Bulletin of Pure and Applied Sciences
Vol.38A (Zoology), No.2,
July-December 2019: P.102-109
Original Research Article

Print version ISSN 0970 0765
Online version ISSN 2320 3188
DOI 10.5958/2320-3188.2019.00011.1
Available online at www.bpasjournals.com

In vitro Antitubercular Activity of Dihydropyridine-Dicarboxamide and Pyrazole Derivatives against *Mycobacterium tuberculosis*

[1]Sushil Kumar Upadhyay[#]*
[2]Raj Singh
[3]Parveen Kumar
[4]Manoj Singh
[5]Mukesh Yadav
[6]Vikas Kumar
[7]Diwakar Aggarwal
[8]Nirmala Sehrawat

Author's Affiliation:
[1,2,4-8]Department of Biotechnology, Maharishi Markandeshwar (Deemed to be University), Mullana-Ambala (Haryana) - 133207, India
[3]Department of Chemistry, Meerut College, Meerut, Uttar Pradesh, India.

***Corresponding Author:**
Dr S. K. Upadhyay, Department of Biotechnology, Maharishi Markandeshwar (Deemed to be University), Mullana-Ambala (Haryana) - 133207, India.

[#]E-mail: sushil.upadhyay@mmumullana.org

Received on 19.09.2019
Accepted on 27.11.2019

Abstract:

The 1,4-dihydropyridine (DHP) is the most feasible heterocyclic ring with various substitutions at several positions. The DHP and pyrazole are ever-growing due to their varied biological, pharmaceutical and therapeutic applications. The antitubercular activity of some DHP and pyrozole derivatives (BPD, CMBPD, NMBPD, DPD, DNDDP, DMDDP, BEMPMP, OMEMPMP, KPEMPMP, CEMTDP and NEPMCP) have been documented in the present study. The *in vitro* activity of selected compounds as antitubercular agents have been measured in term of zone of inhibition (ZI) and minimum inhibitory concentration value (MIC, MIC_{50} and MIC_{90}). The compounds BPD, DNDDP, CEMTDP, NEPMCP showed good antitubercular activity against *Mycobacterium tuberculosis* with zone of inhibition 20-21mm and MIC value ranged between 5-12. The other derivatives DPD, CMBPD, OMEMPMP demonstrated moderate antitubercular activity against *M. tuberculosis* with zone of inhibition 14 to 17mm with MIC value 10 to 14. However, compounds NMBPD, DMDDP showed remarkable antitubercular activity against *M. tuberculosis* with zone of inhibition 21mm and MIC value 4-5. While the compounds BEMPMP, KPEMPMP showed mild activity against *M. tuberculosis* with zone of inhibition 11-12mm and MIC value between 17-20.

Keywords: Antitubercular, Dihydropyridine, Pyrazole, *Mycobacterium tuberculosis,* MIC and ZI.

INTRODUCTION

Tuberculosis (TB) is the foremost grounds of death by contagious disease with 1/3rd of the world population infected. The number of patients infected with RB disease is escalating world-wide because of multi-drug resistant (MDR) strains of causative agent, *Mycobacterium* and high disease prevalence percentage of tuberculosis in patients having acquired human-immunodeficiency syndrome (AIDS) (Newton et al., 2000). The unique structure of *Mycobacterium tuberculosis* cell wall is responsible for resistance of mycobacters. The acid-fast bacillus *M. tuberculosis*, the causative agent of tuberculosis (TB), possesses a cell wall that differs significantly in structure from both Gram-negative and Gram-positive bacteria. This cell wall consists of mycolyl-arabinogalactan units which are bonded to peptidoglycan nucleus through covalent bonds (Dover, et al., 2004). Because of the global

