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Halicyclamine A, a marine spongean alkaloid as a lead for anti-tuberculosis agent

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ABSTRACT

In the course of our search for anti-microbial agents against dormant *Mycobacterium tuberculosis*, halicyclamine A was re-discovered as a lead for anti-tuberculosis agent from a marine sponge of *Haliclona* sp. on the guidance of the constructed bioassay. Halicyclamine A showed growth inhibition against *Mycobacterium smegmatis*, *Mycobacterium bovis* BCG, and *M. tuberculosis* H37Ra with MICs in the range of 1.0–5.0 μg/ml under both aerobic condition and hypoxic condition inducing dormant state. The growth-inhibitory activity of halicyclamine A was bactericidal, and halicyclamine A did not exhibit cross-resistance with the currently used anti-tuberculosis drugs of isoniazid, ethambutol, rifampicin, and streptomycin. Halicyclamine A has been isolated originally as one of the active constituents inhibiting inosine 5′-monophosphate dehydrogenase (IMPDH). Then, in order to elucidate action-mechanism of halicyclamine A, we prepared IMPDH over-expressing strains of *M. smegmatis*. However, IMPDH was not target for halicyclamine A, because halicyclamine A showed same MIC value against the wild-type *M. smegmatis* and IMPDH over-expressing strains.

1. Introduction

Tuberculosis (TB) is one of the most common causes of morbidity and death in HIV-positive adults living in less-developed countries.[1] Eight million new TB cases and two million deaths by TB are estimated each year.[2] It is now generally accepted that the requirement for minimum 6 months treatment for TB is due to the difficulty in eradicating non-replicating persistent *Mycobacterium tuberculosis*. Although physiology of the latent *M. tuberculosis* infections is still unclear, hypoxic condition was found to induce dormant state of *Mycobacterium* sp., which has a drug susceptibility profile resembling that of latent *M. tuberculosis* infections.[3–6] Therefore, the lead compounds, which are effective to *M. tuberculosis* in both active state and dormant state, are urgently needed.

More than 350 of natural products or their derivatives have been reported as anti-*mycobacterial* agents from 2003 to 2005.[7] However, most of the publications focused on growth-inhibitory activity against *M. tuberculosis* in active state. To explore new leads of anti-*mycobacterial* agent, which is effective to *M. tuberculosis* in both active and dormant states, we established a screening system in hypoxic condition inducing dormant state.[8] On the guidance of this bioassay, we have re-discovered halicyclamine A as anti-*M. tuberculosis* agent in dormant state from Indonesian marine sponge of *Haliclona* sp. Halicyclamine A has been isolated originally from a marine sponge of *Haliclona* sp. as one of the active constituents having inhibitory activity of inosine 5′-monophosphate dehydrogenase (IMPDH).[9] In order to elucidate whether the target molecule of halicyclamine A for anti-tuberculosis activity is IMPDH or not, we constructed IMPDH over-expressing strains by using *Mycobacterium smegmatis*. In this paper, we describe anti-*mycobacterial* activity and biological property of halicyclamine A.

2. Result

2.1. MIC of halicyclamine A and isoniazid against *Mycobacterium* sp. under hypoxic condition inducing dormant state

The dormant *M. tuberculosis* was highly resistant against isoniazid, which inhibits inhA of type II fatty acid biosynthetic enzyme.[6,10,11] The MIC values of isoniazid against *M. smegmatis*, *Mycobacterium bovis* BCG, and *M. tuberculosis* H37Ra were 2.5 μg/ml, 0.03 μg/ml, and 0.05 μg/ml under aerobic condition,

