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Novel pyrazoles: *M. Tuberculosis* growth inhibition and synergistic study

Sunil B. Jadhav[a,b], Samreen Fatema[a,b], Muftedar Ahmed Iftekhar Patel[a,b], Mazahar Farooqui[a,b]*

[a]*Post Graduate and Research Centre, Department of Chemistry, Maulana Azad College, Aurangabad-431001, Maharashtra, India.*
[b]*Rafiq Zakaria College for Women, Naukhanda, Aurangabad-431001, Maharashtra, India*
E-mail: mazahar_64@rediffmail.com; Mobile +919422214785
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Abstract: The prevalence of tuberculosis (TB) and multidrug-resistant tuberculosis (MDR-TB) has been increasing which ultimately leading to serious infections, high mortality, and a pose a global health risk. Here, we report the identification of a novel class of pyrazole derivatives as potent growth inhibitors for *M. tuberculosis* H37Rv. A series of twelve novel compounds were synthesized, characterised with spectral data and elemental analysis and subsequently, tested for their growth inhibition against *M. smegmatis* mc^2 155 initially. Anti-tubercular drug rifampicin was used as reference standard. In the next level, selected compounds **2** and **5** were tested for growth inhibition against *M. tuberculosis* H37Rv as well as for their synergistic activity along with first and second line current anti-tubercular drugs. Compound **2** failed to show effective synergism with INH and RIF wherein compound **5** showed synergism everywhere and was identified as best compound among the series of twelve compounds.

Keywords: Fused pyrimidones, pyrazole aldehydes, *Mycobacterium* Tuberculosis, Synergism

Introduction

Pulmonary Tuberculosis (TB), a disease of bacterial infections caused by Mycobacterium *tuberculosis* (MTB) and affects primarily to the respiratory system [1]. This disease was a much prevalent disease in the past than it is today, and was found to be responsible for the deaths of around 1 billion people during last a couple of century [2]. The strategy of vaccination and current antibiotic therapy to treat the disease is not effective in current scenario due to multi-drug resistance strains of *M. tuberculosis* [3]. There are approximately 1.6 million deaths annually reported due to *M. tuberculosis* infections and has infected one-third of the world population, translating TB as a second most mortal infectious disease after HIV/AIDS [4]. In the early 1950s,

there was a gradual decline in the number of cases of TB, but there has been resurgence since from1984 [5]. This upturn of TB throughout the recent years was mainly due to HIV-1 infection, immigration, increased trade, and globalization [6]. Moreover the occurrence of a drug resistant TB, especially multidrug-resistant TB (MDR-TB) is particularly increasing the risk. On the other hand MDR-TB has already caused several lethal out breaks [7] and poses a significant risk to the treatment and control of the disease in some parts of the globe, where the occurrence of MDR-TB can be as high as 14%. In addition, the TB situation may become even poorer with the spread of HIV-1 worldwide, a virus which weakens the patient's immune system, permits latent TB to reactivate, and makes the patient more liable to reinfection with either drug-susceptible or drug-resistant strains. The lethal combination of drug-resistant TB and HIV-1 infection is an upcoming issue that presents serious contests for effective TB control. Due to this situation, in 1993, the WHO declared TB a global emergency [8].

Tuberculosis has been treated with combination therapy for over fifty years. Currently, single drug therapy is ineffective strategy because of existing MDR strains of *M. tuberculosis* hence combination of drugs are normally prescribed to combat MDR-TB infections [9]. Rifampicin and Isoniazid are prescribed as first line of treatment, whereas Ofloxacin, Amikacin, Moxifloxacin are given as second line of treatment [10]. To cure a TB patient successfully a severe clinical supervision along with continuous drugs dosing for a long period of time is essential. Due to inherent resistant mechanism of causative agent, the extended combination therapy has posed another major threat where the organism has become resistant to most of the existing anti-TB drugs (XDR-TB) [11]. Clinical scenario has no more new anti-TB agent in last forty years except Bedaquiline, which was approved in year 2012 by USFDA [12]. Hence advancement in anti-TB drugs is a clinical need to reduce toxicity as well as treatment time.

