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Short Communication

In vitro activity of DNF-3 against drug-resistant *Mycobacterium tuberculosis*

Md Imtiazul Islam[a,1], Choi Moon Han[a,b,1], Hoonhee Seo[c], Sukyung Kim[c], Hafij Al Mahmud[c], Kung-Woo Nam[d], Byung-Eui Lee[c], Venkata S. Sadu[e], Kee-In Lee[e,f], Ho-Yeon Song[a,c,*]

[a] *Department of Microbiology and Immunology, School of Medicine, Soonchunhyang University, Cheonan, Chungnam 31151, South Korea*
[b] *Department of Internal Medicine, Soonchunhyang University Gumi Hospital, Gumi, Gyeongbuk 39371, South Korea*
[c] *Regional Innovation Center, Soonchunhyang University, Asan, Chungnam 31538, South Korea*
[d] *Department of Life Science and Biotechnology, Soonchunhyang University, Asan, Chungnam 31538, South Korea*
[e] *Green Chemistry Division, Korea Research Institute of Chemical Technology, Daejeon 34114, South Korea*
[f] *Major of Green Chemistry and Environmental Biotechnology, University of Science & Technology, Daejeon 34113, South Korea*

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ABSTRACT

Due to the emergence of multidrug-resistant and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis*, new antituberculosis drugs are urgently required to improve the efficacy of current tuberculosis (TB) treatment. To achieve this goal, ca. 1000 chemical compounds were screened for potential antimycobacterial activity, among which methyl 5-(2-diethylaminoethoxy)-7,12-dioxo-7,12 dihydrodinaphtho[1,2-*b*;2′,3′-*d*]furan-6-carboxylate (DNF-3) showed strong activity against all of the tested drug-susceptible and -resistant *M. tuberculosis* strains, with 50% minimum inhibitory concentrations (MIC_{50} values) of 0.02–0.39 μg/mL both in culture broth and within murine RAW 264.7 macrophage cells. When DNF-3 was used in combination with rifampicin or streptomycin, it exhibited direct synergy against XDR-TB and an additive effect against *M. tuberculosis* H37Rv. DNF-3 displayed a long post-antibiotic effect (PAE) that was comparable with rifampicin but was superior to isoniazid, streptomycin and ethambutol. Importantly, DNF-3 showed no cytotoxicity to any cell line tested, with a selectivity index (SI) of >32. DNF-3 was also active against 27 nontuberculous mycobacteria (NTM) strains, *Staphylococcus* spp. and *Streptococcus* spp. Taken together, these results indicate that DNF-3 is a promising new candidate drug for treating TB. Further studies are warranted to establish the in vivo effect and therapeutic potential of DNF-3.

1. Introduction

Although tuberculosis (TB) is one of the oldest known human diseases, it is still a leading cause of death globally. Around 10.4 million people were diagnosed with TB worldwide and an estimated 1.67 million people [1.3 million human immunodeficiency virus (HIV)-negative and 0.374 million HIV-positive] died of TB in 2016 [1]. The chemotherapy currently used for the treatment of TB is more than 40 years old. It is facing significant compliance challenges owing to the long duration of therapy and associated toxicities [2]. The emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) has taken this global concern to a new height [2]. Thus, new drug candidates that can shorten current first-line drug regimens and provide effective therapies are urgently required for drug-resistant TB [3].

Naphthoquinones are natural compounds found mainly in plants (Plumbaginaceae, Juglandaceae, Ebenaceae, Boraginaceae, Dioncophyllaceae, Ancistrocladaceae, Euphorbiaceae, etc.) as well as in some algae, fungi, animals and bacteria as secondary metabolites [4]. They can serve as important intermediates for the production of various synthetic and bioactive compounds with antibacterial, antifungal, antiviral and anticancer activities [4]. The antimycobacterial activity of naphthoquinones has also been demonstrated [5], suggesting that these compounds might be promising candidates as new anti-TB drugs.

Recently, we searched the antimycobacterial activity of a chemical library containing ca. 1000 different small molecules with high

* Corresponding author. Present address: Department of Microbiology and Immunology, School of Medicine, Soonchunhyang University, Cheonan, Chungnam 31151, South Korea. Tel.: +82 41 570 2412; fax: +82 41 577 2415.
E-mail address: songmic@sch.ac.kr (H.-Y. Song).
[1] These two authors contributed equally to this study.

