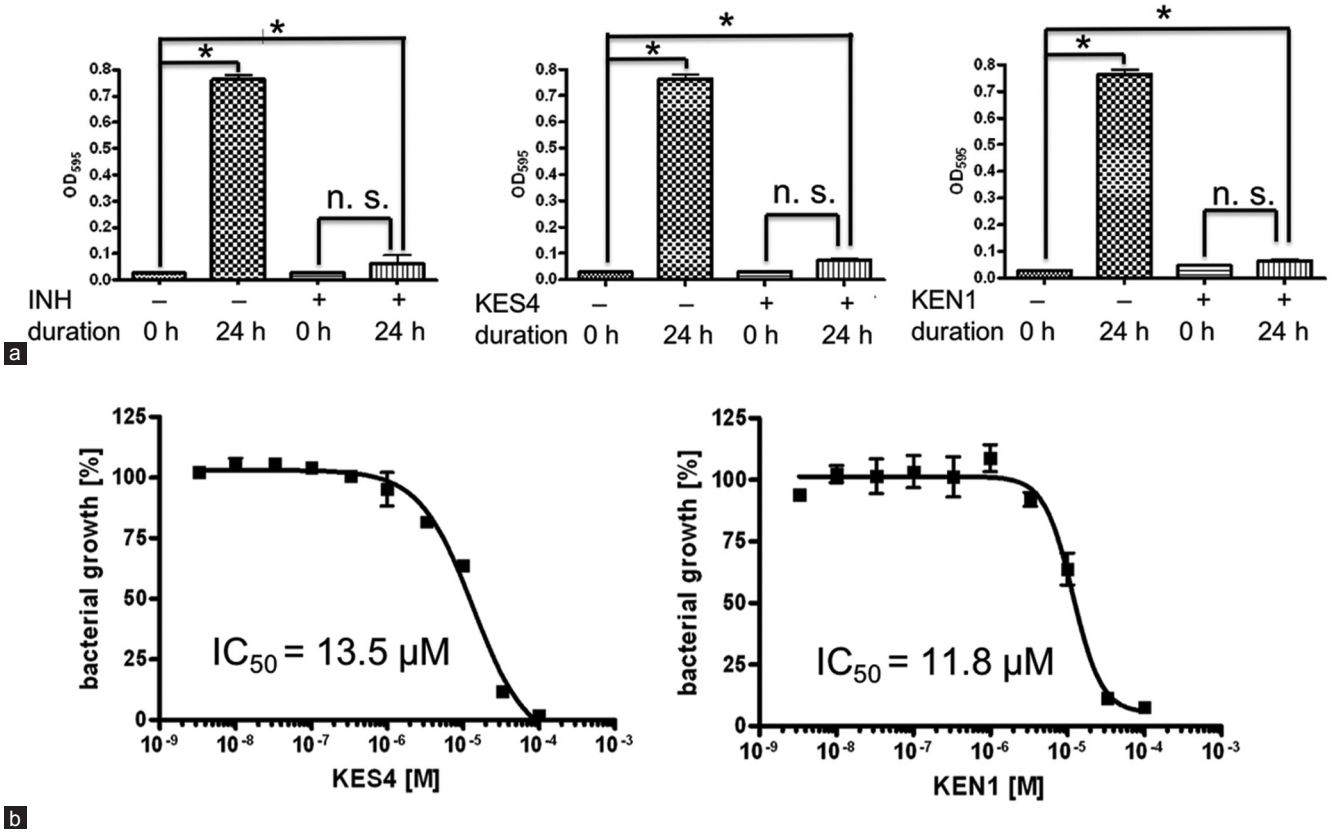

Figure 4: Inhibitory activity of the compounds on the growth of *Mycobacterium smegmatis*. (a) The growth inhibition was monitored by OD_{595} at 0 and 24 h after the treatment using compounds: left, INH (as a positive control); center, KES4; and right, KEN1. The concentration of the tested compounds was 100 μM. All experiments were performed in quadruplicate: nS: Not significant; *$P < 0.05$. (b) Dose-dependent effects of KES4 (left) and KEN1 (right) on the growth of *Mycobacterium smegmatis*. The inhibition of mycobacterial growth was monitored by OD_{595} at 24 h after treatment with the compounds. The IC_{50} value of isoniazid (positive control) is 5.4 μM

