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In vitro evaluation of triazoles and pyrimidines as novel antitubercular agents

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ABSTRACT

Novel triazole and pyrimidine derivatives were efficiently evaluated for antibacterial activity against Mycobacterium tuberculosis strain H37Rv by the Resazurin MIC assay. This new structural class of compounds showed high activity against the bacilli. The activity depends on the substituent's present in chalcones of 1,2,4-triazole and pyrimidine core. Compounds having a 4-ClC_6H_4, 3-$NO_2C_6H_4$, 2,4-$MeOC_6H_4$, 2-ClC_6H_4, 4-$MeOC_6H_4$ and 4-BrC_6H_4 group as the substituent on pyrimidine and triazole ring were active. The highest activity was registered for compounds having 4-ClC6H4, 3-$NO_2C_6H_4$ and 2,4-$MeOC_6H_4$ as substituents. The new compounds showed high potency and promising antitubercular activity and should be regarded as new hits for further development as a novel class of Anti-Mycobacterium tuberculosis agents.

Keywords: *Triazole, pyrimidine, chalcones, Mycobacterium tuberculosis.*

1. INTRODUCTION

Tuberculosis is a chronic infectious disease caused by *Mycobacterium tuberculosis*, an acid-fast aerobic bacillus, considered one of the most significant threats to global health. It is estimated that today one-third to one-half of the world population is infected with *M. tuberculosis* leading to approximately 6% of all death worldwide. Each year, it is estimated that 9.2 million new cases appear, of which many lead to death [1]. The World Health Organization (WHO) has estimated that approximately 2 billion people worldwide are latently infected with *M. tuberculosis* and approximately 10% will develop the active disease during their lifetime. In addition, tuberculosis is a frequent HIV co-infection and a major cause of death among people with HIV-AIDS. In the recent years, multidrug-resistant (MDR-TB) and extensively drug-resistant (XDR-TB) tuberculosis strains emerged. The current TB treatment takes 6-12 months and requires a combination of three or four drugs like Isoniazid, Streptomycin, Rifampin and Pyrazinamide [2]. The narrow choice of antibiotics, lengthy treatment regimens, and patient noncompliance have provided conditions for acquired antibiotic resistance that led to worldwide emergence of strains resistant to virtually all available drugs. Currently, there is still an urgent demand for new and more effective anti-TB drugs possessing new modes of action.

Among azoles, 1,2,4-triazoles have been reported to exhibit anticancer [3], antibacterial [4,5], anticonvulsant [6], anti-inflammatory, analgesic [7], antifungal [8,9], antidepressant [10], antitubercular [11], antimalarial [12] and hypoglycemic [13] activities. Literature survey reveals that pyrimidine derivatives are well known to have antimicrobial [14-16], antimalarial [17],

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anticonvulsant [18], anticancer [19], anti-inflammatory, analgesic [20,21], antitubercular [22] activities. We have reported that 6-(substituted aryl)-4-(3,5-diphenyl-1H-1,2,4-triazol-1-yl)-1, 6-dihydropyrimidine-2-thiol (4a-j) had significant anticancer, anticonvulsant, antimicrobial [23] and analgesic activity [24]. More recently, we have also found that 1-(3,5-diphenyl- 1H-1,2,4-triazol-1-yl)-3-(substituted aryl) prop-2-en- 1-one (Chalcones, 3a-j) have better analgesic [24] and antimicrobial [25] activity. To the best of our knowledge very few references are available on the chalcones of triazole and triazole clubbed with pyrimidines as antitubercular agents, either as a core or incorporated as substituents in other base structures. Herein we describe the *in vitro* activity against *M. tuberculosis* strain $H_{37}Rv$ of this novel structural class of pyrimidines and triazoles.