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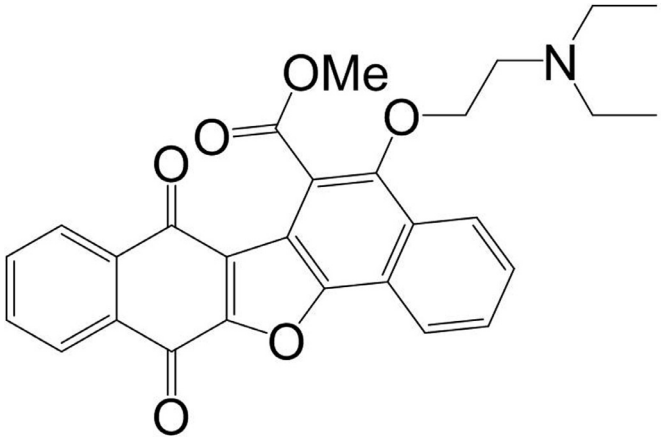

Fig. 1. Chemical structure of the dinaphthofuroquinone methyl 5-(2-diethylaminoethoxy)-7,12-dioxo-7,12-dihydrodinaphtho[1,2-*b*;2′,3′-*d*]furan-6-carboxylate (DNF-3).

chemical and structural diversity. Finally, a compound from the dinaphthofurandione class, named methyl 5-(2-diethylaminoethoxy)-7,12-dioxo-7,12 dihydrodinaphtho[1,2-*b*;2′,3′-*d*]furan-6-carboxylate (DNF-3), was identified to have strong activity against *Mycobacterium tuberculosis* strains. Thus, DNF-3 was selected for further study to determine whether it might have characteristics suitable as a potential antituberculosis drug.

2. Materials and methods

2.1. Synthesis of DNF-3

DNF-3 was synthesised at the Korean Chemical Bank of the Korea Research Institute of Chemical Technology (Daejeon, South Korea) as described in the Supplementary material and was finally collected as an orange solid with molecular formula $C_{28}H_{25}NO_6$ (Fig. 1).

2.2. Commercial drugs and chemicals

Isoniazid (INH), rifampicin (RIF), streptomycin (STR), ethambutol (EMB), pyrazinamide (PZA), vancomycin (VAN), methicillin (MET), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) and resazurin sodium salt were purchased from Sigma-Aldrich (St Louis, MO).

2.3. Mycobacterium tuberculosis strains

Clinically isolated MDR-TB strains (KMRC 00116-00023, KMRC 00116-00072, KMRC 00116-00082, KMRC 00116-00087, KMRC 00116-00107, KMRC 00116-00111, KMRC 00116-00122, KMRC 00116-00150, KMRC 00116-00181, KMRC 00116-00185 and KMRC 00116-00232) and XDR-TB strains (KMRC 00203-00197, KMRC 00203-00101, KMRC 00203-00052, KMRC 00203-00060, KMRC 00203-00063, KMRC 00203-00085, KMRC 00203-00092, KMRC 00203-00117, KMRC 00203-00128, KMRC 00203- 00169, KMRC 00120-00137 and KMRC 00121-00341) were purchased from the Korean Mycobacterium Resource Center (KMRC) (Cheongju, Chungbuk, South Korea). *Mycobacterium tuberculosis* H37Rv (ATCC 27294) was purchased from the American Type Culture Collection (Manassas, VA).

2.4. Antimycobacterial susceptibility assay

The antimycobacterial activity of DNF-3 was determined by the resazurin microtitre assay, luminescent microbial cell viability assay and standard plate count assays as described previously [6], with minor modifications provided in the Supplementary material.

2.5. Chequerboard synergy assay

The activities of DNF-3 in combination with INH, RIF and STR were evaluated against *M. tuberculosis* H37Rv (ATCC 27294) and an XDR-TB strain (KMRC 00203-00197) in 96-well plates using the chequerboard titration method [6] provided in the Supplementary material.

2.6. Post-antibiotic effect (PAE)

PAEs for DNF-3 and control drugs were determined using a previously described approach [7] with slight modifications to accommodate the doubling time of *M. tuberculosis*, as described in the Supplementary material.

