Nosiheptide Harbors Potent In Vitro and Intracellular Inhibitory Activities against Mycobacterium tuberculosis

Xia Yu,a Rui Zhu,a Zhi Geng,b Yaoyao Kong,a Fen Wang,a Lingling Dong,a Liping Zhao,a Yi Xue,a Xiaochi Ma,c Hairong Huanga

aNational Clinical Laboratory on Tuberculosis, Beijing Key Laboratory for Drug-Resistant Tuberculosis Research, Beijing Chest Hospital, Capital Medical University, Beijing, China
bBeijing Synchrotron Radiation Facility, Institute of High Energy Physics, Chinese Academy of Sciences, Beijing, China
cPharmaceutical Research Center, Second Affiliated Hospital, Dalian Medical University, Dalian, China

ABSTRACT Multidrug-resistant tuberculosis (MDR-TB) is often associated with poor clinical outcomes. In this study, we evaluated the potential of nosiheptide (NOS) as a new drug candidate for treating Mycobacterium tuberculosis infections, including MDR-TB. The antimicrobial susceptibility testing was performed to determine the MICs of NOS against 18 reference strains of slowly growing mycobacteria (SGM) and 128 clinical isolates of M. tuberculosis. The postantibiotic effects (PAE) and interaction with other antituberculosis drugs of NOS were also evaluated using M. tuberculosis H37Rv. Fifteen out of the 18 tested reference strains of SGM had MICs far below 1 μg/mL. From the 128 M. tuberculosis clinical isolates, the MIC50 and MIC90 were 0.25 μg/mL and 1 μg/mL, respectively; the tentative epidemiological cutoff (ECOFF) was defined at 1 μg/mL. Furthermore, a Lys89Thr mutation was found in one M. tuberculosis isolate with a MIC of NOS 8 μg/mL. After 24 h of incubation, NOS at 1 μg/mL inhibited 25.79 ± 1.22% of intracellular bacterial growth, which was comparable with the inhibitory rate of 25.71 ± 3.67% achieved by rifampin at 2 μg/mL. Compared to rifampicin and isoniazid (INH), NOS had a much longer PAE, i.e., a value of about 16 days. In addition, a partial synergy between NOS and INH was observed. NOS has potent inhibitory activities against M. tuberculosis in vitro as well as in macrophages. Furthermore, the long PAE and partial synergistic effect with INH, in addition to the added safety of long-term use as a feed additive in husbandry, provide support for NOS being a promising drug candidate for tuberculosis treatment.

IMPORTANCE This study is aimed at chemotherapy for MDR-TB, mainly to explore the anti-TB activity of the existing chemotherapeutic reagent. We found that NOS has potent inhibitory activities against M. tuberculosis in vitro regardless of the drug-resistant profile. Furthermore, NOS also showed the long PAE and partial synergistic effect with INH and is nontoxic, providing support for its promise as a drug candidate for drug-resistant tuberculosis treatment.

KEYWORDS Mycobacterium tuberculosis, nosiheptide, antimicrobial activity, intracellular activity

Tuberculosis (TB) is a big challenge to public health, and 10 million new cases were estimated in 2020 worldwide by World Health Organization (WHO) (1). Multidrug-resistant tuberculosis (MDR-TB), defined as resistance to isoniazid (INH) and rifampin (RIF) simultaneously, is increasing worldwide. MDR-TB is often associated with poor clinical outcomes (2–4). However, very limited new drugs (i.e., bedaquiline, delamanid, and pretomanid) have been developed for the treatment of drug-resistant TB in the past 50 years (5, 6), which underlines the difficulties in developing new anti-TB drugs. Therefore, exploring the anti-TB activity of the existing chemotherapeutic reagent is a more efficient and time-saving strategy for improving anti-TB drug development.
Nosipeptide (NOS) is a sulfur-containing polypeptide and belongs to a family of thiazole antibiotics, which was initially isolated from *Streptomyces actuosus* (7). The antibacterial mechanism of NOS is inhibiting protein synthesis by binding between ribosomal proteins L11 (rplK) and 23S rRNA on the 50S subunit of the ribosome and inhibiting the GTP hydrolysis catalyzed by elongation factor EF-G (8–10). The mechanism of this action is unique and distinct from those of all the other current chemotherapeutics targeting the bacterial ribosome. Therefore, cross-resistance between them would be less expected. NOS is proven to have inhibitory activities against several Gram-positive bacteria and is considered a promising drug candidate (11). Furthermore, a previous study (12) also showed that NOS could significantly inhibit the growth of *Mycobacterium avium* complex (a common pathogenic slowly growing mycobacteria [SGM]) in vitro and in the silkworm model.

