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In vitro evaluation of tetrazoles as a novel class of Antimycobacterium tuberculosis agents

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ABSTRACT

***Purpose*:** We report here the antimycobacterial activity of some already synthesized tetrazole derivatives containing tetrazole against Mycobacterium tuberculosis strain H37Rv. ***Methods*:** In vitro evaluation of the antitubercular activity was carried out within the Tuberculosis Antimicrobial Acquisition & Coordinating Facility (TAACF) screening program for the discovery of novel drugs for the treatment of tuberculosis. Under the direction of the US National Institute of Allergy and Infectious Diseases (NIAID), Southern Research Institute that coordinates the overall program. The method of TAACF was followed for evaluation of activity. ***Results*:** This new structural class of compounds showed high activity against the bacilli. The activity depends on the substituent's present in azatidinone core. Compounds having a 4-$MeOC_6H_4$ 4-$N(CH_3)_2C_6H_4$ group as the substituent on β-lactam ring were active. The highest activity was registered for compounds having 4-$MeOC_6H_4$ as substituent. ***Conclusion*:** 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 Antimycobacterium tuberculosis agents.

Introduction

Infection with Mycobacterium tuberculosis affects much of the world population, despite the fact that drugs for treating tuberculosis (TB) were available for over half a century. Each year, it is estimated that 9.2 million new cases appear, of which many lead to death[1-4]. 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 living with HIV-AIDS. In recent years, multidrug-resistant (MDR-TB) and extensively drug-resistant (XDR-TB) tuberculosis strains emerged and tuberculosis can be considered one of the most significant threats to global health. [5, 6] The current TB treatment takes 6e12 months and requires a combination of three or four drugs that were developed almost half a century ago: isoniazid, streptomycin, rifampin and pyrazinamide (Figure 1). The narrow choice of antibiotics, lengthy treatment regimens, and patient noncompliance has provided conditions for acquired antibiotic resistance that led to worldwide emergence of strains resistant to virtually all available drugs. [5-7]

Since mid-1985s a renewed interest in the discovery of new antitubercular drugs led to the appearance of new classes of compounds active against M. tuberculosis. [8-10] Nowadays several agents belonging to the fluoroquinolone, oxazolidinone, diarylquinoline, and nitroimidazo-oxazole/-oxazine classes are in various stages of development. [2-4, 11] However, new clusters of extensively drug resistant tuberculosis may always appear and, currently, there is still an urgent demand for new and more effective anti-TB drugs possessing new modes of action. According to the literature, tetrazole can be synthesized from the from benzonitrile and sodium azide in presence of ammonium chloride.[12, 13] They can also be synthesized from hydrazoic acid. During the last few years, our research group developed a simple and efficient method to synthesize 5-phenyl tetrazole and its different derivative. Similarly tetrazole are found to possess different pharmacological activities like antimicrobial[14], antibacterial[15], antifungal[16], analgesic[17], anti-inflammatory[18], Antinociceptive[19], antitubercular activity[20], anticancer.[21]

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To the best of our knowledge the tetrazole containing azatidinones were never evaluated as antitubercular agents, either as a core or incorporated as substituent's in other base structures. Herein we describe the in vitro activity against M. tuberculosis strain H37Rv of this novel structural class of azatidinones containing tetrazoles which were previously synthesized and reported. Thus, a single molecule containing more than one pharmacophore, each with different mechanism of action could be beneficial for the treatment of tuberculosis. Taking inspiration from the above, and as a part of our enduring research on the "chemistry-driven" approach of tetrazoles, we have struck a rich lode of novel bioactive agents and report herein the influence tetrazole containing azatidinone scaffold combination on the antimycobacterial effect.
These compounds showed high activity against the bacilli.

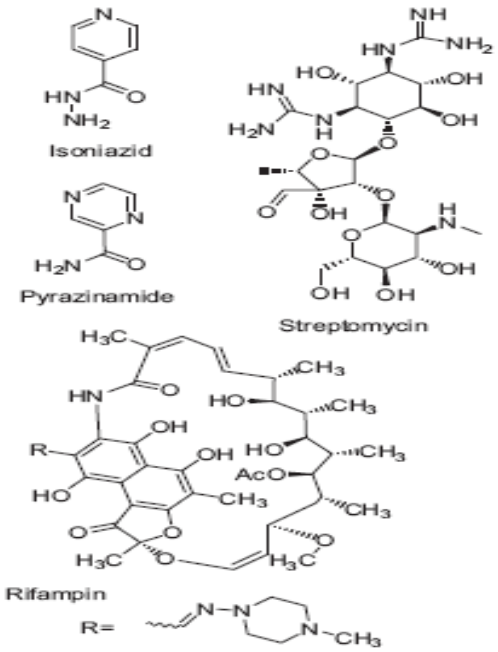

Figure 1. Drugs currently used for TB treatment

Materials and Methods

The scaffold analogues of tetrazole viz.A1-A7 were synthesized and characterized as reported earlier [22] by our research group (Figure 2).