2.7. Cytotoxicity test

The cytotoxicity of DNF-3 was evaluated at a wide range of concentrations (0.02–200 μg/mL) against seven different mammalian cell lines (RAW 264.7, L929, A549, HEK-293, HEPG2, SH-SY5Y and THP-1) by the MTT assay as described in the Supplementary material. The 50% cytotoxic concentration (CC_{50}) determined by the MTT assay was used to calculate the selectivity index (SI) by dividing the CC_{50} by the minimum inhibitory concentration (MIC) of DNF-3 [8].

2.8. Intracellular antimycobacterial activity

The intracellular killing activity of DNF-3 was determined in RAW 264.7 cell monolayers as described in the Supplementary material.

2.9. Determination of activity against nontuberculous mycobacteria (NTM) and clinically significant bacteria

A total of 27 NTM strains and 24 clinically important bacterial strains were purchased from the KMRC and the National Culture Collection for Pathogens (Chungbuk, South Korea), respectively. The MIC of each strain was determined following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (2015) provided in the Supplementary material.

2.10. Statistical analysis

All experiments were done in triplicate. Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA). The means of the drug-free control and drug-treated group were compared using unpaired Student's *t*-test.

3. Results

3.1. Activity of DNF-3 against drug-susceptible and -resistant strains of Mycobacterium tuberculosis

The 50% MIC (MIC_{50}) for DNF-3 was 0.19 μg/mL (Table 1; Fig. 2A,B) both against *M. tuberculosis* H37Rv (ATCC 27294) and XDR-TB (KMRC 00203-00197) by the resazurin assay. The microbial cell viability assay also showed that DNF-3 had activity against both strains at similar MIC_{50} values of 0.19 μg/mL (Fig. 2C,D). The activity of DNF-3 was also evaluated by CFU enumeration on Middlebrook 7H10 agar plates (Becton Dickinson, Franklin Lakes, NJ)