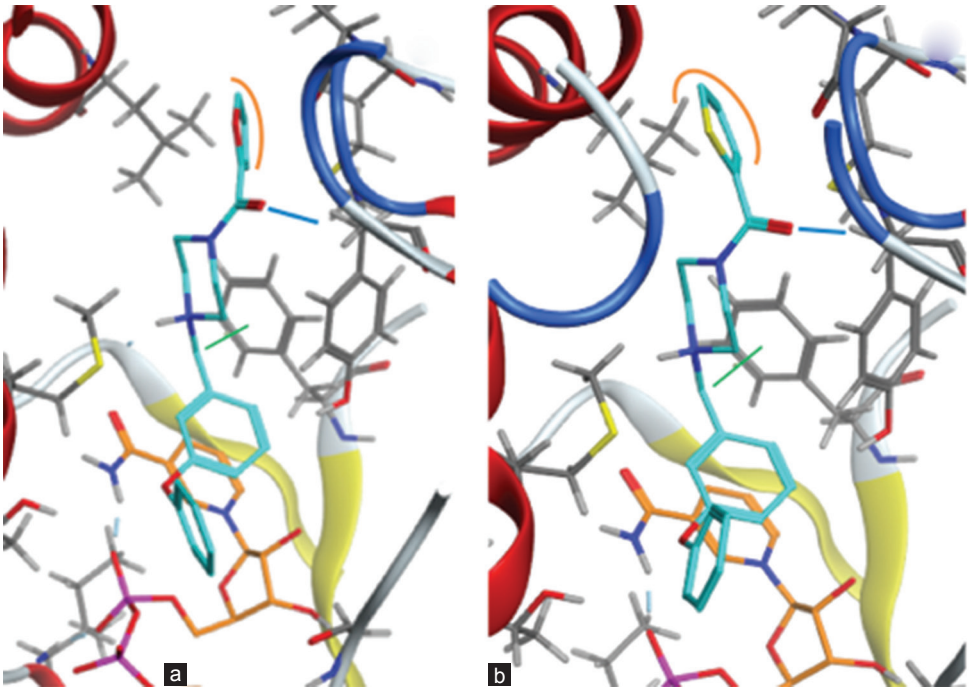

Figure 5: Predicted specific interactions of KEM4 (a) and KEN1 (b) with the active site of mtInhA: The green lines between the hydrogen atoms (between the C-ring and B-ring) and Phe149 indicate CH-π interaction. The orange curves surrounding the 2-furoyl group of KES4 or the 2-thienyl group of KEN1 indicate hydrophobic interactions with Leu218. The blue lines between the D-ring proximal carbonyl groups and Tyr158 indicate hydrogen bonds

cost-, and labor-saving technique for improving lead compounds compared with the conventional structure–activity relationship approach. In addition, the synthesis of a number of compounds can be avoided without deep insights into protein-molecular interaction and organic synthesis.

Acknowledgments

We are grateful to Prof. James C. Sacchettini of Texas A and M University for the preparation of recombinant mtInhA.

Financial support and sponsorship

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (26460145) to SA and (18K05358) to HS from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Conflicts of interest

There are no conflicts of interest.

