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Original Research Article

In vitro evaluation of the inhibitory effect of 3, 5-dichloro-2-pyridone on *Mycobacterium tuberculosis* H37Rv

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Abstract

Purpose: *To investigate the anti-tuberculosis potential of twelve commercially available pyridone analogues against Mycobacterium tuberculosis H37Rv strain.*
Methods: *Twelve commercially available pyridone-based compounds were screened against M. tuberculosis H37Rv using different susceptibility tests. The most active or lead compound was further evaluated in detail for its anti-tuberculosis (anti-TB) potential. Kill kinetics was used to determine the dynamics of its anti-TB activity in vitro.*
Results: *Compounds **d**, **j** and **k** were potent against M. tuberculosis H37Rv, with minimum inhibitory concentrations (MICs) of 10, 5 and 10 µg/mL, respectively. The standard anti-TB drugs used in this study (positive control drugs) demonstrated MIC of 2.5 µg/mL. The anti-TB effect of compound **j** was comparable with those of the standard drugs (RIF, LVX, AMK, EMB and INH). The minimum bactericidal concentration (MBC) of compound **j** was 10 µg/mL. It produced an MIC of 5 µg/mL in agar proportion method (APM). However, its MIC in Middlebrook 7H9 broth supplemented with 10 % fetal bovine serum (FBS) and 4 % bovine serum albumin (BSA) increased 4- and 8-fold, respectively. The bactericidal effect of compound **j** was time- and concentration-dependent at dilutions above 2x MIC. Combination of compound **j** with RIF, LVX or AMK exhibited fractional inhibitory concentration index (ΣFIC) of 1, indicative of additive drug-drug interactions. However, combination with INH or EMB produced a ΣFIC of 2. None of the tested drug combinations was antagonistic.*
Conclusion: *Compound **j** exhibits potent time- and concentration-dependent antimicrobial effect against M. tuberculosis H37Rv. Thus, it may be suitable as an adjunct to current treatment of drug sensitive and drug-resistant TB.*

Keywords: *Tuberculosis, Mycobacterium tuberculosis, Pyridone analogs, Antimicrobial activity, Antibiotics*

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INTRODUCTION

Tuberculosis (TB) is a chronic bacterial infectious disease caused by *Mycobacterium tuberculosis*. It remains a major health challenge globally [1]. Mortality due to TB is put at approximately 2 million annually. About one-third of the world's population harbouring *Tubercle bacilli* in an asymptomatic latent state are at high risk of disease reactivation during their life time [2]. It is

estimated that 5 – 10 % of persons with latent TB infection (LTBI) may develop active TB in their lifetime, and the incidence may be higher in immune-compromised individuals such as those with acquired immunodeficiency syndrome (AIDS) [3].

Despite the progress made so far in TB treatment, recurrence remains a major problem in the developed world [4]. Within the last few decades, in-depth work on TB has led to the discovery of new drug candidates that are currently undergoing pre-clinical and clinical trials. The high lipophilic nature of these drugs makes them potent *in vitro,* with poor *in vivo* pharmacokinetic properties. Thus, they are not effective enough to meet the current requirements in TB drug development [5]. Presently, work on TB drug development is focused on the identification of new chemical moieties and therapeutic scaffolds that can sufficiently improve the pharmacological effects of the drugs [3]. Certain therapeutic scaffolds of pyridones have shown promise as potent anti-TB agents [4,6,7]. This study investigated the inhibitory effects of pyridone-based compounds on *M. tuberculosis* H37Rv.