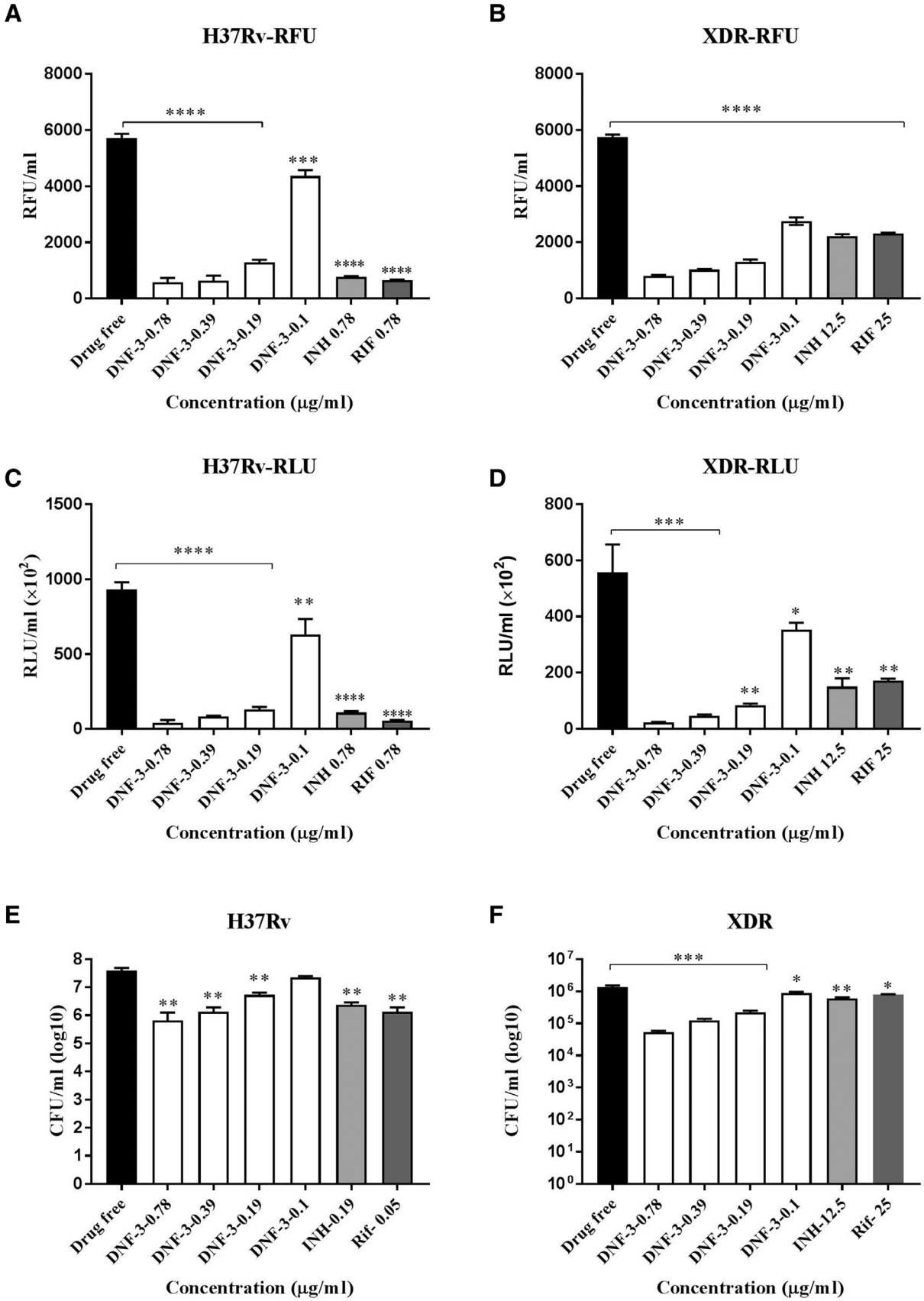

Fig. 2. Antitubercular activities of DNF-3 and the control drugs including isoniazid (INH) and rifampicin (RIF) against (A,C,E) *Mycobacterium tuberculosis* H37Rv (ATCC 27294) and (B,D,F) extensively drug-resistant (XDR) *M. tuberculosis* (KMRC 00203-00197). Mycobacterial viability was determined by the resazurin assay as RFU/mL (A,B), the microbial cell viability assay as RLU/mL (C,D) and the colony-forming unit method as CFU/mL (E,F). Experiments were performed in triplicate. Data are the mean ± standard deviation. Asterisks indicate statistical significance versus the drug-free control using an unpaired Student's *t*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. RFU, relative fluorescence units; RLU, relative luminescence unit.

Table 1
Antitubercular activity of DNF-3 and control drugs against drug-susceptible and -resistant strains of *Mycobacterium tuberculosis*.

Strain	MIC (μg/mL)					
	DNF-3	INH	RIF	STR	EMB	PZA
H37Rv	0.19	0.19	0.049	0.19	0.78	>200
MDR 1	0.19	12.5	>200	100	6.25	>200
MDR 2	0.02	12.5	>200	1.56	6.25	>200
MDR 3	0.19	3.13	>200	0.39	3.13	>200
MDR 4	0.19	25	>200	0.78	6.25	>200
MDR 5	0.19	12.5	3.13	3.13	6.25	>200
MDR 6	0.02	25	>200	25	6.25	>200
MDR 7	0.02	50	>200	200	6.25	>200
MDR 8	0.39	12.5	>200	3.13	6.25	>200
MDR 9	0.02	25	>200	25	3.13	>200
MDR 10	0.02	3.13	3.13	>200	0.78	>200
MDR 11	0.39	12.5	3.13	3.13	6.25	>200
XDR-TB[a]	0.19	25	100	0.78	6.25	>200
XDR 1	0.02	12.5	>200	0.78	6.25	>200
XDR 2	0.02	3.13	>200	50	6.25	>200
XDR 3	0.19	3.13	>200	>200	6.25	>200
XDR 4	0.19	12.5	6.25	0.78	6.25	>200
XDR 5	0.19	12.5	>200	50	6.25	>200
XDR 6	0.19	12.5	100	50	6.25	>200
XDR 7	0.19	6.25	>200	0.78	12.5	>200
XDR 8	0.19	12.5	>200	>200	6.25	>200
XDR 9	0.19	50	>200	200	6.25	>200
XDR 10	0.02	>200	6.25	<0.09	6.25	>200
XDR 11	0.19	6.25	>200	200	6.25	>200

MIC, minimum inhibitory concentration; INH, isoniazid; RIF, rifampicin; STR, streptomycin; EMB, ethambutol; PZA, pyrazinamide; MDR, multidrug-resistant; XDR, extensively drug-resistant.
[a] XDR-TB strain KMRC 00203-00197.

where it showed a bacterial reduction in a dose-dependent manner (Fig. 2E,F). Compared with INH and RIF, DNF-3 significantly reduced the number of XDR-TB at a concentration of 0.19 μg/mL. Antitubercular activities of DNF-3 against 22 additional clinical MDR- and XDR-TB isolates were also tested, with MICs ranging from 0.02–0.39 μg/mL (Table 1).