health tribulations of tuberculosis in human population, the design and development of efficient new anti-tuberculosis drugs lacking cross resistance with known antimycobacterium agents is urgently needed. Antitubercular drugs available for treatment were discovered in the period of 1945 to 1965. Moreover the recent emergence of outbreaks of multidrug resistant tuberculosis, MDR-TB, to the first-line drugs: isoniazid (INH), rifampicin (RIF), ethambutol (ETH), streptomycin (STR), and pyrazinamide (PYR) have made the disease hard to be cured. This obstacle in treatment of tuberculosis and the statistical facts about its prevalence tell us about the necessity of searching and synthesizing new more potent and less prone to resistance compounds with less side-effect (Fassihi et al., 2009; Kumar et al., 2018). The earlier finding on the same plan reflected that 1,4-dihydropyridines class of compounds (DHPs) are excellent opening for the development of anti-tubercular agents (Gavariya et al., 2001, 2002; Eharkar et al., 2002; Foroumadi et al., 2006; Daryabari et al., 2007). The 1,4-dihydropyridine (1,4-DHP) is a molecule based upon pyridine, and the parent of a class of molecules that have been semi-saturated with two substituents replacing one double bond. They are particularly well known and potent as L-type calcium channel blockers, in pharmacology for the treatment of hypertension. The 1, 4-dihydropyridine is ever-growing due to their varied biological pharmaceutical applications (Kumar et al., 2018). The DHP (1,4-dihydropyridine) is an aromatic ring saturated at 1^{st} and 4^{th} position. The most practicable position for substitution is 4^{th} which exhibit various activities i.e., as the calcium channel antagonists and the heterocyclic ring is the universal feature for a range of pharmacological actions such as antihypertensive, antianginal, antitumor, anti-inflammatory activity, antitubercular activity, analgesic activity, antithrombotic (Wachter and Davis, 1998; Bahekar and Shinde, 2002; Gullapalli and Ramarao, 2002; David, 2007; Swarnalatha et al., 2011). The *in vitro* study of the efficacy of a compound as an antitubercular agent can be measured in term of MIC (Minimum Inhibitory Concentration), MIC_{50} and MIC_{90} value (Eby et al., 1984). The MIC value of a drug is the minimum concentration of drug to prevent the growth of microorganism (Spinu et al., 2008). The MIC_{50} value of a drug is the unit concentration of drug to prevent the 50% growth of microorganism. However, MIC_{90}, value of a drug is the unitary concentration of a drug to prevent 90% growth of micro-organism (Chohan and Kausar, 2000).

MATERIALS AND METHODS

The *in vitro* antitubercular activity and efficacy of synthesized compounds was tested against the *Mycobacterium tuberculosis*. The screening results were compared with Moxifloxacin (Zone of inhibition, ZI = 24 to 26mm) as a reference drug (Andrews et al., 1999). The screening culture medium was nutrients agar (Bacteriological grade, Qualigen fine chem. Mumbai, India) after Upadhyay (2016) and antitubercular screening was performed by filter paper disc method or disc diffusion assay (Chang et al., 2000). The solvent used was 10% of Dimethyl sulfoxide (DMSO) in methanol and biological screening result was mentioned in mm (millimeter). The diameter of inhibition zone was categorized mild, moderate and effective for 6mm, 7–13mm and 14–26mm respectively (Harikrishna et al., 2017; Xu et al., 2017). The nutrient broth was prepared with meat extract broth consisted watery extract of buffalo meat to which peptone water was added that has digested with a proteolytic enzyme (Mishra et al., 1995). For meat extract preparation all the fat content of buffalo meat was removed at first and minced. The minced meat was added to water allowed to thorough mixing for 24hrs at cold (4**°C**). After this fat layer was removed and it was allowed to boil for 15minutes, cooled and filtered, thus a clearly meat extract was obtained (Satoskar and Itokawa, 1933). The peptone water was prepared by dissolving 1g of peptone powder and 0.5g of NaCl in 100ml distilled water and sterilized in autoclave at 121°C for 30minutes. The 100ml meat extract broth consisting peptone (10g), meat extract (10g) and sodium chloride (10g) with pH 7.5–7.6 and autoclaved at 121°C for 15 minutes (Das, 1990).