2. EXPERIMENTAL SECTION

2.1. Antitubercular activity. *In vitro* evaluation of the antitubercular activity was carried out within the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) screening program for the discovery of novel drugs for the treatment of tuberculosis [26], under the direction of the US National Institute of Allergy and Infectious Diseases (NIAID), Southern Research Institute that coordinates the overall program. All the new pyrimidines and triazoles were screened for antitubercular activity [27]. All the compounds 3a-3j and 4a-4j were initially submitted to TAACF for initial approval and selection for antitubercular activity. The compounds were submitted by using compound submission form (CSF), which includes the information regarding the structure, physical and chemical properties including molecular weight.

2.2. Procedure for the Resazurin MIC Assay. The Resazurin MIC assay [28] developed by Collins and Franzblau, is a colorimetric assay used to test compounds for anti-mycobacterium activity. A color change from blue to pink was observed when growth occurs. Compounds were initially tested at a single point concentration of 10 µg/mL against *M. tuberculosis* $H_{37}Rv$, obtained from Colorado State University, Fort Collins, CO. If compounds were active at the 10 µg/mL, they were further tested in an MIC assay at 8 concentrations in a dose ranging between 10 to 0.078 µg/mL as shown in Table 1.

2.3. Receipt and Preparation of Test Compounds. Upon receipt, test compounds 3a-3j and 4a-4j were logged into the inventory spreadsheet and placed in a -20ºC freezer. The day of the experiment, one vial from each compound was reconstituted using the DMSO as solvent to achieve a stock concentration of 3.2 mg/mL

2.4. Inoculums Preparation. $H_{37}Rv$ was grown in Middle brook 7H9 broth medium (7H9 medium) supplemented with 0.2% (v/v) glycerol, 10% (v/v) ADC (albumin, dextrose, catalase), and 0.05% (v/v) Tween 80. The bacteria were inoculated in 50 mL of 7H9 medium in 1 liter roller bottles that are placed on a roller bottle apparatus in an ambient 37^{o}C incubator. When the cells reach an OD600 of 0.150 (equivalent to ~1.5 x 107 CFU/mL), they are diluted 200-fold in 7H9 medium.

2.5. Single Point Concentration Procedure. The procedure is the same as that used for the MIC procedure described below, but only the first 2 fold dilutions were made to reach the 1.6 mg/mL concentration. An additional 1:10 dilution was made in water (Step 3 below) to reach the 0.16 mg/mL concentration. Addition of 6.25 µl of the 1:10 dilution to the wells in a final volume of 100 µl will give rise to a concentration equivalent to 10 µg/mL (Step 2 below).

MIC procedure: 1) 20 µl of the 3.2.mg/mL of test was added to 96 -well micro titer plate; 2) Two fold dilutions were made by the addition of 20 µl of diluents; 3) Each dilution was further diluted 1:10 in sterile water (10 µl of dilution in 90ul of sterile water); 4) 6.25 µl of each dilution was transferred to duplicate 96-well test plates; 5) 93.75 µl of the cell suspension (~ 10^4 bacteria) in 7H9

medium is added to the test plates; 6) Positive, negative, sterility and Resazurin controls were tested (Positive controls include: Rifampicin and Isoniazid; Negative controls include: cell culture with solvent DMSO and water, Cell culture only; Sterility controls include: media only, media with solvent DMSO and water); 7) Resazurin control includes one plate containing the diluted compounds with Resazurin only. No bacterial suspension was added. This control plate is needed to verify whether the compound reacts with Resazurin that could possibly elicit fluorescence; 8) The 96 well test plates were incubated in an ambient 37ºC incubator for 6 days; 9) After the 6 day incubation, 5 µl of a 0.05% sterile Resazurin solution was added to each well of the 96-well plate. The plates were placed in an ambient 37ºC incubator for 2 days; 10) After the 2 day incubation, a visual evaluation and fluorimetric read-out is performed. The results are expressed as µg/mL (visual evaluation) and as IC_{50} and IC_{90} (fluorimetric readout) [29]. So, all the compounds 3a-3j and 4a-4j were evaluated for antitubercular activity and results are represented in the form of MIC (Table 2).