To better understand the capacity of NOS against *Mycobacterium tuberculosis* and other SGM, we determined its MICs against 18 SGM reference strains and 128 clinical isolates of *M. tuberculosis* collected in Beijing, China. We investigated the sequences of the reported NOS-resistant genes of *rplK* and 23S rRNA among the clinical isolates to identify their relationships with NOS resistance. The bactericidal activity against *M. tuberculosis* in macrophages was also analyzed. Furthermore, the postantibiotic effect (PAE) of NOS and interactions between NOS and other main anti-TB drugs were also characterized.

**RESULTS**

**MICs of NOS against the reference strains.** The MICs of the 18 SGM reference strains for NOS are presented in Table 1. NOS demonstrated consistently strong antibacterial activity against 83.33% (15/18) of all the tested SGM species, with MICs far below 1 μg/mL, except for *Mycobacterium asiaticum* (MIC = 2 μg/mL), *Mycobacterium marinum*, and *Mycobacterium nonchromogenicum* (MIC >8 μg/mL).

**MICs of NOS against *M. tuberculosis* isolates.** A total of 128 clinical *M. tuberculosis* isolates, including 84 MDR-TB strains and 44 non-MDR-TB, were randomly selected to determine in vitro susceptibility. Among the MDR strains, 35 strains were defined as “simple MDR-TB” with susceptibility to fluoroquinolone or three second-line injectable drugs (capreomycin, kanamycin, and amikacin), whereas the other 49 strains were “MDR-TB plus,” which had additional resistance to any fluoroquinolone and at least one of three second-line injectable drugs.

NOS exhibited potent activity against all the tested *M. tuberculosis* isolates, including MDR-TB strains; the MICs of NOS against *M. tuberculosis* isolates are summarized in Table 2. Overall, most of the isolates tested (96.09%, 123/128) had MICs ≤1 μg/mL; the MIC$_{50}$ and MIC$_{90}$ of the *M. tuberculosis* isolates were 0.125 μg/mL and 1 μg/mL, respectively.

Against MDR-TB isolates, the MIC$_{90}$ of NOS (0.5 μg/mL) was lower than non-MDR isolates (1.0 μg/mL). Among the tested MDR-TB strains, the MIC$_{90}$ of NOS in the simple MDR-TB group (0.25 μg/mL) was lower than the MDR-TB plus group (1.0 μg/mL). According to the distribution of MICs against NOS, we proposed a tentative ECOFF at 1 μg/mL to define a NOS-resistant isolate.

**Mutations conferring NOS resistance and protein alignment.** Multiple amino acid alignments for the *rplK* homologs of different bacterial species and the topology of proteins are shown in Fig. 1A. The protein sequences of *rplK* in different mycobacterial species were highly conserved. Except for *Escherichia coli* with Ser89, the amino acid at position 89 of the homologous proteins of *rplK* in the tested bacterium were all lysine. To determine the relationship between mutations in *rplK* homologous genes and NOS resistance, full-length *rplK* homologs of *M. tuberculosis* were sequenced. According to the tentative ECOFF of NOS, MIC ≥2 μg/mL was defined as resistance to NOS. Therefore, five isolates acquired MIC ≥2 μg/mL. A Lys89Thr mutation was identified in an MDR isolate with MIC >8 μg/mL, whereas no single nucleotide polymorphisms (SNPs) were detected in the remaining 127 clinical isolates. *M. tuberculosis* rplK Lys89 located in the loop was not adjacent to the NOS analog, while it was contacting U1060 of 23S rRNA (Fig. 1B). Notably, hydrogen bonds strengthened the intermolecular interaction between *M. tuberculosis* rplK Lys89 and RNA
and disrupted by rplK mutation Lys89Thr (Fig. 1C and D). In addition, no mutation was detected in the NOS binding region of the 23S rRNA.