The MIC (Minimum Inhibitory Concentration), MIC_{50} and MIC_{90} value (Eby et al., 1984) of synthesised metal complexes and ligands was evaluated by filter paper disc method (Gould and Bowie, 1952) and disc diffusion assay (Chang et al., 2000). The antitubercular activity of methanolic solution of compounds and standard drug was performed by 6.5mm size disc of blank whatmann filter paper grade-1 which was sterilized by dry heat method at 140°C for 1hr. It was saturated with

test solution and the known standard reference antibiotic solution separately. These discs were air dried at room temperature to remove residual solvent which might interfere with determination. The discs were placed on the surface of sterilized agar nutrient medium that had been inoculated with test organism (by using sterile swab). The depth of the agar medium was kept equal in all petri-dishes and standard disc of moxiploxacin was used in each plate as a control. Before incubation petri-dishes were placed for 1hr in cold room (4^{o}C) to allow diffusion of compounds from disc into the agar plate. These discs were further incubated at 37°C for 20–24hrs after which the zone of inhibition or depressed growth was measured (Chohan and Kausar, 2000).

RESULTS

Antitubercular activity of BPD, CMBPD and NMBPD: The compound N, N'-bis (1, 3-benzothiozolyl)-2,6-pyridine dicarboxamide (BPD) showed good antitubercular activity against *M. tuberculosis* with zone of inhibition 20mm. On further observation it was analyzed that minimum 8mg weight of BPD required for inhibiting growth of *M. tuberculosis* (MIC value). The subsequent quantitative augmentation of synthesized compound up to 15mg and 26mg inhibited the 50% (MIC_{50} value) and 90% (MIC_{90} value) growth of microorganism respectively. The compound N, N'-bis [(2-chloro-5-methyl)-N-(1, 3-benzothiozolyl)-benzene sulphonamide]-2, 6-pyridine dicarboxamide (CMBPD) showed moderate antitubercular activity against *M. tuberculosis* with zone of inhibition 17mm. On further observation it was analyzed that minimum 10mg weight of CMBPD was required to inhibit growth of *M. tuberculosis* (MIC-value). The 17mg and 30mg dosage of CMBPD compound inhibited 50% (MIC_{50} value) and 90% (MIC_{90} value) growth of microorganism respectively. However, compound N, N'-bis [(2-nitro-5-methoxy)-N-(1, 3-benzothiozolyl)-benzene sulphonamide]-2,6-pyridine dicarboxamide (NMBPD) showed remarkable antitubercular activity against *M. tuberculosis* with zone of inhibition 21mm (Table 1).

Table 1: The *in vitro* antitubercular activities as ZI (mm) and MIC values of BPD, CMBPD and NMBPD

Synthesized compound	ZI (mm)	MIC	MIC_{50}	MIC_{90}
BPD	20	08	15	26
CMBPD	17	10	17	30
NMBPD	21	04	15	35

Antitubercular activity of DPD, DNDDP and DMDDP: The compound N, N'–diphenyl-4, (5-dichloroimidazole-2yl)-1, 4-dihydro-2, 6-dimetyl-3, 5-pyridine dicarboxamide (DPD) showed moderate antitubercular activity against *M. tuberculosis* with zone of inhibition 14mm. On further workout it was noticed that minimum 12mg of DPD needed to inhibit growth of *M. tuberculosis* (MIC value). The 18mg and 30mg weight of DPD was sufficient to inhibit the 50% (MIC_{50}) and 90% (MIC_{90}) growth of microorganism respectively. The other derived compound N, N'-di-3-Nitrophenyl-4-(4, 5-dichloroimidazole-2yl)-1, 4-dihydro-2, 6-dimethyl-3, 5-pyridine dicarboxamide (DNDDP) reflected good antitubercular activity against *M. tuberculosis* with zone of inhibition 20mm and MIC value 10 (Table 2). However, the compound N, N'-di-3-methoxyphenyl-4 (4, 5-dichloroimidazol-2yl)-1, 4-dihydro-2, 6-dimethyl-3, 5-pyridine dicarboxamide (DMDDP) showed remarkable antitubercular activity against *M. tuberculosis* with zone of inhibition 21mm. On further observation it was analyzed that minimum 5mg weight of DMDDP mandatory to inhibit growth of *M. tuberculosis* (MIC value). The MIC_{50} and MIC_{90} value of DMDDP was analyzed as 10mg and 25mg respectively during experiments.