Previous reports disclosed the most of compounds containing five membered pyrazole showed interesting anti-mycobacterial properties [13]. BM 212 [1,5-diaryl-2-methyl-3-(4-methylpiperazin-1-yl)methyl-pyrrole] is a lead compound among a series of pyrrole derivatives with good in vitro activity against mycobacteria [14]. The articles on BM212 disclosed the importance of the *p*-halophenyl substituent at both N1/C5 on pyrrole for the potency [15]. In the lack of information about both the drug target and the biologically activity of the earlier anti-tubercular agents, in the current context, ligand-based drug design method had utilised for more potent molecules [16] and hybrid compounds were designed comprising four chemical features represented by a hydrophobic region (HY), a hydrogen-binding domain along with one fused phenyl ring (HBD), two aromatic rings (RA1 and RA2) (**Figure 1**). A previously published antitubercular compounds suggested that a phenyl ring bearing a lipophilic group (such as a methyl, ethyl, etc.) at the para position of pyrrole could have favourable interactions with the HY feature of the pharmacophoric model, with a subsequent improvement in activity [17]. On the basis of such a literature and with continuation with our research activities [18-21], we synthesized new pyrazole hybrid derivatives keeping in mind the structure of BM212, ligand based designing approach and the presence a fluoro, methyl, methoxy, and bromo groups into one of the phenyl rings either at N1 or C3, to allow for a better superposition with the corresponding pharmacophoric properties HY.

Hence, our novel hybrid molecules comprises (i) a *p*-substituted phenyl ring at N1 found to be significant for antitubercular activity; (ii) an additional phenyl group (at position C3) bearing OMe, Br and Me substituents with different

substitution patterns, to test the hypothesis that a more lipophilic group (methyl) could improve activity, perhaps on the base of the fact that the mycobacterial cell wall structure is very waxy, hydrophobic, and characterized by a high lipid content, and thus, it requires a hydrophobic compound to be penetrated [22]; (iii) a *trans-stilbene* skeleton to support previously anti-tubular moiety, a naturally occurring resveratrol [23, 24], (iv) a hydrogen bonding domain unit [HBD] (**Figure 1**). Fused pyrimidone moiety which is reported for various biological activity including anti-tubercular [25]. The synthesized molecules were subsequently tested for their growth inhibition potential against *M.smegmatis* mc^2155. Later, the compounds which displayed comparable inhibition with standard Rifampicin were taken ahead for growth inhibition study against *M. tuberculosis* H37Rv and synergistic study with first and second line antibiotics.

Materials and methods

Biology

Assay for growth Inhibition against *M.smegmatis* mc^2155.

Evaluation of anti-tubercular activity against *M. smegmatis* mc^2 155 was carried out for synthesized compounds (**1-12**) by using Rifampicin as standard drug. Compounds were dissolved in the solvent at the concentration of 1.0 mg/ml and were further serially two fold diluted. From each dilution, a 100 µl was incorporated into 2ml of nutrient agar medium. Same procedure was followed for Rifampicin. After adding compounds, the medium was allowed to solidify in the tubes to make slant. Culture of *M. smegmatis*mc^2155 were grown nutrient agar was harvested inn 0.9% Saline to make single cell suspension of $2x10^5$cfu/mL. To each slant 10µL of suspension was spread on the surface of the medium and the tubes were kept at 37ºC for 24 hours for the appearance of colonies. Tubes containing no drug served as control. The minimum concentration of the drug Rifampicin and test compounds were recorded, which completely inhibited the growth of *M. smegmatis* and was termed as Minimum Inhibitory Concentration (MIC) of bacteria (**Table 2**).

In-vitro Cytotoxicity assay

From primary screening against *M. smegmatis,* the results indicated that compound **2** and **5** are having superior anti-tubercular activity and hence were employed for in-vitro Cytotoxicity assay along with compound **1** to assess their cytotoxic potential. The macrophage derived cell suspension was plated in 96- well tissue culture plates having approximate $2x10^3$ cell in each well in minimal essential medium (MEM) with antibiotics + 10% fetal bovine serum (FBS) and incubated overnight at 37 ºC and 5% CO2 for allowing adherence of cells. Compounds of different concentrations were added in MEM + 10% FBS. As a positive control, a known toxic compound was used. DMSO was used as negative control. After 24h incubation, 20 µL of MTT solution (tetrazolium compound) was added to each well and incubated for 2h at 37 ºC, 5% CO_2. Reading was taken at 490 nm using a plate reader. Absorbance shown by DMSO containing wells is taken as 100% survivors.