References

1. Dye C, Williams BG. The population dynamics and control of tuberculosis. Science 2010;328:856-61.
2. Das P, Horton R. Tuberculosis-time to accelerate progress. Lancet 2010;375:1755-7.
3. Das P, Horton R. Tuberculosis-getting to zero. Lancet 2015;386:2231-2.
4. Lönnroth K, Castro KG, Chakaya JM, Chauhan LS, Floyd K, Glaziou P, *et al*. Tuberculosis control and elimination 2010-50: Cure, care, and social development. Lancet 2010;375:1814-29.
5. Bhatt A, Molle V, Besra GS, Jacobs WR Jr., Kremer L. The *Mycobacterium tuberculosis* FAS-II condensing enzymes: Their role in mycolic acid biosynthesis, acid-fastness, pathogenesis and in future drug development. Mol Microbiol 2007;64:1442-54.
6. Tonge PJ, Kisker C, Slayden RA. Development of modern InhA inhibitors to combat drug resistant strains of *Mycobacterium tuberculosis*. Curr Top Med Chem 2007;7:489-98.
7. Rotta M, Pissinate K, Villela AD, Back DF, Timmers LF, Bachega JF, *et al*. Piperazine derivatives: Synthesis, inhibition of the *Mycobacterium tuberculosis* enoyl-acyl carrier protein reductase and SAR studies. Eur J Med Chem 2015;90:436-47.
8. Vilchèze C, Morbidoni HR, Weisbrod TR, Iwamoto H, Kuo M, Sacchettini JC, *et al*. Inactivation of the inhA-encoded fatty acid synthase II (FASII) enoyl-acyl carrier protein reductase induces accumulation of the FASI end products and cell lysis of *Mycobacterium smegmatis*. J Bacteriol 2000;182:4059-67.
9. Brennan PJ, Nikaido H. The envelope of mycobacteria. Annu Rev Biochem 1995;64:29-63.
10. Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, *et al*. inhA, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science 1994;263:227-30.
11. Parikh SL, Xiao G, Tonge PJ. Inhibition of InhA, the enoyl reductase from *Mycobacterium tuberculosis*, by triclosan and isoniazid. Biochemistry 2000;39:7645-50.
12. McMurry LM, McDermott PF, Levy SB. Genetic evidence that InhA of *Mycobacterium smegmatis* is a target for triclosan. Antimicrob Agents Chemother 1999;43:711-3.
13. Quémard A, Sacchettini JC, Dessen A, Vilcheze C, Bittman R, Jacobs WR Jr., *et al*. Enzymatic characterization of the target for isoniazid in *Mycobacterium tuberculosis*. Biochemistry 1995;34:8235-41.
14. Kinjo T, Koseki Y, Kobayashi M, Yamada A, Morita K, Yamaguchi K, *et al*. Identification of compounds with potential antibacterial activity against Mycobacterium through structure-based drug screening. J Chem Inf Model 2013;53:1200-12.
15. Kanetaka H, Koseki Y, Taira J, Umei T, Komatsu H, Sakamoto H, *et al*. Discovery of InhA inhibitors with anti-mycobacterial activity through a matched molecular pair approach. Eur J Med Chem 2015;94:378-85.
16. Taira J, Ito T, Nakatani H, Umei T, Baba H, Kawashima S, *et al*. *In silico* structure-based drug screening of novel antimycobacterial pharmacophores by DOCK-GOLD tandem screening. Int J Mycobacteriol 2017;6:142-8.
17. Taira J, Morita K, Kawashima S, Umei T, Baba H, Maruoka T, *et al*. Identification of a novel class of small compounds with anti-tuberculosis activity by *in silico* structure-based drug screening. J Antibiot (Tokyo) 2017;70:1057-64.
18. He X, Alian A, Stroud R, Ortiz de Montellano PR. Pyrrolidine carboxamides as a novel class of inhibitors of enoyl acyl carrier protein reductase from *Mycobacterium tuberculosis*. J Med Chem 2006;49:6308-23.
19. Delaine T, Bernardes-Génisson V, Quémard A, Constant P, Meunier B, Bernadou J. Development of isoniazid-NAD truncated adducts embedding a lipophilic fragment as potential bi-substrate InhA inhibitors and antimycobacterial agents. Eur J Med Chem 2010;45:4554-61.
20. Jones G, Willett P, Glen RC. Molecular recognition of receptor sites using a genetic algorithm with a description of desolvation. J Mol Biol 1995;245:43-53.
21. Jones G, Willett P, Glen RC, Leach AR, Taylor R. Development and validation of a genetic algorithm for flexible docking. J Mol Biol 1997;267:727-48.
22. Sotgiu G, Centis R, D'ambrosio L, Migliori GB. Tuberculosis treatment and drug regimens. Cold Spring Harb Perspect Med 2015;5:a017822.