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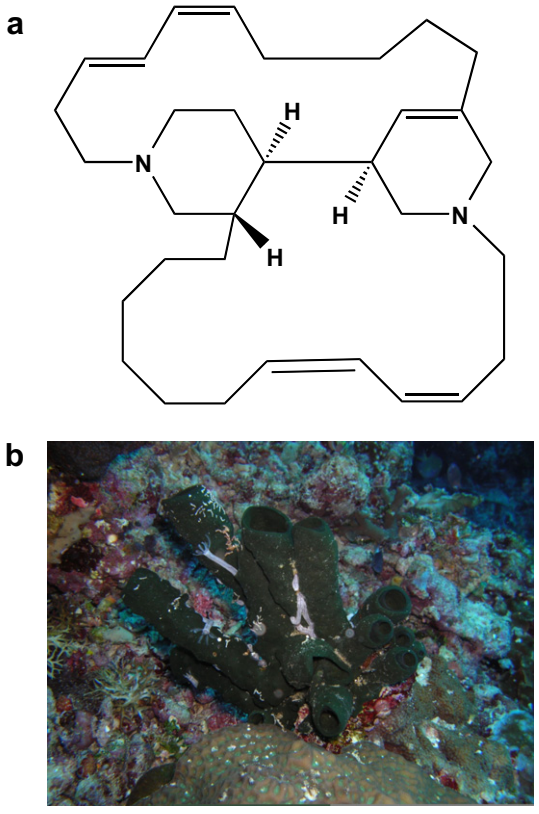

Figure 1. Structure of halicyclamine A (a) and picture of Indonesian marine sponge *Haliclona* sp. 05A08 (b).

respectively. While the MIC value of isoniazid against these strains was more than 25 μg/ml under nitrogen atmosphere containing 0.2% oxygen. On the other hand, the MIC values of halicyclamine A (Fig. 1) were 2.5 μg/ml, 1.0 μg/ml, and 5.0 μg/ml against *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* H37Ra under both aerobic and hypoxic conditions, respectively (Table 1). These results indicated that halicyclamine A was effective against *Mycobacterium* sp. in both actively growing and dormant states.

2.2. Effect of halicyclamine A against various pathogenic tubercle bacilli

Next, we investigated effect of halicyclamine A against various pathogenic *Mycobacterium* strains (Table 2). The MIC value of halicyclamine A against *M. tuberculosis* H37Rv was 6.25 μg/ml, and halicyclamine A showed similar MIC values in the range of 3.13–6.25 μg/ml against drug resistant *M. tuberculosis* H37Rv strains. These findings indicated that halicyclamine A would have a different action-mechanism with isoniazid, ethambutol, rifampicin, and streptomycin. Halicyclamine A also inhibited growth of *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, and *Mycobacterium aurum* as well as *M. tuberculosis* H37Rv with

Table 1
Growth-inhibitory effect of halicyclamine A and isoniazid under hypoxic condition

Strains	MIC (μg/ml)			
	Halicyclamine A		Isoniazid	
	Aerobic	Hypoxic	Aerobic	Hypoxic
M. smegmatis	2.5	2.5	2.5	25
M. bovis BCG	1.0	1.0	0.03	>100
M. tuberculosis H37Ra	5.0	5.0	0.05	>100

Table 2
Growth-inhibitory effect of halicyclamine A against various pathogenic mycobacterial bacilli

Strain	MIC (μg/ml)
M. tuberculosis H37Rv ATCC25618	6.25
M. tuberculosis H37Rv ETH^r ATCC35837	3.13
M. tuberculosis H37Rv INH^r ATCC35822	3.13
M. tuberculosis H37Rv RIF^r ATCC35838	3.13
M. tuberculosis H37Rv STR^r ATCC35820	6.25
M. tuberculosis Kurono ATCC35812	3.13
M. avium ATCC35712	6.25
M. intracellulare ATCC35761	6.25
M. kansasii ATCC35775	3.13
M. fortuitum ATCC9820	25
M. aurum ATCC23366	6.25

ETH^r: ethambutol resistant, INH^r: isoniazid resistant. RIF^r: rifampicin, STR^r: streptomycin resistant.

MIC values of 3.13–6.25 μg/ml. While, halicyclamine A showed moderate inhibitory activity against *M. fortuitum* with MIC value of 25 μg/ml. It is expected that halicyclamine A would also be effective to non-tuberculous mycobacteriosis.