Anti-tubercular assay against *M. tuberculosis* H37Rv

This study was conducted by using standard procedure of Middlebrook (MB) 7H10 agar medium. A 100 µl of serial two fold dilutions of the stock (1.0 mg/mL in DMSO, Dimethyl Sulphoxide) of test compounds **2** and **5** along with standard anti-tubercular drug Rifampicin were incorporated in the medium (final volume, 2 ml/tube) supplemented with OADC (oleic acid, albumin fraction IV, dextrose and catalase). Compounds/drug containing tubes were kept

in slanting position till the medium solidified. Culture of *M. tuberculosis* H37Rv were grown on Lowenstein-Jensen (L-J) was harvested in N-saline containing 0.05% Tween-80. The culture was agitated with glass beads to make a single cell suspension. A working inoculum containing $2x10^7$cfu/Ml in 10µL was spread on surface of media of each tube and were incubated at 37ºC for 4 weeks for the appearance of colonies. Tubes containing no drug served as control. The minimum concentration of the drug Rifampicin and test compounds 2 and 5 that completely inhibited the growth of mycobacterium was recorded as Minimum Inhibitory Concentration (MIC) (**Table 3**).

Chemistry

All the reagents and solvents were purchased from commercial sources and were dried and purified when necessary by standard methods. The melting points of the synthesized compound were determined in open capillary tubes using VMP-D melting point apparatus (Veego Instrument Corporation, Mumbai, India) and are uncorrected. The ^{1}H NMR for the compound synthesized were recorded on Varian Mercury plus 400 using TMS as an internal standard and $CDCl_3$/DMSO-d_6 solvent and chemical values are given δ scales. The splitting patterns were assigned as follow: s: singlet, d: doublet, dd: doublet of doublet, t: triplet, m: multiplet. ^{13}C NMR spectra were recorded on Varian Mercury plus 100 spectrometer in DMSO-d_6 and were measured in δ scales. The spectra of mass were recorded on Waters mass spectrometer (Acquity, Waters Corporation). Microanalyses were carried out on elementar (Vario Micro Cube, Germany). The follow up of reactions was monitored by thin layer chromatography (TLC) on silica gel-precoatedaluminum sheets (Type 60, F254, Merck, Germany) and the spots were detected by exposer to UV lamp at λ 254 nm for 20-30 seconds.

General Procedure for synthesis of pyrazole aldehyde D

A mixture of different acetophenone (1.0 mole) and phenyl hydrazine (1.0 mole) were heated in ethanol (10 volumes) in presence of (1 ml) acetic acid at reflux temperature for 30 minutes. Resulting yellow solid product was separated by the filtration. This solid was washed by cold ethanol (minimum) and was suck dried under vacuum.

A mixture of N,N-dimethylformamide (2.5 mole) and $POCl_3$ (2.5 mole) was stirred together at 0 ^{0}C for 30 minutes. To this reaction mixture the above solid was added at 0 ^{0}C with constant stirring. The reaction mixture was then allowed to warm up to room temperature and stirred for 12-14 hrs. After completion of reaction, reaction mixture was poured on crushed ice where upon the solid was separated. It was filtered and washed by saturated aq. $NaHCO_3$ solution followed by water. Solid was crystalized from ethanol to get white crystalline product in an average 70% yield.

2-methyl-9-thia-1,4a-diaza-fluoren-4-one (G)

To a stirred solution of benzothiazol-2-amine (**E**) (20 g, 0.133 mmol) and acetic acid (120 ml) was added a solution of ethyl acetoacetate (**F**) (26 g, 0.199 mmol) at room temperature. It was refluxed for 12-14 hrs, after complete conversion on mass and TLC, the reaction mixture was cooled and acetic acid was removed under reduced pressure. The obtained residue was basified slowly with aqueous $NaHCO_3$ solution, and was peritonised in ethyl acetate (150 ml). Layers were separated; aqueous layer was washed by ethyl acetate (150 ml). Combined organic layer was washed by brine and dried on anhydrous Na_2SO_4. Solvent was removed under reduced pressure and afforded dark brown solid was further purified by column chromatography using methanol chloroform as eluents to give 8.2

g desired product (**G**) as faint yellow solid. ^{1}H NMR ($DCCl_3$), δ ppm: 2.42 (s, 3H, pyrimidone ring C<u>H</u>$_3$), 6.17 (s, 1H, pyrimidone ring <u>H</u>), 6.98 (d, J=5.12Hz, 2 X Ar<u>H</u>), 7.91-7.96 (m, 2H, Ar<u>H</u>).