Bactericidal or bacteriostatic activity of NOS in vitro. NOS displayed a MIC of 0.125 μg/mL against M. tuberculosis H37Rv and significantly inhibited bacterial growth at the concentration of 1 × MIC after 4 days of incubation compared with the initial bacterial load. Except for the bacterial load that increased at 1 × MIC after 8 days of incubation, the remaining conditions treated with NOS showed slight inhibitory activity compared to the initial group, and there was no statistical significance. The main reason for the regrowth may have been caused by the decay of NOS at 37°C, as the concentration of NOS in 1 × MIC group after 8 days of incubation was decreased from 0.125 μg/mL to 0.012 μg/mL (Fig. S1). Compared to the DMSO group, the NOS-treated groups all presented significantly inhibited bacterial growth, whether the time of incubation was 4 or 8 days. Because NOS could not decrease the bacterial load by 3 log even at the concentration of 10 × MIC, it seemed to display bacteriostatic activity in vitro at moderate and high concentrations (Fig. 2).

Intracellular killing and concentration-kill assay. Intracellular killing of NOS was presented in Fig. 3. At multiplicity of infection (MOI) = 1.1, reductions in colony-forming units (CFU) number were observed when treated with NOS compared to the initial control. After 24 h of incubation, NOS at 1 μg/mL, 2 μg/mL, and 4 μg/mL inhibited 25.65 ± 3.64%, 25.79 ± 1.22%, and 28.45 ± 4.64% of intracellular bacterial growth, which was comparable with that of RIF at 2 μg/mL with the inhibitory rate of 25.71 ± 1.22%. The inhibitory effects were slightly increased at day 3 postinfection, and the inhibitory rates of NOS at 2 μg/mL seemed to display bacteriostatic activity in vitro at moderate and high concentrations (Fig. 2).

### Table 1: MICs of NOS against reference strains of 18 SGM species

| Strain by Mycobacterium type | Species (strain)          | MIC (μg/mL) by antimicrobial agent |
|-------------------------------|---------------------------|-----------------------------------|
| SGM                           |                           | Nosiheptide                       |
| ATCC 25276                    | Mycobacterium asiaticum   | 0.125                             |
| ATCC 25291                    | Mycobacterium avium       | 0.125                             |
| DSM 44243                     | Mycobacterium celatum     | 0.125                             |
| DSM 44622                     | Mycobacterium chimaera    | 0.031                             |
| ATCC 15754                    | Mycobacterium gasti       | 0.03                              |
| ATCC 14470                    | Mycobacterium gordonae    | 0.008                             |
| ATCC 13950                    | Mycobacterium intracellular| 0.25                              |
| ATCC 12478                    | Mycobacterium kanssii     | 0.125                             |
| ATCC 927                      | Mycobacterium marinum     | >8                                |
| ATCC 19422                    | Mycobacterium microti     | 0.031                             |
| ATCC 19530                    | Mycobacterium nonchromogenic | >8                           |
| DSM 44648                     | Mycobacterium parascrofulaceum | 0.25                          |
| ATCC 27024                    | Mycobacterium rhodesiae   | 0.125                             |
| ATCC 19981                    | Mycobacterium scrofulaceum | 0.25                          |
| ATCC 35799                    | Mycobacterium szulgai     | 0.063                             |
| ATCC 23292                    | Mycobacterium triviale    | 0.25                              |
| ATCC 27294                    | Mycobacterium tuberculosis(H37Rv) | 0.125                      |
| ATCC 19250                    | Mycobacterium xenopi      | 0.008                             |

### Table 2: Distribution of NOS MICs among M. tuberculosis isolates

| Classification | No. (%) of strains with MIC (μg/mL) | MIC<sub>50</sub> | MIC<sub>90</sub> |
|----------------|-------------------------------------|------------------|------------------|
|                | 0.016 | 0.031 | 0.062 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | >8 | Total | MIC<sub>50</sub> | MIC<sub>90</sub> |
| Non-MDR        | 44    | 1     |      |      |      |      |    |   |   |   |   |   | 0.125 | 1              |
| MDR            | 84    | 0.125 | 0.5  |      |      |      |    |   |   |   |   |   |      |                |
| Simple MDR-TB  | 35    | 0.125 | 0.25 |      |      |      |    |   |   |   |   |   |      |                |
| MDR-TB plus    | 49    | 0.25  | 1    |      |      |      |    |   |   |   |   |   |      |                |
| Total          | 128   | 0.125 | 1    |      |      |      |    |   |   |   |   |   |      |                |