2.3. Bactericidal effect of halicyclamine A against *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* H37Rv

To examine whether the anti-mycobacterial activity of halicyclamine A is bactericidal or bacteriostatic, colony forming unit (CFU) assay was executed in the aerobic condition. As shown in Fig. 2, the CFU of *M. smegmatis* decreased in time dependent fashion in the presence of 10 μg/ml halicyclamine A, and the colony was not detected after 48 h incubation. The same effect was observed against *M. bovis* BCG. The colony of *M. bovis* BCG was not detected after 6 days incubation. In the case of *M. tuberculosis* H37Rv, the growth-inhibitory effect was observed by the 20 μg/ml treatment of halicyclamine A, and the CFU of *M. tuberculosis* H37Rv was less than 10 CFU/ml from day 11 to day 28. These data indicated that halicyclamine A exhibits bactericidal effect against these mycobacterial bacilli.

2.4. Computer analysis of IMPDH gene in *M. tuberculosis* and *M. smegmatis*

From BLAST search on the human IMPDH-1 gene, *M. tuberculosis* H37Rv was clarified to have three IMPDH-related genes, which have been designed as *guaB1*, *guaB2*, and *guaB3*. Similar genes with higher homology named *MSMEG_3633*, *MSMEG_1602 (guaB)*, *and MSMEG_1603* were also found in the genome of *M. smegmatis*. *GuaB1*, *guaB2,* and *guaB3* in the genome of *M. tuberculosis* H37Rv were supposed to correspond to *MSMEG_3633*, *MSMEG_1602 (guaB),* and *MSMEG_1603* in genome of *M. smegmatis*, respectively (Fig. 3). These findings suggested that *M. tuberculosis* and *M. smegmatis* exhibit IMPDH gene as well as mammalian cells.

2.5. MIC of halicyclamine A against guaBs genes over-expressed *M. smegmatis*

In order to confirm whether the target protein of halicyclamine A is IMPDH of *M. smegmatis* or not, over-expressed strains of *guaBs* genes, which were expected to be resistant against halicyclamine A, were generated by using *M. smegmatis*. MIC values of halicyclamine A against ICHO1002, ICHO1003, and ICHO1004 were same with that for the wild-type of *M. smegmatis* and ICHO1001, respectively (Table 3). These data indicated that the target molecule of halicyclamine A was not IMPDH.