General method for preparation of 1-12

To a stirred solution of 2-methyl-9-thia-1,4a-diaza-fluoren-4-one (**G**) (1 mol) and pyrazole aldehyde (**D**) (1.5 mole) in ethanol (10 volume), was added Sodium ethoxide (2 mole). Resulting reaction mixture was refluxed for 13 hrs. Reaction mixture (transparent solution) was cooled to room temperature. The product (precipitated solid) was obtained after filtration. Crude solid was purified in diethyl ether (**Table 1**).

2-[2-(1,3-Diphenyl-1H-pyrazol-4-yl)-vinyl]-9-thia-1,4a-diaza-fluoren-4-one 1: Yield 61%, M.P. 218-220 ^{0}C, ^{1}H NMR (DMSO-d_6), δ ppm: 6.40 (s, 1H, pyrimidone ring <u>H</u>), 7.15 (d, 1H, J=16Hz, olefinic <u>H</u> near to pyrazole ring), 7.19-7.47 (m, 4H, Ar<u>H</u>), 7.50-7.60 (m, 7H, Ar<u>H</u>)), 7.65-7.80 (m, 3H, Ar<u>HH</u> near to pyrimidone ring), 7.99 (d, 1H, J=4Hz, Ar<u>H</u>), 8.90 (s, 1H, pyrazole ring <u>H</u>); ES-MS, (m/z) 447.3 (M+1); Anal.Calcd.for $C_{27}H_{18}N_4OS$ (446.3): C, 72.63; H, 4.06; N, 12.55. Found C, 72.48; H, 4.59; N, 11.99.

2{2-[1-(4-Fluoro-phenyl)-3-phenyl-1H-pyrazol-4-yl]-vinyl}-9-thia-1,4a-diaza-fluoren-4-one2: Yield 61%, M.P. 222-224^{0}C, ^{1}H NMR (DMSO-d_6), δ ppm: 6.40 (s, 1H, pyrimidone ring <u>H</u>), 7.12 (d, 1H, J=15.8Hz, olefinic<u>H</u> near to pyrazole ring), 7.30-7.42 (m, 4H, Ar<u>H</u>), 7.55-7.60 (m, 6H, Ar<u>H</u>)), 7.65-7.82 (m, 3H, Ar<u>H</u> and olefinic <u>H</u> near to pyrimidine ring), 7.99 (d, 1H, J=4Hz, Ar<u>H</u>), 8.90 (s, 1H, pyrazole ring <u>H</u>); ES-MS, (m/z) 465.5 (M+1); Anal.Calcd.for $C_{27}H_{17}FN_4OS$ (464.4): C, 69.81; H, 3.69; N, 12.06. Found C, 70.01; H, 4.09; N, 11.95.

2{2-[1-(4-methoxy-phenyl)-3-phenyl-1H-pyrazol-4-yl]-vinyl}-9-thia-1,4a-diaza-fluoren-4-one 3: Yield 62%, M.P. 228-230 ^{0}C, ^{1}H NMR (DMSO-d_6), δ ppm: 3.81 (s, 3H, Ar-OC<u>H</u>$_3$), 6.42 (s, 1H, pyrimidone ring <u>H</u>), 7.10 (d, 1H, J=16Hz, olefinic <u>H</u> near to pyrazole ring), 7.28 (t, 2H, ArH), 7.38-7.48 (m, 2H, Ar<u>H</u>), 7.52-7.62 (m, 6H, Ar<u>H</u>), 7.68-7.75 (m, 3H, Ar<u>H</u> and olefinic H near to pyrimidine ring), 8.00 (d, 1H, J=3.8Hz, Ar<u>H</u>), 8.90 (s, 1H, pyrazole ring <u>H</u>); ES-MS, (m/z) 477.5 (M+1); Anal.Calcd. for $C_{28}H_{20}N_4O_2S$ (476.4): C, 70.57; H, 4.23; N, 11.76. Found C, 70.11; H, 4.19; N, 11.78.