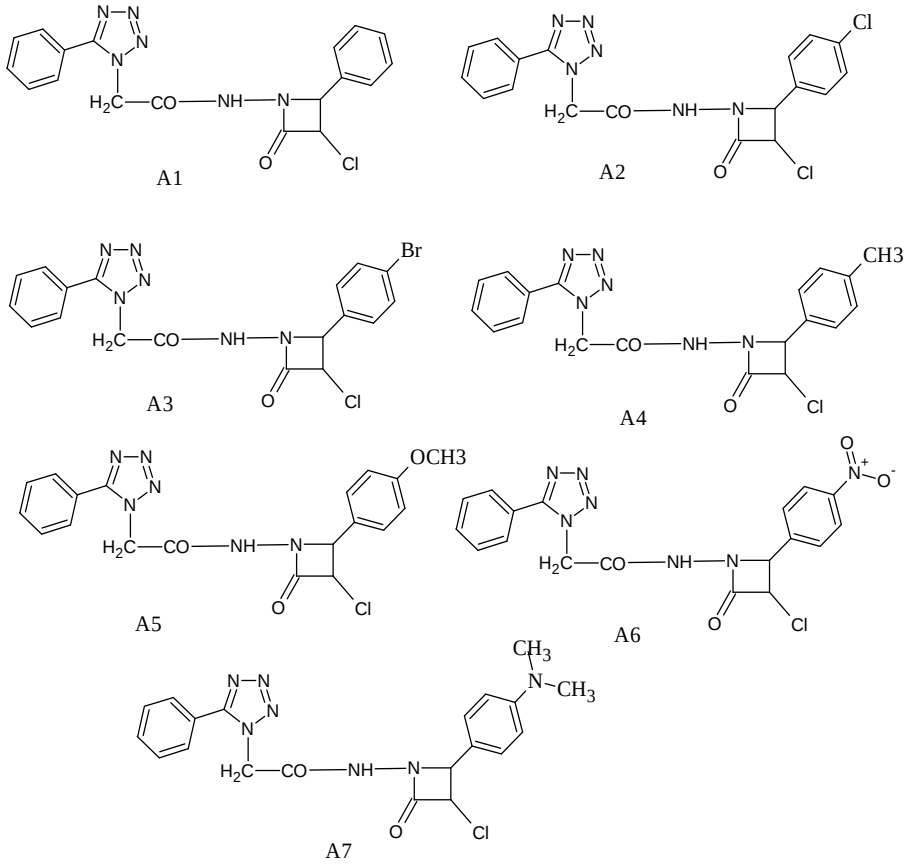

Figure 2. Structure of different tetrazole derivatives (A1-A7).

In vitro antitubercular activity

In vitro evaluation of the antitubercular activity was carried out within the Tuberculosis Antimicrobial Acquisition & Coordinating Facility (TAACF) screening program for the discovery of novel drugs for the treatment of tuberculosis. 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 tetrazole were screened for antimycobacterial activity on the M. tuberculosis strain H37Rv according to procedures previously published by the TAACF organization[23]. All the new tetrazole were screened for antimycobacterial According to the information on the TAACF webpage[24] and NIAID webpage[25] the IC90 of the compounds is determined as a primary screen. Any compound having an IC90 ≥10 mg/mL is considered active for antitubercular activity.

Procedure for the Resazurin MIC Assay

The resazurin MIC assay, developed by Collins and Franzblau (1997), is a colorimetric assay used to test compounds for antimycobacterial activity. A color change from blue to pink is observed when growth occurs. Compounds are initially tested at a single point concentration of 10 μg/ml against *Mycobacteruim tuberculosis* H37Rv (H37Rv), obtained from Colorado State University, Fort Collins, CO. If compounds are active at the 10 μg/ml level, they are further tested in an MIC assay at 8 concentrations in a dose range between 10 to 0.078 μg/ml.

Receipt and Preparation of Test Compounds

Upon receipt, test compounds [A1-A7] are logged into the inventory spreadsheet and placed in a -20ºC freezer. The day of the experiment, one vial from each compound is reconstituted using the supplier's recommended solvent to achieve a stock concentration of 3.2 mg/ml.

Inoculum Preparation

H37Rv is grown in Middlebrook 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 are inoculated in 50 ml of 7H9 medium in 1 liter roller bottles that are placed on a roller bottle apparatus in an ambient 37oC 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.

Single Point Concentration Procedure

The procedure is the same as that used for the MIC procedure described below, but only the first 2 fold dilution is made that reduces the stock solution to 1.6 mg/ml. An additional 1:10 dilution is made in water (see Step 3 below) which reduces the stock solution further to 0.16 mg/ml. 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 (see Step 2 below).

