JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

In vitro bactericidal activities of two novel dihydropyridine derivatives against *Mycobacterium tuberculosis*

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Abstract

Introduction: Introducing new and effective antitubercular agents is important in tuberculosis control programs. In this study, the *in vitro* antitubercular activity of two novel 1,4-dihydropyridine derivatives (F-27, Cl-33) were screened against a total of 113 different strains of *Mycobacterium tuberculosis* (77 susceptible and 36 resistant clinical isolates).

Methodology: The *in vitro* activities of these compounds were evaluated based on the modified broth macro-dilution assay.

Results: Compound F-27 showed more than 90% growth inhibition at the range of 2 to 8 µg/mL (minimum inhibitory concentration $[MIC]_{90}$: 4.13 ± 0.45 µg/mL; $p < 0.01$), and complete growth inhibition was observed at the range of 8 to 32 µg/mL (minimum bactericidal concentration [MBC]: 11.2 ± 1.65 µg/mL; $p < 0.01$) against susceptible strains. However, 92% of the resistant strains showed some degree of susceptibility against this compound (MIC_{90} range: 16 to 64 µg/mL; mean: 40.4 ± 8 µg/mL; $p < 0.01$). It was found that although there is a linear relationship between the inhibitory activity of F-27 and isoniazid against resistant strains at low concentrations ($r = 0.484$, $p < 0.001$), there was no relationship between resistance to isoniazid and F-27 at higher concentrations ($r = 0.019$, $p > 0.1$). This may emphasize no cross-resistance between F-27 and isoniazid.

Conclusions: Considering the sufficient sample size of the study and based on the excellent antimycobacterial activity of F-27, it could be concluded that F-27 is a potent candidate as a lead compound, and may be considered for development of a new antitubercular agent.

Key words: drug discovery; dihydropyridines; antitubercular agents; drug evaluation, preclinical.

J Infect Dev Ctries 2017; 11(6):453-458. doi:10.3855/jidc.7966

(Received 01 December 2015 – Accepted 17 February 2016)

Introduction

Tuberculosis (TB) remains one of the most important health problems. In 2015, about 10.4 million people developed tuberculosis, of which 1.4 million died. At the same time, about 480,000 cases of multidrug-resistant TB (MDR-TB) developed and 125,000 patients died from MDR-TB. The treatment for MDR-TB takes longer and requires more expensive and toxic drugs. Global tuberculosis control needs new, effective, cheap, and well-tolerated anti-tuberculosis drugs [1]. Therefore, it is necessary to introduce new antitubercular agents that are more effective, selective, and less toxic.

Isoniazid is an excellent anti-tuberculosis drug that binds covalently to the nicotinamide moiety of the NADH coenzyme of the enoyl acyl carrier protein reductase (InhA); it blocks the synthesis of mycolic acid in *Mycobacterium tuberculosis*. Various analogues and derivatives of isoniazid have been synthesized by scientists in the past decades, and their efficacies for TB treatment have been explored [2].

Derivatives of 1,4-dihydropyridine (1,4-DHPs) are also known as calcium channel blockers and are used for the treatment of several cardiovascular diseases. Studies have shown close structural similarity between 1,4-DHPs and isoniazid, as well as a probable similarity in the inhibition site of InhA [3,4]. These led to an evaluation of the antitubercular activities of various 1,4-DHPs substituents. A previous study showed that two novel substitutions of 1,4-DHPs (N,N-Bisphenyl-4-[1-(4-fluorobenzyl)-2-methylthioimidazole-5-yl]-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxamide ($C_{32}H_{30}FN_5O_2S$) (called F-27) and N, N-bisphenyl-4-[1-(2-chlorobenzyl)-2-methylthioimidazole-5-yl]-2, 6-dimethyl-1, 4-dihydropyridine-3, 5-dicarboxamide ($C_{32}H_{30}ClN_5O_2S$) (called Cl-33) (Figure 1) have

acceptable inhibitory activities against non-pathogenic mycobacteria [5]. These compounds were found to be the most active among many tested compounds. One of them (F-27) also showed excellent antitubercular activity against the standard *M. tuberculosis* H37Rv strain, and mild cytotoxicity on normal and malignant cell lines [6]. Previous studies have suggested that susceptibility may relate in part to differences between species, subspecies, and biovariants [7]. Therefore, in this study, we investigated the antitubercular activity of both compounds (F-27 and Cl-33) with statistically sufficient clinical samples. The present study was designed to evaluate the antimycobacterial activity of two novel 1,4-DHPs (F-27 and Cl-33) against 113 *M. tuberculosis* clinical isolates, and to determine their susceptibility differences.