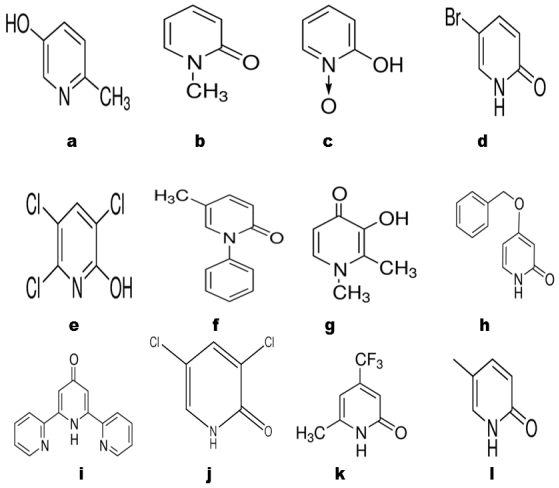

Figure 1: Structures of some commercially-available pyridone analogues

EXPERIMENTAL

Materials

Mycobacterium tuberculosis H37Rv was obtained from American Type Culture Collection (ATCC 27294). Standard anti-TB drugs (rifampin (RIF), ethambutol (EMB), isoniazid (INH), amikacin (AMK) and levofloxacin (LVX)), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (USA). Middlebrook 7H9 broth base, Middlebrook 7H10 agar base, OADC and ADC media supplements were obtained from BD Difco (USA). Fetal bovine serum (FBS) and BSA were products of Gibco (USA).

Preparation of antibiotics

Stock solutions of INH, EMB, AMK and LVX were prepared using sterile Middlebrook 7H9 broth. Stock solutions of test compound and rifampin were prepared using 99 % DMSO. The stock solutions were refrigerated at -40 °C till required.

Mycobacterium tuberculosis cell culture

Mycobacterium tuberculosis was cultured in 100 mL of Middlebrook 7H9 broth supplemented with 10 % ADC solution and 0.2 % glycerol at 37 ˚C for 24 h in a humidified atmosphere of 5 % CO_2 and 95 % air. Cells in logarithmic growth phase were used for the study.

Susceptibility tests

Broth macrodilution method

Broth macrodilution method (BMM) was employed for the assessment of anti-mycobacterial effect of the test compound, with minor modification [8]. Graded concentrations of INH (0.312 - 80 µg/mL), RIF (0.039 - 5 µg/mL), EMB (0.019 - 10 µg/mL) and LVX (0.039 - 20 µg/mL) were prepared in 20 mL falcon tube. The various antibiotics concentrations were subsequently serially diluted in broth medium and added to the tubes at a final cell density of 1 x 10^5 CFU/mL. Colony forming units (CFU) of inoculums were determined by plating appropriate dilutions from the inoculum culture on Middlebrook 7H11 agar supplemented with 10 % OADC in triplicate. Colony-forming unit (CFU) on agar plates were counted after 28 days of incubation. The procedures were carried out for free growth control, positive control and standard anti-TB drugs control. All the tubes were incubated at 37 °C and monitored after 2 weeks of incubation for microbial growth. The minimum inhibitory concentration (MIC) was taken as the lowest antimicrobial concentration at which mycobacterial growth was completely inhibited.

Agar proportion method

Agar proportion method (APM) was performed with slight modification [9]. The test compound or standard was added to Middlebrook 7H10 agar at 2-fold serial dilution. Agar plates free of antibiotics were taken as control plates. The inoculums containing 0.5 mL of *M. tuberculosis* (1 x 10^5 CFU /mL) in logarithmic growth phase were plated on drug or control plates. The inoculated CFUs were determined as described

previously. The plates were then incubated for 4 weeks at 37 °C, and CFUs were scored.

Determination of MIC in the presence of albumin/serum

Minimum inhibitory concentration (MIC) of test compound or standard in the presence of albumin/serum was determined according to the procedure described in literature [10]. Broth microdilution of the test compound or control antibiotics (INH) was performed in 96-well plates with U-shaped bottom. Minimum inhibitory concentration (MIC) of the lead compound and INH were determined in Middlebrook 7H9 broth supplemented with 4 % BSA or 10 % FBS or without protein supplementation.

Determination of minimum bactericidal concentration

Minimum bactericidal concentration (MBC) refers to the lowest antimicrobial concentration which kills 99.99 % of initial bacterial population [11]. The MBCs for the standard anti-TB drugs or test compound were calculated immediately after MIC determination. They were determined after 16-fold serial dilution of the MIC with normal saline. Exactly 0.5 mL aliquots were drawn from each dilution per antibiotic and mixed with Middle brook 7H10 agar supplemented with 10 % OADC. The plates were incubated at 37 °C for 28 days in a humidified atmosphere of 5 % CO_2 and 95 % air, and CFU was thereafter calculated.

Kill curve study

Antibiotics kill curve is a dose-response study in which mammalian cells are subjected to increasing concentrations of selection antibiotics to determine the minimum concentration of an antibiotic that can kill all the cells within a specified period of time.