2-[2-(3-Phenyl-1-p-tolyl-1H-pyrazol-4-yl)-vinyl]-9-thia-1,4a-diaza-fluoren-4-one4: Yield 68%, M.P. 224-226 ^{0}C, ^{1}H NMR (DMSO-d_6), δ ppm: 2.41 (s, 3H, Ar-C<u>H</u>$_3$), 6.62 (s, 1H, pyrimidone ring <u>H</u>), 7.08 (d, 1H, J=16Hz, olefinic <u>H</u> near to pyrazole ring), 7.30-7.43 (m, 4H, Ar<u>H</u>), 7.50-7.58 (m, 6H, Ar<u>H</u>), 7.68-7.73 (m, 3H, Ar<u>H</u> and olefinic <u>H</u> near to pyrimidine ring), 8.01 (d, 1H, J=4Hz, Ar<u>H</u>), 8.94 (s, 1H, pyrazole ring <u>H</u>); ES-MS, (m/z) 461.4 (M+1); Anal.Calcd.for $C_{28}H_{20}N_4OS$ (460.4): C, 73.20; H, 4.38; N, 12.16. Found C, 73.18; H, 4.18; N, 12.40.

2{2-[1-(4-bromo-phenyl)-3-phenyl-1H-pyrazol-4-yl]-vinyl}-9-thia-1,4a-diaza-fluoren-4-one 5: Yield-58%, M.P. 227-229 ^{0}C, ^{1}H NMR (DMSO-d_6), δ ppm: 6.58 (s, 1H, pyrimidone ring <u>H</u>), 7.12 (d, 1H, J=16Hz, olefinic <u>H</u> near to pyrazole ring), 7.24 (t, 2H, Ar<u>H</u>), 7.33-7.37 (m, 2H, Ar<u>H</u>), 7.46-7.51 (m, 6H, Ar<u>H</u>), 7.70-7.72 (m, 4H, Ar<u>H</u>), 7.76 (d, J=16Hz, olefinic <u>H</u> near to pyrimidine ring), 8.00 (d, 1H, J=4Hz, Ar<u>H</u>), 8.99 (s, 1H, pyrazole ring <u>H</u>); ES-MS, (m/z) 525.4 (M^+), 527.4 (M+2); Anal.Calcd.for $C_{27}H_{19}BrN_4OS$ (525.4): C, 61.72; H, 3.26; N, 10.66. Found C, 62.08; H, 3.47; N, 10.23.

2-[2-(1-phenyl-3-o-tolyl-1H-pyrazol-4-yl)-vinyl]-9-thia-1,4a-diaza-fluoren-4-one 6: Yield-71%, M.P. 241-243^{0}C, ^{1}H NMR (DMSO-d_6), δ ppm: 2.43 (s, 3H, Ar-C<u>H</u>$_3$), 6.64 (s, 1H, pyrimidone ring <u>H</u>), 7.00 (d, 1H, J=16Hz, olefinic <u>H</u> near to pyrazole ring), 7.20 (t, 3H, Ar<u>H</u>), 7.48-7.52 (m, 7H, Ar<u>H</u>), 7.81 (d, J=16Hz, olefinic <u>H</u> near to pyrimidine ring), 7.81-7.84 (m, 3H, Ar<u>H</u>), 8.00 (d, 1H, J=4Hz, Ar<u>H</u>), 8.99 (s, 1H, pyrazole ring <u>H</u>); ES-MS, (m/z) 461.3 (M+1); Anal.Calcd.for $C_{28}H_{20}N_4OS$ (460.4): C, 73.02; H, 4.38; N, 12.16. Found C, 73.41; H, 4.47; N, 12.98.

2-[2-(1-phenyl-3-m-tolyl-1H-pyrazol-4-yl)-vinyl]-9-thia-1,4a-diaza-fluoren-4-one 7: Yield-71%, M.P. 241-243^{0}C, ^{1}H NMR (DMSO-d_6), δ ppm:2.41 (s, 3H, Ar-C<u>H</u>$_3$), 6.60 (s, 1H, pyrimidone ring <u>H</u>), 7.04 (d, 1H, J=16Hz, olefinic <u>H</u> near to pyrazole ring), 7.18 (t, 2H, Ar<u>H</u>), 7.46-7.50 (m, 7H, Ar<u>H</u>), 7.74 (d, J=16Hz, olefinic <u>H</u> near to pyrimidine ring), 7.78-7.80 (m, 3H, Ar<u>H</u>), 8.01 (d, 1H, J=4Hz, Ar<u>H</u>), 8.95 (s, 1H, pyrazole ring <u>H</u>); ES-MS, (m/z) 461.3 (M+1); Anal.Calcd.for $C_{28}H_{20}N_4OS$ (460.4): C, 73.02; H, 4.38; N, 12.16. Found C, 73.44; H, 4.41; N, 12.67.