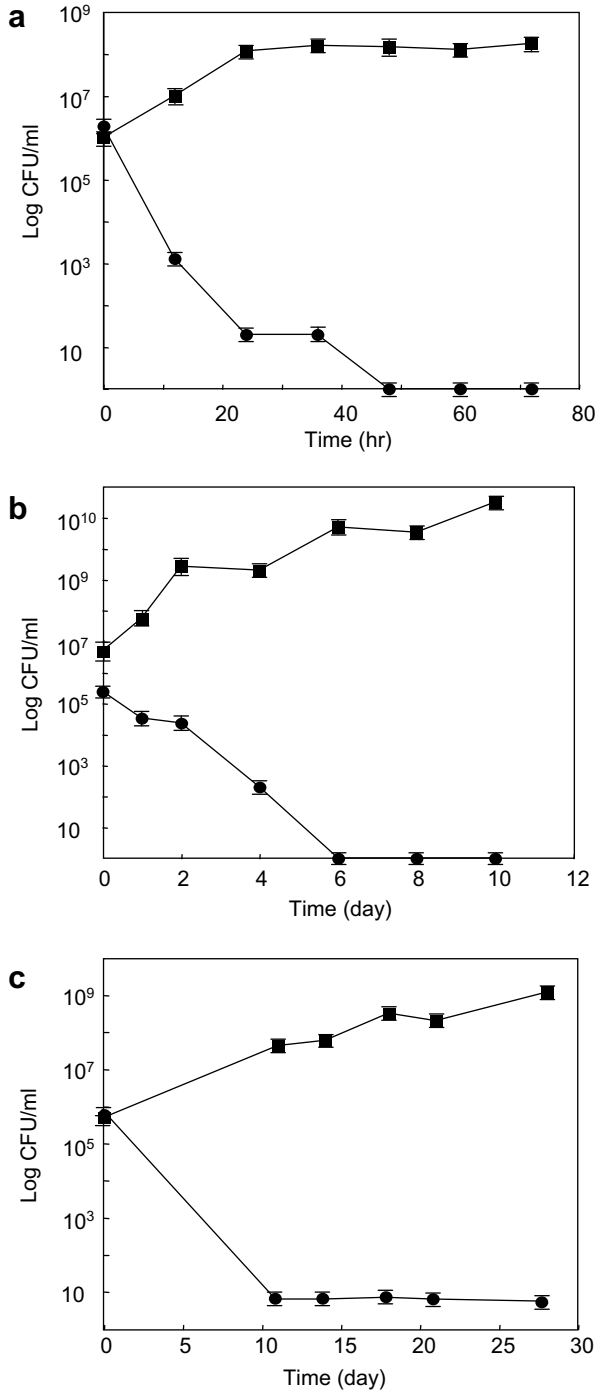

Figure 2. Bactericidal effect of halicyclamine A against *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* H37Rv. Mycobacterial bacilli were adjusted to 1×10^6 CFU/ml by Middlebrook 7H9 broth. The culture was incubated in the presence (closed circle) or absence (closed square) of halicyclamine A at 37 °C for indicated time. The portion (100 μl) was collected at each time point, and serial diluted culture was plated on the Middlebrook 7H10 agar. Number of colony was counted after 4 days incubation for *M. smegmatis* (a) or 4 weeks incubation for *M. bovis* BCG (b) and *M. tuberculosis* H37Rv (c).

3. Discussion

Although a lot of progresses regarding characterization of the dormant state of *M. tuberculosis*, the search for anti-tuberculosis agents against dormant *M. tuberculosis* is not paid much attention. In fact, among the compounds under clinical study, only nitroimidazopyran PA-824 was clarified to inhibit growth of dormant *M. tuberculosis*, which was induced by hypoxia condition.[12] In our search for anti-dormant mycobacterial agent, we constructed a bioassay system using 96-well plate. We screened the library of the sponge extracts and isolated a tetracyclic diamine alkaloid, halicyclamine A, as an active substance from Indonesian marine sponge of *Haliclona* sp. on the basis of bioassay-guided separation.

Halicyclamine A showed growth inhibition against *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* H37Ra with MICs in the range of 1.0–5.0 μg/ml under both aerobic condition and hypoxic condition inducing dormant state. While isoniazid exhibiting strong growth-inhibitory activity against these mycobacterial bacilli showed only weak MIC values (more than 25 μg/ml) against dormant bacilli (Table 1). As shown in Fig. 2, the CFU of *M. smegmatis* and *M. bovis* BCG decreased in time dependent fashion in the presence of 10 μg/ml halicyclamine A, and the colony was not detected after 48 h incubation. In the case of *M. tuberculosis* H37Rv, the growth-inhibitory effect was observed by the 20 μg/ml treatment of halicyclamine A, and the CFU of *M. tuberculosis* H37Rv was less than 10 CFU/ml from day 11 to day 28. These data indicated that halicyclamine A exhibits bactericidal effect against these mycobacterial bacilli. Furthermore, halicyclamine A did not exhibited cross-resistance with the currently used anti-tuberculosis drugs of isoniazid, ethambutol, rifampicin, and streptomycin (Table 2).

Jaspars et al. isolated halicyclamine A from a marine sponge of *Haliclona* sp., whose extract showed inhibitory activity against inosine 5′-monophosphate dehydrogenase (IMPDH). IMPDH catalyzes conversion of inosine monophosphate (IMP) to xanthosine monophosphate (XMP) in the de novo synthesis of guanine nucleotides (Fig. 3).[9] In order to clarify whether the target molecule concerning with anti-mycobacterial activity of halicyclamine A is also IMPDH or not, the following study has been executed. From BLAST search, we found *M. tuberculosis* (and *M. smegmatis*) has three homologous genes with human IMPDH-1, which were named as *guaB1* (*MSMEG_3633* for *M. smegmatis*), *guaB2* (*MSMEG_1602* for *M. smegmatis*), and *guaB3* (*MSMEG_1603* for *M. smegmatis*), with approximately 40% sequence similarity. The over-expressed strains of guaBs proteins were expected to become resistant against halicyclamine A. Then, we constructed *guaB1*, *guaB2*, or *guaB3* over-expressed strains of *M. smegmatis*, respectively, and compared MIC values with that of the wild-type strain. As shown in Table 3, halicyclamine A showed same MIC value against the wild-type *M. smegmatis* and the *guaB1*, *guaB2*, or *guaB3* genes over-expressed strains (ICHO1002, ICHO1003, and ICHO1004). Furthermore, halicyclamine A also showed same MIC value against the wild-type *M. smegmatis* in the presence of xanthosine monophosphate (XMP), which is a substrate for the enzyme in the downstream of IMPDH (data not shown). This result indicated that the target molecule of anti-mycobacterial activity of halicyclamine A is not IMPDH. The further analysis of molecular target of halicyclamine A is currently under way.