Table 2: The *in vitro* antitubercular activities as ZI (mm) and MIC values of DPD, DNDDP and DMDDP

Synthesized compound	ZI (mm)	MIC	MIC_{50}	MIC_{90}
DPD	14	12	18	30
DNDDP	20	10	15	30
DMDDP	21	05	10	25

Antitubercular activity of BEMPMP, OMEMPMP and KPEMPMP: The compound 3–(4–bromo phenyl)–6–N–ethoxypthalimido–2–isonicotinoyl–4–methyl–3,3–dihydropyrazolo- [3,4–C]- pyrazole (BEMPMP) showed mild activity against *M. tuberculosis* with zone of inhibition 12mm. The MIC, MIC_{50} and MIC_{90} value of the compound was poor to inhibit growth of *M. tuberculosis* (Table 3). However, compound 3–(4–methoxyphenyl)–6–N-ethoxypthalmido–2–isonicotinyl–4–methyl–3,3–dihydro pyrazolo-[3,4–C]-pyrazole (OMEMPMP) demonstrated moderate antitubercular activity against *M. tuberculosis* with zone of inhibition 15mm and the minimum inhibitory concentration was 14mg. While 18mg and 35mg of OMEMPMP was competent to inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of causative agents. The other compound 3–(4–Ketophenyl)–6–N–ethoxyphthalimido–2–isonicotinyl methyl–3,3–dinydropyrazolo-[3,4-C]-pyrazole (KPEMPMP) also showed mild activity against *M. tuberculosis* with zone of inhibition 11mm with MIC value 20mg. The MIC_{50} and MIC_{90} value of same compound was 35 and 60mg respectively which suggested that the KPEMPMP compound as poorly active to inhibit the growth of *M. tuberculosis* (Table 3).

Table 3: The *in vitro* antitubercular activities as ZI (mm) and MIC values of BEMPMP, OMEMPMP and KPEMPMP

Synthesized compound	ZI (mm)	MIC	MIC_{50}	MIC_{90}
BEMPMP	12	17	30	50
OMEMPMP	15	14	18	35
KPEMPMP	11	20	35	60

Antitubercular activity of CEMTDP and NEPMCP: The compound 3-(4-(chlorophenyl-6-N-ethoxyphthalimido-4-methyl-2-thiocarbamoyl-3, 3-dihydropyrazolo- [3,4-C]- Pyrazole (CEMTDP) showed good activity against *M. tuberculosis* through zone of inhibition 20mm. The MIC, MIC_{50} and MIC_{90} value of compound was 10, 18 and 28mg respectively which suggested the compound as great antitubercular against *M. tuberculosis*. The other compound 3-(4-nitro phenyl)-6-N-ethoxy-phthalimido-4-methyl-2-thiocarbamoyl-3,3-dihydropyrazolo-[3,4-C]-pyrazole (NEPMCP) was also demonstrated good activity against *M. tuberculosis* by means of zone of inhibition 21mm. The compound was very active and remarkable for antitubercular activity at quite lower dosages like MIC, $MIC_{50}$and MIC_{90} at 5, 15 and 20mg respectively.

DISCUSSION

The commencement of a reproducible screening modus operandi for tuberculosis infection allowed to launch 1, 4-DHPs as a prototype of anti-tuberculosis agents (Gaveriya et al., 2002). It could be seen from earlier literature that an aryl-amide chain at 3 and 5-position of 1, 4-DHP ring influenced the anti-tubercular activity (Desai et al., 2001). Numerous aryl six member ring and some aliphatic substitution in 4-position of 3, 5-dicarboxamid-1, 4-DHP were synthesized. Some of them showed a moderate to good activity against *M. tuberculosis* (Shafii et al., 2008; Fassihi et al., 2009; Swarnalatha et al., 2011). In view of the imperative biological properties of 1,4-dihydropyridine it was therefore of our interest to examine further DHP and pyrazole derivatives which possess a linkage between isoxazolyl ring and 1, 4-DHPs ring with diaryl-carboxamide moiety in 3,5-position. The 1/3rd of the world's population infected tuberculosis (TB), with more than 1 million deaths annually (Kumar et al., 2018). The ample continuums of biological potential and successful exploitation of pyrazole-containing drugs in clinic have inspired more and more attention towards this kind of heterocycles.