2-[2-(1-phenyl-3-p-tolyl-1H-pyrazol-4-yl)-vinyl]-9-thia-1,4a-diaza-fluoren-4-one 8: Yield-71%, M.P. 248-250^{0}C, ^{1}H NMR (DMSO-d_6), δ ppm:2.44 (s, 3H, Ar-C<u>H</u>$_3$), 6.62 (s, 1H, pyrimidone ring <u>H</u>), 7.10 (d, 1H, J=16Hz, olefinic <u>H</u> near to pyrazole ring), 7.23 (t, 2H, Ar<u>H</u>), 7.48-7.51 (m, 7H, Ar<u>H</u>), 7.53 (t, 1H, Ar<u>H</u>), 7.79 (d, J=16Hz, olefinic <u>H</u> near to pyrimidine ring), 7.78-7.80 (m, 2H, Ar<u>H</u>), 8.01 (d, 1H, J=4Hz, Ar<u>H</u>), 8.95 (s, 1H, pyrazole ring <u>H</u>); ES-MS, (m/z) 461.3 (M+1); Anal.Calcd. for $C_{28}H_{20}N_4OS$ (460.4): C, 73.02; H, 4.38; N, 12.16. Found C, 73.44; H, 4.41; N, 12.67.

2{2-[3-(2-methoxy-phenyl)-1-phenyl-1H-pyrazol-4-yl]-vinyl}-9-thia-1,4a-diaza-fluoren-4-one 9: Yield-68%, M.P. 238-240 ^{0}C, ^{1}H NMR (DMSO-d_6), δ ppm:3.80 (s, 3H, Ar-OC<u>H</u>$_3$), 6.80 (s, 1H, pyrimidone ring <u>H</u>), 7.20 (d, 1H, J=16Hz, olefinic <u>H</u> near to pyrazole ring), 7.23 (t, 2H, Ar<u>H</u>), 7.44-7.49 (m, 7H, Ar<u>H</u>),7.80 (d, J=16Hz, olefinic <u>H</u> near to pyrimidine ring), 7.78-7.80 (m, 3H, Ar<u>H</u>), 8.10 (d, 1H, J=4Hz, Ar<u>H</u>), 8.97 (s, 1H, pyrazole ring <u>H</u>); ES-MS, (m/z) 477.3 (M+1); Anal.Calcd.for $C_{28}H_{20}N_4O_2S$ (476.4): C, 70.57; H, 4.23; N, 11.77. Found C, 71.02; H, 4.11; N, 11.81.

2{2-[3-(3-methoxy-phenyl)-1-phenyl-1H-pyrazol-4-yl]-vinyl}-9-thia-1,4a-diaza-fluoren-4-one 10: Yield-68%, M.P. 235-237^{0}C, ^{1}H NMR (DMSO-d_6), δ ppm:3.86 (s, 3H, Ar-OC<u>H</u>$_3$), 6.68 (s, 1H, pyrimidone ring <u>H</u>), 7.27 (d, 1H, J=16Hz, olefinic <u>H</u> near to pyrazole ring), 7.30 (t, 2H, Ar<u>H</u>), 7.42-7.50 (m, 8H, Ar<u>H</u>), 7.83 (d, J=16Hz, olefinic <u>H</u> near to pyrimidine ring), 7.80-7.82 (m, 2H, Ar<u>H</u>), 8.11 (d, 1H, J=4Hz, Ar<u>H</u>), 8.99 (s, 1H, pyrazole ring <u>H</u>); ES-MS, (m/z) 477.3 (M+1); Anal.Calcd. for $C_{28}H_{20}N_4O_2S$ (476.4): C, 70.57; H, 4.23; N, 11.77. Found C, 71.12; H, 4.24; N, 11.67.