*MIC<sub>50</sub> concentration required to inhibit the growth of 50% of the isolates tested. MIC<sub>90</sub> concentration required to inhibit the growth of 90% of the isolates tested: Simple MDR-TB, MDR-TB with susceptible to fluoroquinolone or three second-line injectable drugs (capreomycin, kanamycin, and amikacin); MDR-TB plus, MDR-TB with additional resistance to any fluoroquinolone and to at least one of three second-line injectable drugs.*
and 4 μg/mL were 27.95 ± 1.21% and 31.34 ± 4.42%, respectively. Compared to day 3, the bacterium presented regrowth on day 5 postinfection in all NOS-treated groups. In contrast, RIF demonstrated more robust inhibitory activities on both day 3 and day 5 post-infection (Fig. 3).

FIG 1 Sequence and structural alignment of rplK homolog proteins. (A) Alignment of the amino acid sequences of rplK in *M. tuberculosis*, *M. smegmatis*, *M. avium*, *M. intracellulare*, *M. marinum*, *M. nonchromogenium*, *M. chelonae*, *M. abscessus*, *M. fortuitum*, *E. coli*, and *S. aureus*. The topology of the rplK encoded protein of *M. tuberculosis* is shown at the top. Red boxes with white letters indicate a single, fully conserved residue. The mutation in rplK of *M. tuberculosis* was highlighted in yellow box. (B) Structure of thiostrepton antibiotic binding to rplK in *M. tuberculosis* from 23S rRNA. rplK in Green and Lys89 highlighted in rainbow stick; thiostrepton antibiotic in magenta; 23SrRNA in orange and blue green gradient; (C) *M. tuberculosis* rplK Lys89 interacts with U1060 of 23S rRNA by hydrogen bond. (D) *M. tuberculosis* rplK Lys89Thr disrupts the interaction with 23S rRNA.

and 4 μg/mL were 27.95 ± 1.21% and 31.34 ± 4.42%, respectively. Compared to day 3, the bacterium presented regrowth on day 5 postinfection in all NOS-treated groups. In contrast, RIF demonstrated more robust inhibitory activities on both day 3 and day 5 post-infection (Fig. 3).

FIG 2 Logarithmically growing *M. tuberculosis* cells were exposed for 4 and 8 days to NOS at 1×, 4×, and 10× MIC values. Rifampicin (RIF) and dimethyl sulfoxide (DMSO) were used as positive and negative controls. Data were generated from three independent experiments and are shown as mean ± SD (*, *P* < 0.05, ***, *P* < 0.001 versus initial control; **, *P* < 0.01, ****, *P* < 0.001 versus DMSO control by unpaired *t* test).
Following 2 h of pulse exposure to 10 μg/mL of NOS and RIF, the growth of *M. tuberculosis* was retarded, as reflected by the long recovery time. The PAE value of NOS was found to be more than 384 h (16 days), which was far superior to RIF with a PAE value of 192 h (8 days) (Fig. 4), whereas INH showed little PAE, which was comparable with the no drug, control group.

**Checkerboard assay for compound interactions.** When used with INH, NOS showed a partial synergistic effect against *M. tuberculosis* H37Rv with fractional inhibitory concentrations (FICI) = 0.75 (Table 3). In addition, it showed an additive effect with bedaquiline (BDQ) and linezolid (LZD) against *M. tuberculosis* H37Rv, with a FICI of 1. When used in combination with RIF, clofazimine (CFZ), and moxifloxacin (MFX), NOS showed indifference against *M. tuberculosis* H37Rv, since the FICI was 1 to 4. No antagonistic interactions were found between NOS and the other tested compounds.

**DISCUSSION**

The activities of NOS against *M. avium* complex and *M. abscessus* have been reported (12, 13), which aroused our interest to evaluate the potential of NOS as an anti-TB drug. To the best of our knowledge, this is the first study that describes the antibacterial activity of NOS against *M. tuberculosis*. In the present study, NOS demonstrated good antimicrobial activities against both the reference and clinical strains of *M. tuberculosis*. The MIC₅₀ and MIC₉₀ of NOS were found to be lower than those of RIF and INH, indicating a more potent activity against *M. tuberculosis*. Moreover, the combined use of NOS with other anti-TB drugs showed both synergistic and additive effects, suggesting that NOS could be a promising candidate for anti-TB therapy.
MIC\textsubscript{90} of NOS against \textit{M. tuberculosis} were 0.125 µg/mL and 1 µg/mL, respectively. Most importantly, NOS demonstrated good antimicrobial activities against both drug-susceptible TB and MDR-TB strains, including the MDR-TB strains with additional resistance to fluoroquinolones and second-line injectable agents. Therefore, these outcomes suggest that NOS might be a promising candidate for the treatment of TB, including MDR-TB.