The kill curve assay was performed according to standard method [11, 12]. *Mycobacterium tuberculosis* cells in logarithmic growth phase (1 x 10^5 CFU/mL) were challenged with the test compound based on its MIC (16-fold dilution) for 16 days in 15 mL falcon tubes. This was performed in duplicate. Then, aliquots were taken from the drug-exposed cultures at different time points (1^{st}, 2^{nd}, 4^{th}, 8^{th}, 12^{th} and 16^{th} days) for determination of bacterial viability. The aliquots were further diluted with Middlebrook 7H9 broth so as to make the concentration of the test antibiotic lower than its MIC. The CFUs on the agar plates were counted after 4 weeks of incubation. The kill curve was obtained by plotting $\log_{10}$ of CFU against time.

Assessment of drug-drug interactions

In vitro drug-drug interaction studies on the first or second line anti-TB drugs were evaluated using standard method with slight modification [4].The test compound was mixed with INH, RIF, EMB, LVX or AMK at 16-fold serial dilution of their respective MICs in 96-well plates. Then, two-fold serial dilutions were made to obtain two drug combinations ranging from 16x to 1/32x concentration of each drug. Antimicrobials were diluted 2-fold separately to obtain a single-drug dilution with similar concentration range as that in two-drug combinations. Subsequently, 96-well plates were inoculated with *M. tuberculosis* H37Rv cells in logarithmic growth phase so that the final cell density in each well was approximately 1 x 10^5 CFU/mL. The plates were sealed in zip lock bags and incubated at 37 °C for 2 weeks. At the end of the 1^{st} and 2^{nd} weeks, the plates were monitored for assessment of microbial growth. The lowest antimicrobial concentration that prevented visible growth of the cells was taken as its inhibitory concentration. Changes in MIC were established for 2 compounds alone and in combination. The combinatorial change in MICs was used to calculate ΣFIC. The fractional inhibitory concentration (ΣFIC) for each drug combination was calculated as shown in Equations 1, 2 and 3:

$$FIC_{DrugA} = MIC_{DrugA} + MIC_{DrugB}/MIC_{DrugA} \ldots. (1)$$

$$FIC_{DrugB} = MIC_{DrugB} + MIC_{DrugA}/MIC_{DrugB} \ldots. (2)$$

$$\Sigma FIC = FIC_{DrugA} \text{ is } + FIC_{DrugB} \ldots\ldots\ldots\ldots\ldots (3)$$

where ΣFIC ≤ 0.5, 0.5 - 4.0 and ≥ 4.0 reflect synergistic, antagonistic and indifferent or additive interaction, respectively [13,14].

RESULTS

Antimycobacterial effects of pyridone analogs

The investigated compounds exhibited potent anti-mycobacterial effects, with MIC values ranging from 5 to 80 µg/mL, as shown in Table 1. The MIC of compound **j** in unsupplemented medium was reduced to 2.5 µg/mL, when compared with that of INH in standard Middlebrook 7H9 broth (Table 2).

The antimycobacterial activity of the test compound at all concentrations plotted as $\log_{10}$ CFU against time (in days) is shown in Figure 2. The *in vitro* drug-drug interaction of compound **j** determined in combination with either the first

line (RIF, INH and EMB) or second line (LVX and AMK) anti-TB drugs is shown in Table 3.

Table 1: Antimycobacterial effects of commercially-available pyridone compounds

Compound	MIC (µg/mL)
A	40
B	20
C	20
D	10
E	80
F	20
G	80
H	40
I	40
J	5
K	10

INH, RIF, EMB, AMK and LVX were used as reference antibiotics in the assay

Table 2: Effect of serum/albumin on MIC of lead compound

MB7H9*	MIC (µg/mL)	
	INH	***Compound j***
Standard media	1.25	5.00
MB7H9 (-OADC)	0.63	2.50
MB7H9 (+ 10 % FBS)	2.50	10.00
MB7H9 (+ 4 % BSA)	2.50	20.00