2{2-[3-(4-methoxy-phenyl)-1-phenyl-1H-pyrazol-4-yl]-vinyl}-9-thia-1,4a-diaza-fluoren-4-one 11: Yield-68%, M.P. 237-239 ^{0}C, ^{1}H NMR (DMSO-d_6), δ ppm:3.87 (s, 3H, Ar-OC<u>H</u>$_3$), 6.76 (s, 1H, pyrimidone ring <u>H</u>), 7.23 (d, 1H, J=15.8Hz, olefinic <u>H</u> near to pyrazole ring), 7.33 (t, 2H, Ar<u>H</u>), 7.42-7.48 (m, 8H, Ar<u>H</u>), 7.82 (d, J=15.6Hz, olefinic <u>H</u> near to pyrimidine ring), 7.80-7.82 (m, 2H, Ar<u>H</u>), 8.11 (d, 1H, J=4Hz, Ar<u>H</u>), 9.01 (s, 1H, pyrazole ring <u>H</u>); ES-MS, (m/z) 477.3 (M+1); Anal.Calcd. for $C_{28}H_{20}N_4O_2S$ (476.4): C, 70.57; H, 4.23; N, 11.77. Found C, 71.22; H, 4.35; N, 11.71.

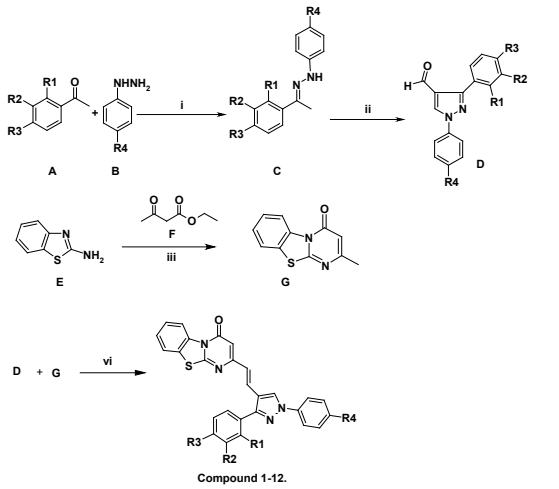
2{2-[3-(4-bromo-phenyl)-1-phenyl-1H-pyrazol-4-yl]-vinyl}-9-thia-1,4a-diaza-fluoren-4-one 12: Yield-68%, M.P. 237-239 ^{0}C, ^{1}H NMR (DMSO-d_6), δ ppm: 6.71 (s, 1H, pyrimidone ring <u>H</u>), 7.08 (d, 1H, J=16Hz, olefinic <u>H</u> near to pyrazole ring), 7.23 (t, 2H, Ar<u>H</u>), 7.44-

7.47 (m, 8H, ArH), 7.67 (d, J=15.9Hz, olefinic H near to pyrimidine ring), 7.81-7.83 (m, 2H, ArH), 8.09 (d, 1H, J=4Hz, ArH), 8.79 (s, 1H, pyrazole ring H); ES-MS, (m/z) 525 (M^+); 527 (M+2)Anal.Calcd.for $C_{27}H_{17}BrN_4OS$ (525.4): C, 61.72; H, 3.26; N, 10.26. Found C, 61.52; H, 3.35; N, 10.78.

Result and Discussion

Chemistry

The synthetic strategies utilized are being mention in the synthetic scheme 1. Synthetic process we initiated with preparations of pyrazole aldehydes **D** with help of literature [26] commercially available different acetophenones **A** when treated with commercially available various phenylhydrazines **B** in acidic medium under refluxing in ethanol gave imine as an intermediate **C**. This intermediate upon reaction with $POCl_3$ in DMF underwent cyclization to give different pyrazole aldehydes **D** in good yield as key precursors. Another important precursor 2-methyl-9-thia-1,4a-diaza-fluoren-4-one (**G**) was synthesized with help of literature [27]. Here, benzothiazole-2-amine was treated with ethyl acetoacetate in acetic acid at reflux condition for overnight. Crude product was purified by column chromatography to afford pure G in moderate yield. Conversion was monitored by TLC and mass analysis.



Synthetic Scheme 1. Reagents and conditions: (i) AcOH/EtOH, reflux, 30 minutes; (ii) DMF/$POCl_3$, 0 ^{0}C-30 ^{0}C, 12-14 h; (iii) AcOH, 12-14 Hrs Reflux; (iv) NaOEt, Ethanol, RT, 12-14Hrs.