3.2. Antimicrobial synergy of DNF-3

When used in combination with RIF and STR, DNF-3 showed synergistic effects against XDR-TB (KMRC 00203-00197), with fractional inhibitory concentration indices (FICIs) of 0.312 and 0.50, respectively (Supplementary Table S1). On the other hand, it showed an additive effect both with RIF and STR against *M. tuberculosis* H37Rv (ATCC 27294), with an FICI of 1. When used in combination with INH, DNF-3 showed no synergy against H37Rv or XDR-TB, although the combination was not antagonistic since the FICI was in the range of 1–2.

3.3. Post-antibiotic effect of DNF-3 against Mycobacterium tuberculosis

In this study, the optical density at 600 nm (OD_{600}) was used to determine the PAE of DNF-3 along with first-line control drugs. Following 2 h of pulse exposure to 10 μg/mL of each of DNF-3, INH, RIF, STR and EMB, growth of *M. tuberculosis* was retarded as reflected by the long recovery time. The PAE value of DNF-3 was found to be 68 h, which was far superior to INH, STR and EMB (PAE values of 20 h, 24 h and 28 h, respectively). Only RIF showed a longer PAE value (144 h) than DNF-3 (Supplementary Fig. S1).

3.4. Cytotoxicity of DNF-3

According to the MTT assay, the CC_{50} for DNF-3 against all seven tested cell lines ranged from 6.25–12.5 μg/mL, resulting in SI values for DNF-3 ranging from 32.9–65.7, which are higher than the minimum acceptable limit (Supplementary Table S2).

3.5. Intracellular antimycobacterial activity of DNF-3 against Mycobacterium tuberculosis in macrophages

The growth of *M. tuberculosis* was significantly inhibited by DNF-3 in a dose-dependent manner during co-cultivation for 3 days (Supplementary Fig. S2A,B). The MIC_{50} value of DNF-3 against *M. tuberculosis* H37Rv (ATCC 27294) and XDR-TB (KMRC 00203-00197) in RAW 264.7 cells was 0.19 μg/mL. The intracellular inhibitory activity of DNF-3 in RAW 264.7 cells at a concentration of 0.19 μg/mL against XDR-TB was far superior to that of INH or RIF.

3.6. Activity of DNF-3 against nontuberculous mycobacteria and clinically significant bacteria

DNF-3 was further tested for in vitro activity against 27 NTM strains and 24 clinically significant non-mycobacterial pathogens. DNF-3 also showed significant activity against all 27 NTM strains, with MICs ranging from 0.39–6.25 μg/mL. Compared with DNF-3, the control drugs showed a variety of activities, with MICs ranging from <0.09 μg/mL to >200 μg/mL. INH, EMB and PZA had a much higher MICs for most of the NTM strains, but RIF and STR had a lower MICs for more than one-half of the 27 NTM strains (Supplementary Table S3).

On the other hand, among the 24 clinically significant bacteria, DNF-3 showed activity only against 7 Gram-positive bacteria, including three *Streptococcus* spp., three *Staphylococcus* spp. and a *Corynebacterium* sp., with MICs ranging from 0.10–6.25 μg/mL. However, DNF-3 did not show any activity against the remaining 17 Gram-negative bacteria. Although the control drugs RIF and STR showed a significant effect with MICs ranging from <0.1 μg/mL to 25 μg/mL against almost all 24 bacteria, VAN and MET showed similar results to DNF-3 (Supplementary Table S4).

4. Discussion

TB kills ca. 3 people every minute around the world [9]. Currently used first-line chemotherapy for TB was adequate until the emergence of MDR- and XDR-TB strains. Moreover, resistance to all fluoroquinolones and other antituberculosis drugs has been reported [10], necessitating novel therapeutic options to combat deadly TB.

The activities of naphthoquinones and their derivatives against drug-susceptible, MDR and XDR *M. tuberculosis* have been reported [11], leading our present effort to investigate the potential of dinaphthofuroquinone derivatives as antituberculosis drugs.