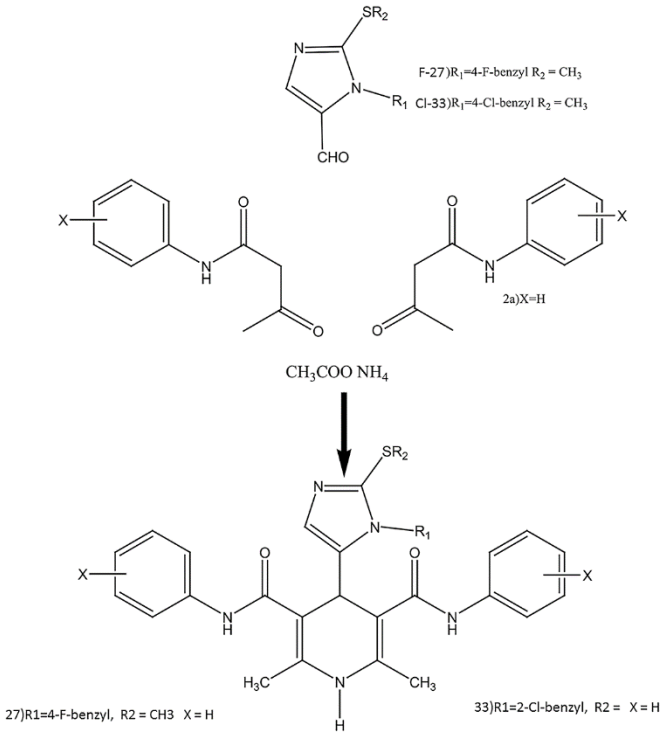
Methodology

Sample size and isolates

To achieve an adequate statistical power for the study, the sample sizes were determined using the following formula: $n = (z^2pq) / d^2$; where $z = 1.96$ (95% confidence interval), $d = 0.05$, and $p = < 0.05$, $q = 1-p = 0.95$ for susceptible strains; and $p = < 0.02$, $q = 1-p = 0.98$ for resistant strains [8].

Accordingly, 113 clinical isolates of *M. tuberculosis* (77 susceptible and 36 resistant) were randomly selected from patients who were referred to the Regional Reference Laboratory of Tuberculosis, Mashhad, Iran. Isolates had been collected during 2011 to 2013 from different TB patients. All isolates were identified as *M. tuberculosis* by Ziehl-Neelsen staining, biochemical and phenotypic methods, as well as polymerase chain reaction (PCR). Their susceptibilities against isoniazid and rifampicin were also determined by standard proportional method, using Löwenstein-Jensen (L-J) medium (Sigma, Poole, UK) as recommended by Canetti *et al*. [9]. The critical concentration in the L-J medium was 0.2 µg/mL for isoniazid and 40 µg/mL for rifampicin, as recommended by the World Health Organization (WHO). Susceptibility was defined as no growth on media containing the critical concentration of the drug, and resistance was defined as growth of 1% or more of the bacterial population [10]. Primary susceptibility tests of isolates (by proportional method on L-J medium) showed that 77 isolates were fully susceptible, while 36 isolates were resistant to both isoniazid and rifampicin.

Figure 1. Synthesis of 1, 4-dihydropyridine3, 5-dicarboxamide derivatives.



Antimycobacterial activity

To determine the inhibitory activity of F-27 and Cl-33 (minimum inhibitory concentration required to inhibit the growth of 90% of organisms [MIC_{90}], minimum bactericidal concentration [MBC]) against clinical isolates, a modified broth macro-dilution-based method was used as described previously [11].

Middlebrook 7H9 (Fluka Chemie, Buchs, Switzerland) broth was prepared and enriched with 10% ADC (albumin-dextrose-catalase) (Sigma, Poole, UK) and 0.05% Tween80. Each compound was dissolved in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) at a concentration of 1,800 µg/mL and sterilized by passage through a syringe filter (BIOFIL .22 µm, Yueqing, China). Subsequently, the final test range (1 to 64 µg/mL) was prepared by twofold serial dilutions in 7H9 broth medium (Sigma, Poole, UK). This range of concentrations was selected based on cytotoxicity and inhibition of *M. tuberculosis* H37Rv in a previous study [6]. Additionally, a drug-free control tube with medium and no additive, a solvent control tube with test medium supplemented with DMSO at the highest concentration (4%, v/v), and 3 tubes containing 0.2, 1, and 2 µg/mL of isoniazid (Sigma, Poole, UK) in 7H9 as positive control tubes (reference drug), were prepared and included in the

study based on Clinical and Laboratory Standards Institute (CLSI) standards [12].