Furthermore, 15 out of 18 reference strains of different SGM species acquired MICs lower than 1 µg/mL, which also supports the usage in mycobacteria inhibition. In addition, a partial synergistic effect of NOS with INH against \textit{M. tuberculosis} H37Rv was found, and additive effects were observed when combined with BDQ or LZD. This drug interaction information might be important in a regimen establishment for treating MDR-TB.

Thiazole antibiotics, including NOS and thiostrepton, affect protein synthesis inhibition by binding 50S ribosomal subunit, which is composed of 5S and 23S rRNAs and 36 riboproteins (L1 through L36). Reportedly, thiazole antibiotics interact with nucleobase A1067 and A1095 (\textit{E. coli} numbering) that are located at helices 43 or 44 of the 23S rRNA, which are the binding sites of the ribosomal protein L11 (\textit{rplK}), and are also the target sites of given methyltransferase and these methylations can lead thiazole resistance (9, 14, 15). However, no mutation was found in the corresponding locus of 23sRNA in the five \textit{M. tuberculosis} isolates with NOS MIC $\geq$ 2 µg/mL. According to the L11-thiostrepton binding model, a proline-rich helix in the L11 protein N terminus (21-PPVGPALQQH-30) interacted with thiostrepton, which was essential for the binding of thiostrepton (9). Furthermore, \textit{in vitro}-induced resistant strains with mutants like P22S, P23L, or G25V in \textit{Bacillus megaterium} could grow at a 5-fold higher drug concentration (0.061 µg/mL/50 nM) than wild-type strains (16), suggesting that the mutation of this residue confers resistance. In our study, a new mutation Lys89Thr in \textit{rplK} was detected in one \textit{M. tuberculosis} isolate with MIC > 8 µg/mL (6,545 nM). In contrast, no mutation was found in NOS susceptible isolates or strains with MIC ranging 1 µg/mL to 8 µg/mL. Additionally, \textit{M. tuberculosis} \textit{rplK} Lys89Thr mutation could disrupt the interaction with U1060 of 23S rRNA by structural alignment. Thus, we speculated that Lys89 may play an important role in the conformation of the complex of \textit{rplK} and 23S rRNA, which consequently affected the binding of thiostrepton antibiotic. Since the appropriate breakpoint to define NOS resistance is unknown, a strain with MIC greater than ECOFF could still be a sensitive strain. Therefore, only the strain with MIC > 8 µg/mL that harbored a mutation in \textit{rplK} is plausible. Even though only a single isolate was identified, more data need to be gathered to clarify the relationship between Lys89Thr mutation in \textit{rplK} and NOS resistance. Furthermore, other mechanisms were possible for the resistance of NOS, such as efflux or methyltransferase activities, which may need further investigation.

Although NOS has not shown bactericidal activity \textit{in vitro}, it manifested strong antibacterial activity in the intracellular bactericidal experiment. Notably, NOS (1 µg/mL) inhibited 24.72% of bacterial growth at MOI = 1 after the incubation time of 24 h, which was comparable with Rif at 2 µg/mL. As potent activity against clinical isolates of TB and strong inhibitory activity against \textit{M. tuberculosis} H37Rv in macrophages were acquired, PAE of NOS was then determined. Surprisingly, our study revealed a

\begin{table}[h]
\centering
\caption{Effectiveness of NOS in combination with antituberculosis drugs against \textit{M. tuberculosis} H37Rv$^a$}
\label{table:1}
\begin{tabular}{|c|c|c|c|c|}
\hline
Antibiotic combination & MIC (µg/mL) & & & \\
\hline & Antibiotics alone & Combinations & FICI & Outcome \\
\hline NOS + INH & 0.125/0.05 & 0.0625/0.0125 & 0.75 & Partial synergy \\
NOS + RIF & 0.125/0.032 & 0.125/0.016 & 1.5 & Indifference \\
NOS + MFX & 0.125/0.0625 & 0.125/0.0625 & 2 & Indifference \\
NOS + LZD & 0.125/0.25 & 0.0625/0.125 & 1 & Additive \\
NOS + CFZ & 0.125/0.062 & 0.125/0.125 & 3 & Indifference \\
NOS + BDQ & 0.125/0.031 & 0.0625/0.016 & 1 & Additive \\
\hline
\end{tabular}
\end{table}