4. Experimental

4.1. General materials

Middlebrook 7H9 broth, Middlebrook 7H10 agar, Middlebrook OADC Enrichment, and Luria–Bertani (LB) broth were obtained from BD (Franklin Lakes, NJ). DNA restriction enzymes and T4 DNA ligase were obtained from New England BioLabs, Inc. (Ipswich, MA). Expand High Fidelity PCR System (Roche Applied Science, Mannheim, Germany) was used for PCR. Isoniazid, kanamycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals were purchased from Sigma (St. Louis, MO).

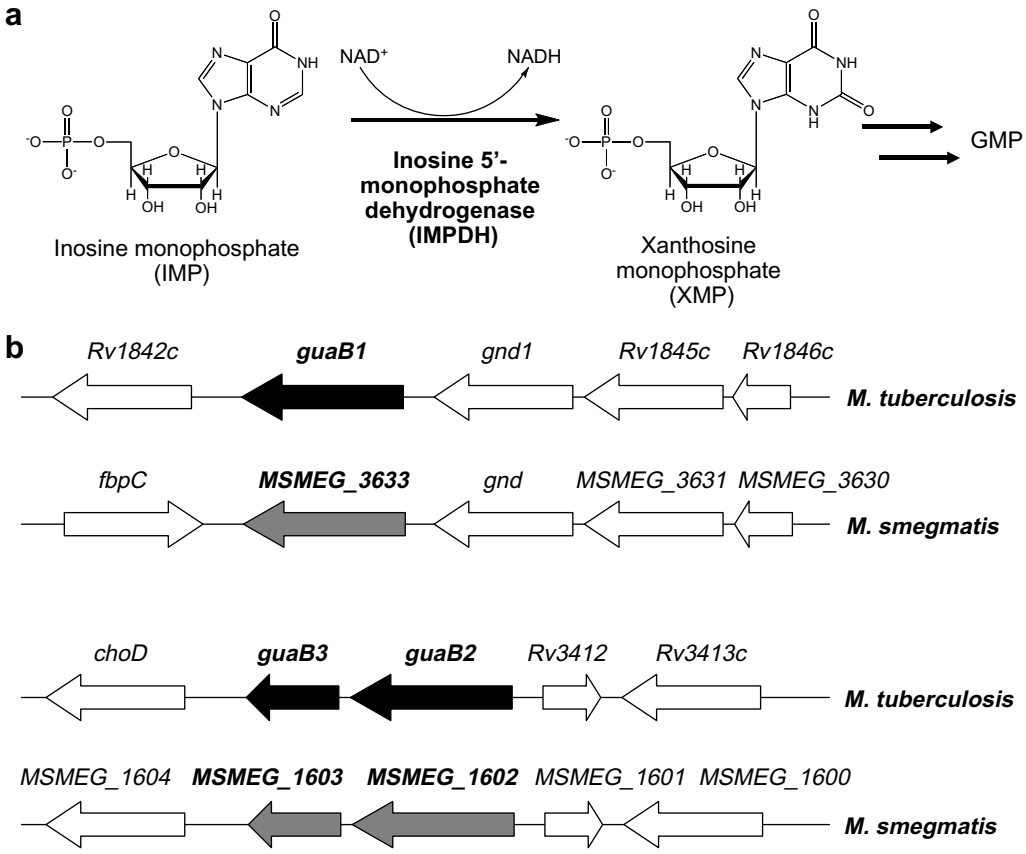

Figure 3. Function of inosine 5′-monophosphate dehydrogenase (IMPDH) and homolog of IMPDH in *M. tuberculosis* and *M. smegmatis*. (a) Enzymatic reaction of IMPDH. (b) Gene region of homolog of IMPDH in *M. tuberculosis* and *M. smegmatis*.