* MB7H9 = Middlebrook 7H9 broth

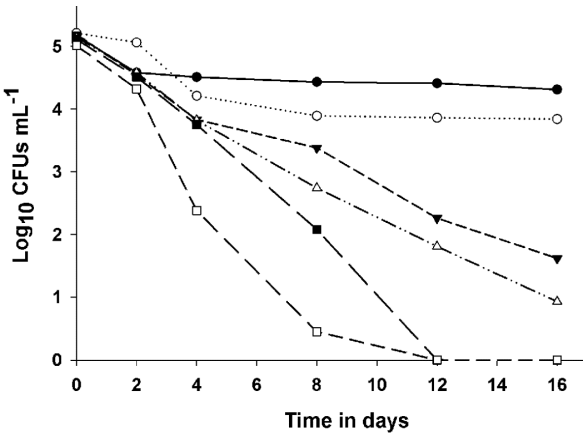

Figure 2: Kill curve data for compound **j**. Cultures of *M. tuberculosis* exposed to compound **j** at 1x (●), 2x (○), 4x (▼), 8x (Δ), 16x (■) and 32x (□) of MIC for 16 days

DISCUSSION

The number of novel anti-TB agents entering the global TB drug development program is scanty. Thus, there is need to identify novel scaffolds for anti-TB drugs. Moreover, the current long duration treatment options, and the emergence of drug resistance in TB have led to renewed interest in discovery of novel and effective anti-tubercular agents or scaffolds. The present study investigated the anti-TB potential of twelve commercially available pyridone analogues against *M. tuberculosis* H37Rv strain. The results showed that compounds **d, j** and **k** were potent against *M. tuberculosis* H37Rv.

Table 3: Drug-drug interaction of compound j with first-line and few second-line anti-TB drugs against *M. tuberculosis* H37RvM

Drug/drug combination	MIC (µg/mL)		ΣFIC	Remarks
	Alone	***Combination***		
INH	1.25	0.31	0.25	
RIF	0.63	0.16		Synergism
Compound j	5.00	5.00	2.00	
INH	0.63	0.63		Additive
Compound j	5.00	2.50	1.00	
RIF	0.63	0.31		Additive
Compound j	5.00	5.00	2.00	Additive
EMB	2.50	2.50		
Compound j	5.00	2.50	1.00	Additive
AMK	2.50	1.25		
Compound j	5.00	2.50	1.00	Additive
LVX	2.50	1.25		

The anti-TB effect of compound **j** was comparable with those of the standard drugs. These results are in agreement with those of previous reports [10]. The minimum bactericidal activity of compound **j** suggests that its MBC is 2 folds of its MIC, and agrees with reports of previous studies [15].

The binding of drug to protein is known to reduce its free and active concentration, thereby reducing the therapeutic effectiveness of the drug [16]. In this study, the MIC of compound **j** was evaluated in the presence of different protein supplements. Its MIC in Middlebrook 7H9 broth supplemented with 10 % FBS and 4 % BSA increased 4-fold and 8- fold, respectively, while INH showed a 2-fold reduction in the two supplement media. Kill kinetics of compound **j** showed that it exhibited up to twice its MIC, and its bacteriostatic effect was exerted with 0.8 and 1 log killing till the end. The other remaining concentrations exerted bactericidal effects that became more prominent from day 4, with constant increase in activity towards the end of the study. On day 12, complete sterilization of the culture medium was achieved at 16 x and 32 x MIC of compound **j**.

The bactericidal effect of compound **j** was time- and concentration dependent at dilutions above 2x MIC, an indication that its antimicrobial activity may be separate from its adverse toxic effects. These results are in agreement with those of previous studies [17]. It is believed that the cavities of the lungs of TB patients contain more than 10^8 *tubercle bacilli.* Therefore, monotherapy of TB is likely to overcome the drug resistance problem associated with *M. tuberculosis* mutants [18].

It is important to validate the type of drug-drug interaction of any test compound with standard drugs in clinics. Isoniazid and RIF, two known anti-TB drugs demonstrated strong synergism as indicated by their ΣFIC values. This phenomenon is the basis of the short-lived effect of modern TB treatment in humans [19,20]. The combination of compound **j** with RIF, LVX or AMK exhibited additive drug-drug interactions. None of the tested drug combinations was antagonistic.

CONCLUSION

Compound **j** exhibits potent time- and concentration-dependent antimicrobial effect against *M. tuberculosis* H37Rv. Thus, it is potentially suitable as an adjunct to current treatment for both drug-sensitive and drug-resistant TB.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

This work was carried out by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by them. All the authors read and approved the manuscript for publication. The authors contributed equally to the conceptualization and execution of this work.

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