$^a$BDQ, bedaquiline; CFZ, clofazimine; FICI, fractional inhibitory concentration index; INH, isoniazid; LZD, linezolid; MFX, moxifloxacin; Rif, rifampicin.
The Activity of Nosipeptide against M. tuberculosis

MATERIALS AND METHODS

sis infections. Important insights into the potential clinical applications of NOS in treating tuberculosis support for NOS being a promising drug candidate for TB treatment. Our data provide addition to the added safety of long-term use as a feed additive in husbandry, provide and macrophages. Furthermore, the long PAE and partial synergistic effect with INH, in overcome the obstacles faced by NOS in the clinical application (18).

Notably, serials of analogs for NOS were semisynthesized, and they probably could major concerns associated with NOS, making it difficult to achieve therapeutic doses. Notably, serials of analogs for NOS were semisynthesized, and they probably could overcome the obstacles faced by NOS in the clinical application (18).

In conclusion, NOS has potent inhibitory activities against M. tuberculosis in vitro and macrophages. Furthermore, the long PAE and partial synergistic effect with INH, in addition to the added safety of long-term use as a feed additive in husbandry, provide support for NOS being a promising drug candidate for TB treatment. Our data provide important insights into the potential clinical applications of NOS in treating tuberculosis infections.

MATERIALS AND METHODS

Ethics statement. Ethical approval was not sought, as the study only involved laboratory testing of mycobacteria without the direct involvement of human subjects.

Reference strains and clinical isolates. The mycobacterial reference strains, including 18 SGM reference species, were obtained either from the American Type Culture Collection (ATCC) or from the German Collection of Microorganisms (DSM). The species constitution of these reference strains is listed in Table 1. The clinical isolates of M. tuberculosis stored in the Biobank in Beijing Chest Hospital (Beijing, China) were tested to investigate their susceptibility to NOS in vitro. The isolates were first cultured positive on Löwenstein-Jensen (LJ) medium, and then classified as tuberculosis preliminarily with negative results using p-nitrobenzoic acid-containing medium (500 µg/mL). All the strains were tested with MPT64 antigen to confirm the presence of M. tuberculosis complex (Hangzhou Genesis Biodetection And Biocontrol Co., Ltd, China). A total of 128 isolates of M. tuberculosis were recruited in Beijing chest hospital from 2019 to 2020, including 44 non-multidrug-resistant strains and 84 MDR-TB strains. More than 90% (116/128) of the isolates were from northern China and may be related genetically and epidemiologically.

MIC testing. NOS was purchased from Targetmol (USA) and was dissolved in dimethyl sulfoxide (DMSO) with a concentration of 8 mg/mL for the stock solution. The broth microdilution method was performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (22). Middlebrook 7H9 broth (Becton, Dickinson) containing 10% oleic acid-albumin-dextrose-catalase (OADC) was used for the MIC test of M. tuberculosis and 5% for the remaining SGM using Cation-adjusted Mueller-Hinton broth. The inoculum was prepared with fresh culture grown on LJ medium. The tested drug concentrations ranged from 0.008 µg/mL (654 nM) to 8 µg/mL (6.54 g/mL). Briefly, M. tuberculosis and the remaining SGM were scraped from the LJ medium, homogenized, and adjusted to 1 and 0.5 McFarland standards. Then, the suspensions were diluted and inoculated into a 96-well microtiter plate to achieve the final bacterial load at 10^5 CFU (CFU) per well. Plates were then incubated at 37°C for 7 days, expect for M. marinum at 30°C. A 70-µL solution containing 50 µL Tween 80 (5%) and 20 µL Alamak Blue (Bio-Rad) was added to each well and incubated for 24 h at 37°C before color development was assessed. A change from blue to pink or purple indicated bacterial growth (23). The MIC was defined as the lowest concentration of antibiotic that prevented a color change from blue to pink.
Mutations conferring NOS resistance and protein alignment. Sequencing of the PCR products against M. tuberculosis isolates was performed using the Sanger method with primers designed to be specific for rplK and partial 23S rRNA region (C800-C1350, M. tuberculosis sequence) covering thiazole antibiotics binding site. The primers were designed as follows: rplK forward primer sequence, TCAAG CCGAAACAGCAAAA; rplK reverse primer sequence, GGTGTAAGGTGTTGTCGGC; 23S rRNA forward primer sequence, CAGCGAAACGGAGTGCTGAAT; and 23S rRNA reverse primer sequence, TGGTCGCTACTCTGCGTGC. Mutations were identified according to the outcomes of the alignments against the M. tuberculosis reference strain (ATCC 27294).