Various pyrazole-containing derivatives have been synthesized for discovery of new anti-tubercular agents against *M. tuberculosis*, and some of them showed promising potency and may have novel mechanism of action (Fig. 1). The findings of the current investigation (Fig. 2) corroborated to the yesteryear achievements in pyrazole-containing derivatives as anti-TB agents and their structure-activity relationship studied by Xu et al. (2017).

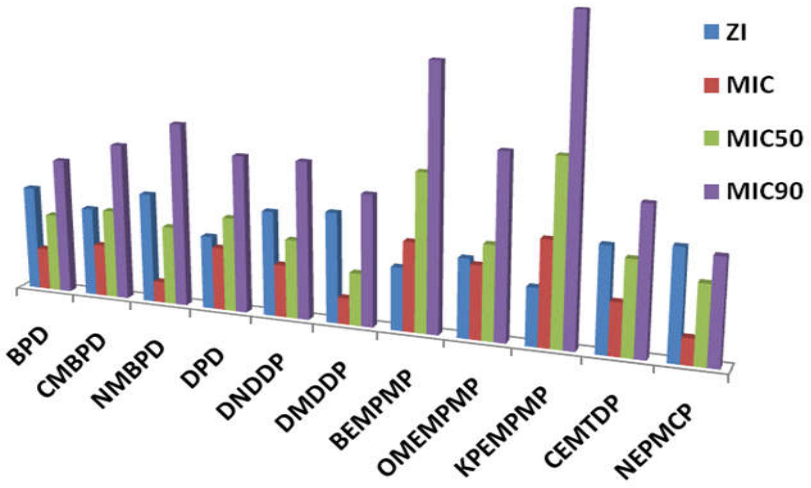

Figure 1: The comparative antitubercular activity and MIC values of DHP and pyrazole derivatives against *M. tuberculosis*

The consecutive experimentation and its numerical analysis were worked out critically which reflected that the area of ZI (zone of inhibition) inversely proportional to the minimum inhibition concentration values (MIC values) for all compounds (Fig. 2) similar to the findings of Xu et al. (2017). The 2,6-dimethyl-3, 5-bis-N-(heteroaryl)-carbamoyl-1, 4-dihydropyridines derivatives with 2-pyridyl at 4-position and 6-methylpyridin-2-yl at 3,5-position having more efficient antitubercular activity than control pyrazinamide and 2-imidazolyl at 4-position and 5-chloropyridin-2-yl at 3, 5-position and more or less equipotent to pyrazinamide (Sirisha et al., 2010). A progression of symmetrical, asymmetrical and unsubstituted 1, 4-dihydropyridines compounds showing differential activity against *M. tuberculosis* and the highest activity was observed for the 4-(4-(dimethylamino) phenyl)-1, 4-dihydro-N3, N5-bis(2-methoxyphenyl)-2, 6-dimethylpyridine-3, 5-dicarboxamide and 4-(2-hydroxyphenyl)-1, 4-dihydro-N3, N5-bis(3-nitro-phenyl)-2, 6-dimethylpyridine-3, 5-dicarboxamide nearly 93% and 92% of inhibition respectively because of the presence of 2-methoxy and 3-nitro groups on the carbonyl moiety and 4-N(CH3)2 and 2-OH on the aryl ring corroborated to the current investigation (Manvar et al., 2010). The similar studies have been performed by Desai et al. (2001) for series of 4-substituted Phenyl-2, 6-dimethyl-3, 5-Bis-N-(substituted Phenyl) carbamoyl-1, 4-dihydropyridines. The compounds substituted with NO_2 group or 2-Cl or OCH_3 at 3, 4-position of phenyl carbamoyl ring exhibit >90% of inhibition against H37 RV in comparison with rifampicin. A series 4-substituted-2, 6-dimethyl-3, 5-bis-N-(heteroaryl)-carbamoyl-1, 4-dihydropyridines have been screened for the antibacterial activity against gram +ve bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and gram –ve bacteria (*Escherichia coli* and *Proteus vulgaris*) (Tanabe et al., 1998; Kawase et al., 2002). Thus the variable antitubercular activities were documented during investigation for DHP and pyrazole derivatives subsequently substantiated and validated with earlier literatures.