MIC procedure

1. 20 μl of the 3.2.mg/ml of test was added to 96 -well microtitre plate.
2. Two fold dilutions were made by the addition of 20 μl of diluents.
3. Each dilution is further diluted 1:10 in sterile water (10 μl of dilution in 90ul of sterile water)
   Note: The additional 10-fold dilution in water is required when DMSO is used as solvent to minimize toxicity to the bacteria. For uniformity in the assay procedure, this dilution step is used even if water or other solvents are used.
4. 6.25 μl of each dilution is transferred to duplicate 96-well test plates.
5. 93.75 μl of the cell suspension (~ 104 bacteria) in 7H9 medium is added to the test plates.
6. Positive, negative, sterility and resazurin controls are tested.
   a. Positive controls include: rifampicin and isoniazid
   b. Negative controls include:
      i. cell culture with solvent and water
      ii. cell culture only
   c. Sterility controls include:
      i. media only
      ii. media with solvent and water
   d. Resazurin control includes one plate containing the diluted compounds with resazurin only. No bacterial suspension is added. This control plate is needed to verify whether the compound reacts with resazurin that could possibly elicit fluorescence.
7. The 96 well test plates are incubated in an ambient 37ºC incubator for 6 days.
8. After the 6 day incubation, 5 μl of a 0.05% sterile resazurin solution is added to each well of the 96-well plate. The plates are placed in an ambient 37ºC incubator for 2 days.
9. 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 IC50 and IC90 (fluoremetric readout)

Results and discussion

The results summarized in Table 1, shows that most of the tetrazole were active against Mycobacterium tuberculosis and the activity depends on substituents present in azatidinone core.

Different tetrazole derivatives of general structure A1-A7 having different aryl substituents on azatidinone core were efficiently synthesized. The activity of these compounds against M. tuberculosis strain H37Rv was assessed and most of the molecules proved to be active. Their activity depends on phenyl ring substituents. Compounds having a 4-methoxyphenyl and 4-dimethylamino phenyl unit, are highly active when compared with isoniazid and rifampin. The compound having 4-chlorophenyl,4-Bromophenyl on azatidinone core are also found to be moderately active. In case of methoxy and dimehtylamino, the IC90 varies from 0.18 (A5) to 0.14 mM (A7).The results of MIC was depicted in figure 3,4.These novel molecules are new promising antitubercular hit compounds.

Table 1. showing dilution of dose level of test compound

Expected final dose level In µg/ml	Test compound =3.2 µg/ml	Dilution
10	1st dilution of 8= 1.6 µg/ml	Dilute 1:2
5	2nd dilution of 8= 8 µg/ml	Dilute 1:2
2.5	3rd dilution of 8= 4 µg/ml	Dilute 1:2
1.25	4th dilution of 8= 2 µg/ml	Dilute 1:2
0.625	5th dilution of 8= 1 µg/ml	Dilute 1:2
0.312	6th dilution of 8= 0.05 µg/ml	Dilute 1:2
0.156	7th dilution of 8= 0.025 µg/ml	Dilute 1:2
0.078	8th dilution of 8= 0.0125 µg/ml	Dilute 1:2

Table 2. Antibacterial activity of compound A1-A7 against M. tuberculosis strain $H_{37}Rv$

Entry	Compound	R	MIC (µg/ml)	IC50 (µg/ml)	IC90 (µg/ml)
1	A1	C_6H_5	>10	NT	NT
2	A2	4-ClC_6H_4	>6.25	NT	NT
3	A3	4-BrC_6H_4	>6.25	NT	NT
4	A4	4-MeC_6H_4	>10	NT	NT
5	A5	4-$MeOC_6H_4$	0.156	0.11	0.18
6	A6	4-$NO_2C_6H_4$	>10	NT	NT
7	A7	4-$N(CH_3)_2C_6H_4$	0.156	0.12	0.14
8	Rifampin	-	0.006	-	-
9	Isoniazid	-	0.063	-	-
10	Control	Growth	-	-	-