The homologous genes of rplK in bacterium were downloaded from the NCBI, including M. smegmatis (ATCC 19420), M. tuberculosis H37Rv (ATCC 27294), M. avium (ATCC 25291), M. intracellulare (ATCC 13930), M. marinum (ATCC 927), M. nonchromogenicum (ATCC 15930), M. chelonae (ATCC 14472), M. abscessus (ATCC 19977), M. fortuitum (ATCC 6841), S. aureus (ATCC 25923), and E coli (ATCC 700926). Multiple sequence alignment of the homologous proteins was performed using the Clustal Omega software. Structure-based multiple sequence alignment was performed with ESPript 3 based on the structure of rplK of M. tuberculosis by AlphaFold AI system from the following website: https://escherichia.co.jp/ESPript/ESPript3/.

Then, the predicted rplK in M. tuberculosis was docking to the model for thiostrepton antibiotic binding to L11 substrate from 50S rRNA (PDB: 13950), (ATCC 19420), M. avium (ATCC 25291), M. intracellulare (ATCC 13930), M. marinum (ATCC 927), M. nonchromogenicum (ATCC 15930), M. chelonae (ATCC 14472), M. abscessus (ATCC 19977), M. fortuitum (ATCC 6841), S. aureus (ATCC 25923), and E. coli (ATCC 700926). The structure of rplK was extracted from the PDB file, and the best alignment was acquired by structural alignment.

Bactericidal or bacteriostatic activity of NOS in vitro. M. tuberculosis H37Rv (ATCC 27294) grows to early logarithmic phase and adjusted to optical density at 600 nm (OD600 = 0.8) and diluted 1:25 with 7H9 medium containing 10% OADC. Then, the corresponding antibiotics were added to obtain 1 × MIC (0.125 μg/mL), 4 × MIC (0.5 μg/mL), and 10 × MIC (1.25 μg/mL) of NOS and RIF (5 μg/mL). The tubes were incubated with shaking at 60 rpm/min for 8 days at 37°C. The bacteria were enumerated at defined time intervals (4 and 8 days) by plating serial dilutions on 7H10 agar plates.

Intracellular killing and concentration-kill assay. THP-1 were seeded at 2 × 10⁵ cells/well in a 24-well plate and differentiated with phorbol myristate acetate. Cells were infected with M. tuberculosis H37Rv (ATCC 27294) at an MOI of 1 (25). At 4 h postinfection, the cells were gently washed three times with prewarmed phosphate-buffered saline (PBS) to remove the extracellular bacteria. For the intracellular killing assay, RPMI complete medium containing NOS (1 μg/mL, 2 μg/mL, and 4 μg/mL) or RIF (0.5 μg/mL, 1 μg/mL, and 2 μg/mL) were added. The culture medium with DMSO was included as a growth control. At the treatment time of 1, 3, and 5 days postinfection, macrophages were extensively washed with PBS and lysed with 0.1% Trion-X. CFU were quantified by plating serial dilutions of lysates on 7H10 agar plates.

Statistical analysis. Experiments were repeated in triplicate, with a minimum of triplicate data points per experiment. Data were analyzed using GraphPad Prism 8.0. The means of the drug-free control and drug-treated group were compared using an unpaired Student’s t test. The epidemiological cut-off (ECOFF) was determined according to the distribution profile of the MIC values. For the unimodal MIC distribution profile, ECOFF was defined as the concentration that could inhibit 95% of the bacterial population; For the bimodal MIC distribution profile, ECOFF was set between the two populations (28).

Data availability. All data relevant to this study are supplied in the manuscript and supplemental material or are available from the corresponding author upon request. Supplemental Table S1 and Fig. S1 and the methods for NOS stability monitored by HPLC are provided in the supplemental material.
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