In this study, the antimycobacterial activities of a chemical library containing ca. 1000 chemical compounds were evaluated. Surprisingly, DNF-3, a member of the dinaphthofurandione class, showed significant antimycobacterial potential in the preliminary screening, whereas other compounds with similar structure showed no activity against *M. tuberculosis* strains (Supplementary material). Thus, DNF-3 was subjected to further studies.

DNF-3 showed strong activity against drug-susceptible *M. tuberculosis* H37Rv (ATCC 27294) and against XDR-TB (KMRC 00203-00197) as well as against 22 additional clinically isolated MDR- and XDR-TB with MICs ranging from 0.02–0.39 μg/mL. Most importantly, the strong activity and low MICs of DNF-3 against all of the tested resistant *M. tuberculosis* suggest that DNF-3 might serve as a potential candidate for the treatment of MDR- and XDR-TB. Similar to the current findings, Yang et al. reported that line-

zolid showed significant activity against MDR- and XDR-TB clinical isolates [12]. In addition, a profound synergistic effect of DNF-3 with RIF and STR against XDR-TB was found, although these combinations showed additive effects against *M. tuberculosis* H37Rv in the chequerboard synergy assay. This finding might be helpful for treating XDR-TB infections. A similar result has been reported for minocycline [13]. A definitive explanation for the profound synergy between RIF and DNF-3 against *M. tuberculosis* is yet to be found.

The current study revealed a prolonged PAE for DNF-3 compared with INH, STR and EMB, but not RIF. An extended PAE can allow wider dosing intervals without loss of therapeutic efficacy [14]. In the case of TB treatment, administration of drugs at wider intervals would reduce costs and toxicities [15]. Thus, DNF-3 is expected to be a deserving candidate for TB treatment. In addition, DNF-3 showed a SI $>$ 10 against all seven types of mammalian cell lines. Thus, DNF-3 might be pharmacologically safe as a suitable candidate for further evaluation [16].

Mycobacterium tuberculosis can enter and replicate within alveolar macrophages [17]. Such intracellular localisation protects it from some antibiotics. Therefore, the effect of DNF-3 in RAW 264.7 cells infected with *M. tuberculosis* H37Rv (ATCC 27294) and XDR-TB (KMRC 00203-00197) was evaluated. The results revealed that growth of *M. tuberculosis* was significantly inhibited by DNF-3, indicating its efficacy as a potential choice for TB treatment.

Along with TB, a new health concern has been raised by NTM because contemporary TB drugs are not very effective against NTM infection [18]. In this study, the effect of DNF-3 was also determined against 27 NTM strains and it 3 showed significant activities against all of the tested strains. Similar activities of quinone derivatives have been reported previously [19]. The activities of TB control drugs against all of the tested NTM strains was not as consistent as DNF-3. At a similar concentration of DNF-3, INH, EMB and PZA showed less activity against most of the NTM strains. Although RIF and STR showed superior activity at a lower concentration than DNF-3 against 15 strains, for the remaining 12 strains their activities were not as good as DNF-3. These inconsistent results of control drugs make DNF-3 far better and clearly indicate that DNF-3 can also be used as an option for NTM treatment along with TB.

Inspired by previously reported antibacterial activities of naphthoquinones and their derivatives, the activity of DNF-3 was determined against 24 clinically significant bacteria. Surprisingly, DNF-3 also displayed activity against seven tested Gram-positive bacteria, including three *Streptococcus* spp., three *Staphylococcus* spp. and a *Corynebacterium* sp., similar to DC-159a, a phase 1 preclinical candidate for TB [20]. Although DNF-3 showed no activity against the remaining 17 Gram-negative bacteria, RIF and STR showed considerable activity against all of the Gram-negative and Gram-positive bacteria. All of the results together signify that DNF-3 not only has a potential antimycobacterial effect but also has broad-spectrum effects against NTM and other Gram-positive bacteria.

In this study, the antimycobacterial effect of DNF-3 was confirmed using different in vitro experiments. However, further studies are required to investigate its efficacy in an animal model as well as its mechanism of action.

5. Conclusions

In summary, DNF-3 alone or in combination with other drugs had excellent activity against MDR- and XDR-TB strains. In addition, DNF-3 had an extended PAE and high SI, clearly demonstrating its strong antitubercular characteristics. Furthermore, DNF-3 showed high activities against NTM and other pathogens, making it a powerful candidate to carry out the next phase of experiments necessary for new drug development, including preclinical and animal efficacy assays.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.02.013.

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