To prepare bacterial inoculum in the exponential phase of growth, frozen stocks were subcultured on L-J medium (Merck, Darmstadt, Germany) for 4 weeks. Fresh colonies were then suspended in Middlebrook 7H9 broth (Sigma, Poole, UK) up to bacterial turbidity corresponding to 1 McFarland standard. Final concentrations of 3×10^7 and 3×10^5 CFU/mL were obtained by adding Middlebrook 7H9.

One hundred (100) µL of each inoculum (3×10^7 and 3×10^5 CFU/mL) were then separately added to each set of tubes for these 2 compounds. Tubes were incubated at 37°C for 7 days. After this time, 100 µL of medium was transferred to L-J medium (without any drug) and incubated at 37°C. After 28 days, visible growth of colonies was interpreted as bacterial growth and if there was no growth of colonies. The L-J medium was incubated for two more weeks, and results were reported after 42 days [9]. The number of colonies in the test tubes (transferred from drug-containing tubes) were compared with the control tubes (transferred from drug-free tubes), and the percentage of inhibition was calculated by the following formula: percentage of inhibition = (1 - [colony count of test sample/colony count of free control]) × 100.

The MIC_{90} is defined as the lowest drug concentration which reduces the bacterial load by 1 log unit (90%), while MBC is defined as the lowest drug concentration which totally prevents colony formation in comparison to the drug-free control.

These antitubercular assays were performed by one researcher in two independent experiments, and the mean values of inhibition are reported in this study. All the procedures for antitubercular assay were carried out in a biosafety cabinet class B2 (Scanlaf, Plymouth, USA).

Statistical data were analyzed by Pearson correlation, independent sample t test, and t-distribution 99% confidence interval for mean, using SPSS software version 16.0.0 (IBM, Armonk, USA). Descriptive data were summarized as mean ± standard deviation (SD). Results with p values < 0.01 were considered statistically significant.

Results

The results of this study are summarized as cumulative percentages of isolates, inhibited at different concentrations of these two compounds, as shown in Tables 1 and 2. In these tables, the MIC_{90}s and MBCs are shown separately among susceptible and resistant strains against isoniazid and rifampicin.

Among these 2 compounds, F-27 exhibited prominent inhibitory activity against the susceptible group, so its descriptive inhibitory activity is presented in more detail in Table 3.

The result obtained for F-27 (Table 1) shows that 100% of the susceptible strains were totally inhibited at a concentration of 32 µg/mL (MBC range: 8 to 32

Table 1. Cumulative percentages of *M. tuberculosis* isolates that were inhibited at the different concentrations of F-27.

Susceptibility	Final concentrations of compound in medium						
	1 µg/mL	2 µg/mL	4 µg/mL	8 µg/mL	16 µg/mL	32 µg/mL	64 µg/mL
Susceptible strains							
MIC_{90}	0%	17%	88%	100%	100%	100%	100%
MBC	0%	0%	0%	68%	96%	100%	100%
MDR strains							
MIC_{90}	0	0	0	0	14%	67%	92%
MBC	0	0	0	0	0	0	0

MIC_{90}: minimum inhibitory concentration required to inhibit the growth of 90% of organisms; MBC: minimum bactericidal concentration; MDR: multidrug resistant.

Table 2. Cumulative percentages of *M. tuberculosis* isolates that were inhibited at the different concentrations of Cl-33.

Susceptibility	Final concentrations of compound in medium						
	1 µg/mL	2 µg/mL	4 µg/mL	8 µg/mL	16 µg/mL	32 µg/mL	64 µg/mL
Susceptible strains							
MIC_{90}	0	0	0	0	0	23%	90%
MBC	0	0	0	0	0	0	0
MDR strains							
MIC_{90}	0	0	0	0	0	0	6%
MBC	0	0	0	0	0	0	0

MIC_{90}: minimum inhibitory concentration required to inhibit the growth of 90% of organisms; MBC: minimum bactericidal concentration; MDR: multidrug resistant.