Table 1: Dilution of test drugs.

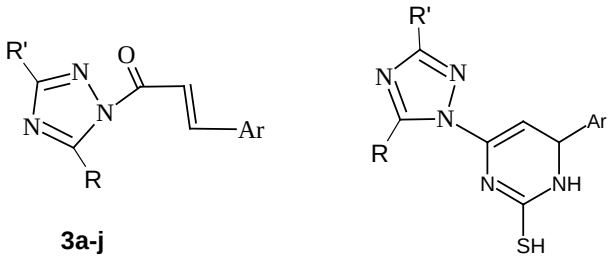
Expected final dose level In µg/mL	Test compound =3.2 µg/mL	Dilution
10.00	1st dilution of 8= 1.600 µg/mL	Dilute 1:2
5.000	2nd dilution of 8= 8.000 µg/mL	Dilute 1:2
2.500	3rd dilution of 8= 4.000 µg/mL	Dilute 1:2
1.250	4th dilution of 8= 2.000 µg/mL	Dilute 1:2
0.625	5th dilution of 8= 1.000 µg/mL	Dilute 1:2
0.312	6th dilution of 8= 0.050 µg/mL	Dilute 1:2
0.156	7th dilution of 8= 0.025 µg/mL	Dilute 1:2
0.078	8th dilution of 8= 0.012 µg/mL	Dilute 1:2

Note: The additional 10-fold dilution in water is required when DMSO is used as solvent to minimize toxicity towards bacteria. For the uniformity in the assay procedure, this dilution step is used even if water or other solvents are used.

3. RESULTS SECTION

The antitubercular activity of compounds 3a-3j and 4a-4j against *M.tuberculosis* strain $H_{37}Rv$ was assessed by Resazurin assay method at Tuberculosis acquisition and antimicrobial coordination facility (TAACF), USA [29]. The activity was evaluated and compared on the basis of substituent at phenyl ring but not on the basis of active lead. The DMSO was used as solvent. The most of the molecules were proved to be active. Their activity depends on the substituent in phenyl ring of the lead structure. Any compound having an $IC_{90} \leq 10$ µg/mL was considered active for antitubercular activity. While the compound having MIC value greater than 10 µg/mL was considered to be inactive when compared with Isoniazid and Rifampin and control. The antitubercular activity for compounds 3a-3j and 4a-4j is shown in Table 2.

The compounds 3a, 3b, 4a, 4b and 4j showing a MIC at 0.156 µg/mL were considered highly active antitubercular agents. They also showed IC_{50} values at 0.14 µg/mL, 0.16 µg/mL, 0.12, µg/mL, 0.14 µg/mL and 0.12 µg/mL respectively. The MIC graph of compound 4a is shown in Figure 2. Compounds 3f, 4d and 4f were also found to be good antitubercular agents with MIC at 0.312µg/mL respectively. Derivatives 3h, 3j, 4e, 4h and 4i exhibited moderate antitubercular potential, with MIC at 0.625µg/mL respectively. The activity result suggests that substitution of EWG like chloro and nitro on phenyl ring (Figure 1) of chalcones and pyrimidine induced excellent activity. The ERG like methoxy and hydroxy substituted analogues showed moderate to good activity, some of them being weak antitubercular agents.