*NT-not tested

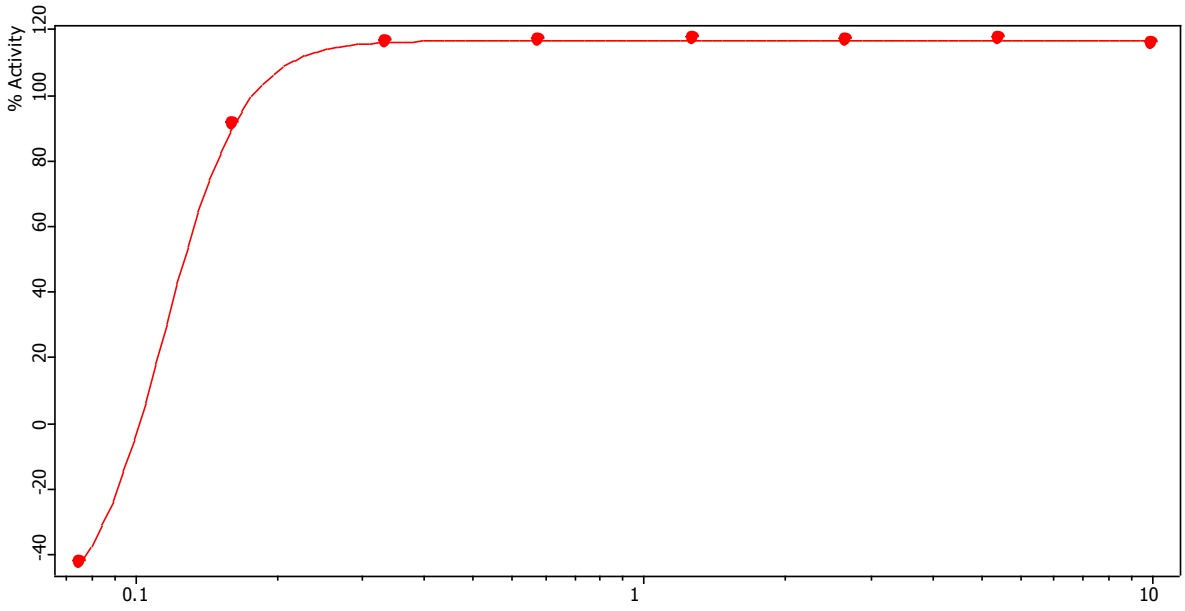

Figure 3. MIC graph of compound A5

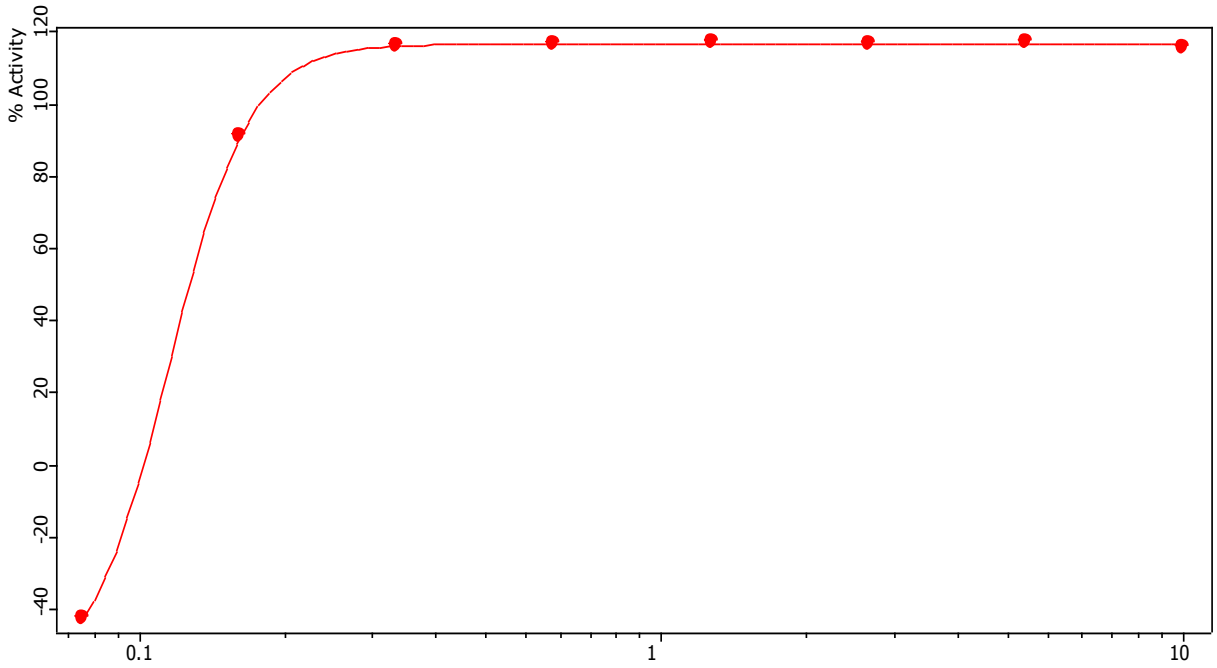

Figure 4. MIC graph of compound A7

Furthermore, as the compounds discussed herein have no structural similarity to any other compounds active against 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.

Conclusion

The different tetrazole derivatives containing azatidinone may serve as good antimycobacterial agents which may help the medicinal chemist in drug discovery and development. The obtained results prove the necessity for further investigations to clarify the features underlying the Antimycobacterial potential of tested compounds.

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Conflict of interest

All the authors report no conflicts of interest.

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