Table 3
MIC of halicyclamine A against *guaBs* genes over-expressed strains

Strains	MIC (μg/ml)
M. smegmatis	2.5
ICHO1001	2.5
ICHO1002	2.5
ICHO1003	2.5
ICHO1004	2.5

4.2. Isolation of halicyclamine A from Indonesian marine sponge of *Haliclona sp.*

We have constructed a bioassay method searching for growth-inhibitory agents against mycobacterial bacilli in dormant state. From the bioassay-guided separation, halicyclamine A was isolated from Indonesian marine sponge of *Haliclona* sp. 05A08 (Fig. 1). Briefly, the methanol extract (43 g) of the dry sponge (200 g) were partitioned by alkaloid extraction procedure. The alkaloid fraction (2.5 g) was subjected to silica gel column chromatography (eluted with $CHCl_3$–MeOH containing 1.0% triethylamine) and HPLC [COSMOSIL Sugar-D column (Nacalai tesque, Kyoto, Japan); eluted with $CHCl_3$–CH_3CN–H_2O] to isolate halicyclamine A (20% yield from the alkaloid fraction). Halicyclamine A was identified by ESI-TOF-MS and 2D-NMR analyses and comparison with authentic spectral data.[9,13]

4.3. Bacterial strains and culture

Escherichia coli DH5alpha was used for cloning and maintaining plasmid and was grown in LB liquid medium. *M. tuberculosis* H37Rv and other mycobacterial strains were grown in Middlebrook 7H9 broth containing 10% OADC, 0.5% glycerol and 0.05% Tyloxapol or on Middlebrook 7H10 agar containing 10% OADC and 0.5% glycerol. *M. smegmatis* mc^2155 was grown in LB liquid medium with 0.05% Tween 80 for competent cell preparation. Kanamycin was added into medium in the following concentration when required; kanamycin 40 μg/ml for *E. coli* and 20 μg/ml for *Mycobacterium* strains.

4.4. Determination of MIC value under aerobic and hypoxic conditions

Determination of minimum growth-inhibitory concentration (MIC) values against *M. smegmatis* mc^2155, *M. bovis* BCG Pasteur, and *M. tuberculosis* H37Ra was performed by the established MTT method.[14] Mid-log phase of *M. smegmatis* (1×10^4 CFU/0.1 ml) or other strains (1×10^5 CFU/0.1 ml) were inoculated in 96-well plate, and then serial diluted sample was added to the 96-well plate. In the case of aerobic condition, the bacteria were incubated at 37 °C for 24 h (for *M. smegmatis*) or for 7 days (for other strains). On the other hand, the hypoxic model was performed based on the description by Rustad et al.[8] The mycobacterial bacilli were grown in Middlebrook 7H9 broth at 37 °C under nitrogen atmosphere containing 0.2% oxygen until the optical density reached 0.8 at 600 nm. Then, the bacilli were inoculated to the 96-well plate in the same density as the aerobic condition and incubated at 37 °C under nitrogen atmosphere containing 0.2% oxygen for 96 h (for *M. smegmatis*) or for 14 days (for other strains). After incubation, 50 μl of MTT solution (0.5 mg/ml) was added into each well and incubated at 37 °C for additional 12 h under aerobic or hypoxic conditions. The optical density at 560 nm was measured to determine MIC value.