µg/mL; mean: 11.2 ± 1.65 µg/mL; $p < 0.01$). The MIC_{90}s of F-27 for susceptible strains were in the range of 2 to 8 µg/mL (mean: 4.13 ± 0.45 µg/mL; $p < 0.01$). In the case of the MDR strains, F-27 did not completely inhibit the growth of resistant isolates in the range of 1 to 64 µg/mL, although > 90% of the isolates showed MIC_{90} in the range of 16 to 64 µg/mL (mean: 40.4 ± 8.05 µg/mL; $p < 0.01$). In general, the antitubercular activity of F-27 was concentration dependent and higher among the susceptible strains than among the MDR strains.

The Cl-33 did not show complete inhibition (MBC) against both susceptible and resistant strains in the range of 1–64 µg/mL, though 90% of susceptible strains had MIC_{90}s in the range of 32–64 µg/mL.

In this study, isoniazid was considered a reference drug, and all susceptible and resistant isolates were examined against it, according to the modified-broth-based method (Table 4).

To ensure that the solvent had no effect on bacterial growth, a solvent control was prepared and included in the experiment, which confirmed no inhibitory activity, as no inhibition was observed in this tube.

The correlation coefficient of the frequency of resistance against F-27 and isoniazid was calculated at different concentrations. A significant correlation was found ($r = 0.484$, $p < 0.001$) between F-27 and isoniazid at a concentration of 4 and 0.2 µg/mL, respectively. However, there was no correlation at higher concentrations ($r = 0.019$, $p > 0.1$) (Table 5).

Discussion

Treatment of tuberculosis poses a challenge in terms of proper selection of antimicrobial agents. Previous studies show that the new 1,4-DHPs have

Table 3. Percent of inhibition of colony formation against susceptible group by different concentrations of F-27.

	Percent of inhibition of colony formation			
	Minimum	**Maximum**	**Mean**	**Std. deviation**
F-27 (1 µg/mL)	49.17%	86.80%	73.55%	8.22
F-27 (2 µg/mL)	69.17%	93.33%	84.84%	4.92
F-27 (4 µg/mL)	82.91%	97.89%	92.12%	2.91
F-27 (8 µg/mL)	93.68%	100%	99.08%	1.67
F-27 (16 µg/mL)	98.4%	100%	99.94%	0.28
F-27 (32 µg/L)	100%	100%	100%	0.00

Table 4. Cumulative percentages of *M. tuberculosis* isolate that was inhibited at the different concentrations of isoniazid.

Susceptibility	**Cumulative % of isolate inhibition**			
	< 0.2 µg/mL	**0.2 µg/mL**	**1 µg/mL**	**2 µg/mL**
		Susceptible strains		
MIC_{90}	100%	100%	100%	100%
MBC	ND	100%	100%	100%
		MDR strains		
MIC_{90}	ND	47%	97%	100%
MBC	0	0	0	0

MIC_{90}: minimum inhibitory concentration required to inhibit the growth of 90% of organisms; MBC: minimum bactericidal concentration; MDR: multidrug resistant; ND: not determined.

Table 5. Correlations between isoniazid and F-27 against multidrug-resistant group.

		INH 0.2 µg/mL	**INH 1 µg/mL**	**INH 2 µg/mL**
F-27 (4 µg/mL)	Pearson correlation	0.484**	0.347*	0.188
	Sig. (1-tailed)	0.001	0.019	0.136
F-27 (8 µg/mL)	Pearson correlation	0.401**	0.255	0.008
	Sig. (1-tailed)	0.008	0.066	0.483
F-27 (16 µg/mL)	Pearson correlation	0.223	0.096	-0.060
	Sig. (1-tailed)	0.095	0.288	0.365
F-27 (32 µg/mL)	Pearson correlation	0.251	-0.031	-0.053
	Sig. (1-tailed)	0.070	0.429	0.379
F-27 (64 µg/mL)	Pearson correlation	0.150	-0.108	0.019
	Sig. (1-tailed)	0.191	0.265	0.457

*Correlation is significant at the 0.05 level (1-tailed); **Correlation is significant at the 0.01 level (1-tailed).

become an alternative in the treatment of *M. tuberculosis* infections [3,4,13-20]. In this study, the bacteriostatic/bactericidal activities of two 1,4-DHPs (F-27, Cl-33) against 113 *M. tuberculosis* clinical isolates were measured. We found that F-27 had MIC_{90}s in the range of 2–8 μg/mL against susceptible isolates, and F-27 completely inhibited the growth of all susceptible isolates in the range of 8–32 μg/mL; these concentrations are one to eightfold lower than the cytotoxicity breakpoint (64 μg/mL) [6]. Growth inhibition of isolates is concentration dependent, so F-27 could be proposed as a promising antitubercular agent [21].