R = R' = Phenyl ring

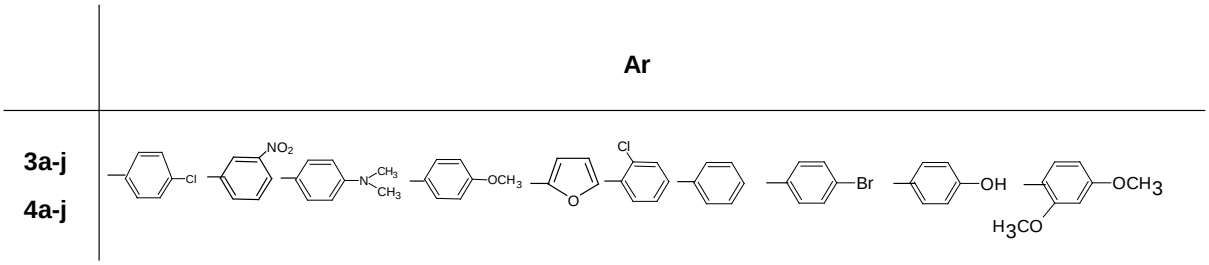


Figure 1: Compounds with their substituents tested for antitubercular activity.

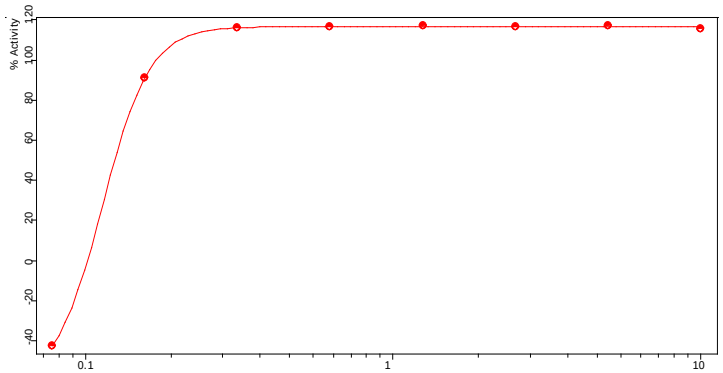


Figure 2: MIC graph of compound 4a for antitubercular activity.

Table 2: Antitubercular activity of compounds 3a-3j and 4a-4j against *M. tuberculosis* strain $H_{37}Rv$.

Sr. no.	Compound	MIC (µg/mL)	IC_{50} (µg/mL)	IC_{90} (µg/mL)	Activity
1	3a	0.156	0.14	0.20	Active
2	3b	0.156	0.16	0.22	Active
3	3c	>10	NT	NT	-
4	3d	5	2.44	2.76	Active
5	3e	>10	NT	NT	-
6	3f	0.312	0.20	0.24	Active
7	3g	>10	NT	NT	-
8	3h	0.625	0.14	0.16	Active
9	3i	5	2.56	2.88	Active
10	3j	0.625	0.14	0.16	Active
11	4a	0.156	0.12	0.14	Active
12	4b	0.156	0.14	0.20	Active
13	4c	>10	NT	NT	-
14	4d	0.312	0.12	0.18	Active
15	4e	0.625	0.11	0.18	Active
16	4f	0.312	0.12	0.18	Active
17	4g	>10	NT	NT	-
18	4h	0.625	0.13	0.19	Active
19	4i	0.625	0.12	0.20	Active
20	4j	0.156	0.12	0.16	Active
21	Isoniazid	0.063	-	-	Active
22	Rifampicin	0.006	-	-	Active
23	Control	No growth	-	-	-

*NT-not tested

4. CONCLUSIONS

Different triazole and pyrimidine derivatives were efficiently screened for their antimycobacterial activity against *M.tuberculosis* strain $H_{37}Rv$ and most of the molecules proved to be active. Their activity depends on the substituents present on phenyl ring of pyrimidine and triazole chalcones. Compounds having a 4-chloro, 3-nitro and 2,4-OCH_3 entity, were highly active. The compounds bearing 4-OCH_3, 4-Br, 4-OH and 2-Cl were also found to be moderately active. These novel molecules are new promising antitubercular hit compounds. Furthermore, as the compounds discussed herein have no structural similarity to any other compounds active on *M. tuberculosis,* this may indicate that they may act by a new mechanism of action. Further structural modifications of the identified hits are in progress in order to enhance the efficacy of this new structural class of compounds active against *M. tuberculosis*.

5. ACKNOWLEDGMENT

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