Susceptibility testing against various pathogenic *Mycobacterium* strains were carried out by using a procedure previously reported.[15] Briefly, bacteria stocks preserved in a deep freezer were dissolved and adjusted to approximately 1×10^6 CFU/ml, respectively. The designated bacterial suspension were inoculated into the test sample-containing plates (approximately 1×10^6 CFU/ml) by using a multipoint inoculator (Sakuma Seisakusho, Tokyo, Japan). Each plate was incubated at 37 °C for 14 days and analyzed to determine MIC value. The MIC value was expressed as the lowest concentration that inhibited visible growth of organism on the agar plate after incubation.

4.5. Bactericidal effect of halicyclamine A

To examine whether the growth-inhibitory activity of halicyclamine A against *M. smegmatis*, *M bovis* BCG, and *M. tuberculosis* H37Rv is bactericidal or bacteriostatic, CFU assay was executed. The cell suspension in Middlebrook 7H9 broth was adjusted to 1×10^6 CFU/ml, and then the indicated concentration of halicyclamine A was added. The 100 μL portion was collected at each time point, and serial diluted culture was plated on the Middlebrook 7H10 agar to measure CFU. Number of colony was counted after 4 days incubation for *M. smegmatis* or 4 weeks incubation for *M. bovis* BCG and *M. tuberculosis* H37Rv.

4.6. Computer analysis of IMPDH gene in *M. tuberculosis* and *M. smegmatis*

The primary sequence of human inosine 5′-monophosphate dehydrogenase (IMPDH)-1 was obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The primary sequences of *guaB1*, *guaB2*, and *guaB3* genes of *M. tuberculosis* were obtained from TubercuList (http://genolist.pasteur.fr/TubercuList/), and the database of Comprehensive Microbial Resource in J. Craig Venter Institute (http://cmr.tigr.org/cgi-bin/CMR/CmrHomePage.cgi) was used for BLAST search and for obtaining genome sequence of *M. smegmatis*.

4.7. Preparation of guaB1 gene, guaB2 gene, or guaB3 gene over-expressed *M. smegmatis*

Genomic DNA from *M. smegmatis* was prepared by hexadecyltrimethylammonium bromide (CTAB) method.[16] The open reading frame of *MSMEG_3633*, *MSMEG_1602*, and *MSMEG_1603* were PCR amplified from genomic DNA of *M. smegmatis* by using the primer pairs S_guaB1_F (5′-AAGCTTTCGACTCCGTGAGGTTTC-3′) and S_guaB1_R (5′-ATCGATCTGGCCTACCCGTCAC-3′) as *MSMEG_3633*, S_guaB2_F (5′-GAATTCGACTCGGCTGTGGAAATGC-3′) and S_guaB2_R (5′-ATCGATTCAGCGGGTGTAGTTGG-3′) as *MSMEG_1602* and S_guaB3_F (5′-AAGCTTCAGTCATGCGTGACATGG-3′) and S_guaB2_R (5′-ATCGATAGCTGTCGGCTCAACTG-3′) as *MSMEG_1603*. PCR was performed using a program of 30 cycles of 95 °C for 1 min, 58 °C for 0.5 min and 72 °C for 1.5 min. Following cloning into pCR2.1-TOPO (Invitrogen) and sequencing, the cloned PCR fragments were excised using the primer-introduced restriction sites and cloned into the mycobacterial shuttle plasmid vector pMV261.

Mycobacterium smegmatis were grown at 37 °C as described above until the optical density reached 0.8–1.0 at 600 nm. The cultures were centrifuged, and the resulting pellets were washed with 10% glycerol twice and re-suspended in same solution (1/10th of the initial culture volume). The cell suspensions were mixed with plasmid DNA and electroporated (2500 V, 25 μF, 1000 Ω). The resulting suspensions were incubated at 37°C for 4 h, and then plated on Middlebrook 7H10 containing 20 μg/ml of kanamycin. In order to confirm whether *M. smegmatis* was transformed with the *guaBs* genes plasmids or not, the plasmid was isolated from the transformant of kanamycin resistant, and then the plasmids were treated by primer-introduced restriction enzymes.

The transformant of *M. smegmatis* with pMV261, *guaB1 gene* over-expressed strain, *guaB2 gene* over-expressed strain, and *guaB3 gene* over-expressed strain were designed ICHO1001, ICHO1002, ICHO1003, and ICHO1004, respectively.

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