Cl-33 was also active against many susceptible isolates (MIC_{90}: 64 mg/mL; 90%), although none of the isolates were completely inhibited (MBC) even at the highest concentration studied (64 mg/mL). Unfortunately, Cl-33 was inactive against most of the resistant strains at tested concentrations.

On the way toward the discovery of new antituberculosis agents, the aim is to identify molecules called leads, which show properties such as safety, selectivity, low molecular weight, good bactericidal activity against *M. tuberculosis*, act at a known site in the organism, and have acceptable physicochemical and pharmacokinetic properties [22]. Therefore, the 1,4-DHPs have some features that are characteristic of lead compounds; they have been safely used for the treatment of several cardiovascular diseases for decades, their physicochemical and pharmacokinetic properties are well known, and their synthesis is relatively cheap [23]. Deshmane *et al*. [3] showed that isoniazid and 1,4-DHPs interact with InhA at a similar conformation binding pocket, so these compounds probably target the mycolic acids synthesis pathway [23]. Mahnam *et al*. [4] reported the binding affinity of some novel 1,4-dihydropyridine-3,5-dicarboxamides to InhA and inhibition of the InhA enzyme. They also concluded that these compounds do not need the KatG enzyme for activation, and even after mutation of this enzyme in drug-resistant strains, they could still exert their pharmacologic effect [4]. The correlation coefficient of the response of MDR strains against isoniazid and F-27 were analyzed. A good linear relationship was found in the resistance against both drugs (isoniazid and F-27) at low concentrations ($r = 0.484$, $p < 0.001$), but there was no relationship at higher concentrations ($r = 0.019$, $p > 0.1$). This may emphasize a similar mechanism of action for F-27 and isoniazid and no cross-resistance of F-27 with isoniazid. Our conclusion confirmed the finding of Mahnam *et al*. [4] that these compounds have a similar mechanism of action with isoniazid, although their resistance mechanisms were different.

The antitubercular activities of some other 1,4-DHPs have been studied only against the *M. tuberculosis* standard strain (H37Rv), and the results were unsatisfactory. For example, Desai *et al*. observed 98% inhibition at a concentration of 12.5 μg/mL, and Virsodia *et al*. reported 63% inhibition at a concentration of 6.25 μg/mL; similarly, Amini *et al*. and Shafii *et al*. reported 50% and 62% inhibition at a concentration of 6.25 μg/mL, respectively [13,14,17,24]. Other researchers studied more active compounds; Trivedi *et al*. reported MIC of 0.02 μg/mL, Fassihi *et al*. reported MIC of 1 μg/mL, and Deshmane *et al*. reported inhibition with MIC of 3.125 μg/mL [3,15,20]. To the best of our knowledge, only Sirisha *et al*. evaluated the *in vitro* activity of 1,4-DHPs against both susceptible and resistant strains and reported the MIC of 125 μg/mL against H37Rv and 1,280 μg/mL for the only resistant strain tested [18].

Conclusions

In a study with adequate statistical power and optimal sample size, the compound F-27 showed appreciable inhibitory activity against a prominent number of *M. tuberculosis* isoniazid-susceptible strains with MBC of 11.2 ± 1.65 μg/mL. As a result, F-27 could be considered as an excellent lead compound in the class of antitubercular agents. The antimycobacterial action of 1,4-DHPs is likely related to the inhibition of InhA. However, definite mechanisms may actually be more complex, and the precise mechanisms of action of 1,4-DHPs remain to be investigated.

Further *in vitro* studies of F-27 could complete these investigations. For example, the mouse bone marrow macrophage infection assay could be performed to determine the inhibition of *M. tuberculosis* in an intracellular environment [25], the synthesis of analogues of this lead compound, and could help to understanding the synergy or interaction with front-line drugs for *M. tuberculosis*.

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Conflict of interests: No conflict of interests is declared.