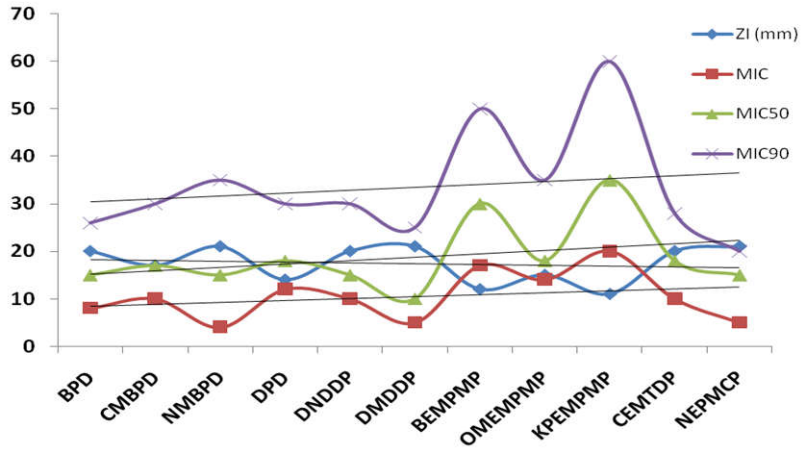

Figure 2: The comparative correlation of zone of inhibition and MIC values of DHP and pyrazole derivatives agaisnt *M. tuberculosis*

CONCLUSION

The tuberculosis (TB) is a communicable air borne bacterial disease. It is one of the fatal disease and the foremost grounds of death of 1/3rd of the world population infected. The count of patients of TB is escalating world-wide due to multi-drug resistant (MDR) strains of pathogen, *Mycobacterium*. The acid-fast bacillus *M. tuberculosis* possesses mycolyl-arabinogalactan units in cell wall which are bonded to peptidoglycan nucleus through covalent bonds. The global health evils in human population due to TB insisted to design and development of proficient new anti-tuberculosis drugs lacking cross resistance with known antimycobacterium. The *in vitro* activity of antitubercular agents measured as ZI (zone of inhibition), MIC (minimum inhibitory concentration), MIC_{50} and MIC_{90} value. The 1, 4-dihydropyridine (DHP) is the most practicable heterocyclic ring with various substitutions at several positions. The DHP and pyrazole are ever-growing due to their varied biological, pharmaceutical and therapeutic applications. The synthesized derivatives of DHP and pyrazole like BPD, DNDDP, CEMTDP, NEPMCP showed good antitubercular activity against *Mycobacterium tuberculosis*. While others like DPD, CMBPD, OMEMPMP demonstrated moderate, however, NMBPD, DMDDP showed remarkable antitubercular activity against *M. tuberculosis*. On the other hand BEMPMP, KPEMPMP having mild antitubercular activity against *M. tuberculosis* during investigation. Thus the synthesized substitutes of dihydropyridine-dicarboxamide and pyrazole derivatives are potent antitubercular agent against *Mycobacterium tuberculosis* with variable activity.

ACKNOWLEDGEMENT

Authors are gratifying to Head Department of Biotechnology, Maharishi Markandeshwar (Deemed to be University), Mullana, Ambala (Haryana), India for his ceaseless prop up to the joint research work.

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