Finally, intermediates **D** and **G** were taken in ethanol, base sodium ethoxide was added and resultant suspension was stirred at reflux temperature for overnight. Transparent solution was cooled to room temperature. Targeted compounds (1-12) were filtered after precipitation.

Pharmacology

Growth inhibition study against*M.smegmatis mc² 155*.

As per strategy, initially, H was kept across R_1-R_4, gave rise to compound **1**. In the assay, compound **1** demonstrated reasonable *M.smegmatis* mc^2155 inhibition (42μg/mL), although this initial result was poor when compared with standard rifampicin (32μg/mL), but it gave motivation to our synthesis. Further, a small structural change was brought at R_4positions. In the compound **2**, small fluoro group was employed at R_4, in the growth inhibition study;itdisplayed excellent improvement(30μg/ml) than compound **1**. This growth inhibitory result was almost equal to standard rifampicin. Taking a decent boost from the result of compound **2** methoxy, methyl and bromo group was introduced at R_4. Unfortunately, the resulting compounds **3** and **4** showed massive drop in the growth inhibition (600μg/ml and 520 μg/ml), whereas compound **5**(29μg/ml) retained as like compound **2**. Further, to explore SAR, we moved towards R_1-R_3substitutions. In this context, methoxy, methyl and bromo groups were employed at R_1-R_3. Resulting compounds **6-12** did not exhibited any improvement in the growth inhibition, indeed the growth inhibitory results were poor than compounds **1-5**. Hence compounds **2** and **5** were found the most active compound from

the series.

Cell cytotoxicity

In cell cytotoxicity study, compound **1**proved toxic (IC_{50}=170µg/ml) than standard rifampicin and compounds **2** (IC_{50}=371µg/mL) and **5** (IC_{50}=378µg/mL) were found to be safer towards human macrophages compare to rifampicin (400µg/mL). Since compound **2** and **5**presented good *M.smegmatis* $mc^2$155 inhibition and safe cytotoxicity profile, hence were taken ahead for growth inhibition study against *M. tuberculosis* H37Rv.

Evaluation of antitubercular activity of compound 2 and 5 in M. *tuberculosis* H37Rv strain.

Based on MIC and cell cytotoxicity results compounds **2** and **5** were selected to establishing their *M. tuberculosis* growth inhibition inhibition study. Here both the compounds were found equipotent (MIC= 2.9µg/mL and MIC= 2.6µg/mL) when compared with standard rifampicin (2.8µg/mL). Hence both these molecules were taken ahead for further study.

Evaluation of Synergism

After screening growth inhibition against *M. tuberculosis* H37Rv, in the next level, compounds **2** and **5** were taken ahead for synergistic study with first line rifampicin (RIF), isoniazide (INH) and second line ofloxacin (OFX),amikacin (AMK) antitubercular drugs. The MICs of RIF, INH, OFX and AMK were calculated in the absence and in the presence of various concentrations of compound **2** and **5**against *M. tuberculosis* H37Rv (**Table 4**).

Compound **2** and **5** were tested at their ½ MIC followed by two-dimensional broth microdilution checkerboard assaywhich is used tocalculate magnitude of synergism.Compound**2** doesn't showed effective synergism with INH and RIF even at 9.60 µg/mL also. Whereas it demonstrated good synergism with OFX, AMK. On the other hand, compound **5** (likewise to rifampicin) showed synergism everywhere. Hence, compound **5** was found to be a good potent compound among the series.

Conclusion

Current article deals with synthesis of novel pyrazole derivatives andtheir anti-tubercular potential against *M. tuberculosis* H37Rv. In the preliminary screening, these novel compounds were tested for their growth inhibitory potential against *M. smegmatis* mc^2 155. Compounds 1, **2** and **5** showed good growth inhibitory results (MIC = 42µg/ml, 30µg/ml and 29µg/ml respectively). Compound **1** showed cell cytotoxicity (IC_{50}=170µg/ml) and **2** and **5** were emerged as safe compound by showing a high IC_{50} values. In the next level, **2** and **5** were found equipotent in anti-tubercular effect against *M. tuberculosis* H37Rv strain as compared to standard rifampicin. Further, compound **2** and **5** were taken ahead for synergistic studies with first and second line anti-tubercular drugs. In conclusion, compound **2** failed to demonstrate a synergism profile with INH and RIF, while compound **5**demonstrated good synergism everywhere and was identified as best compound among the series of twelve compounds.

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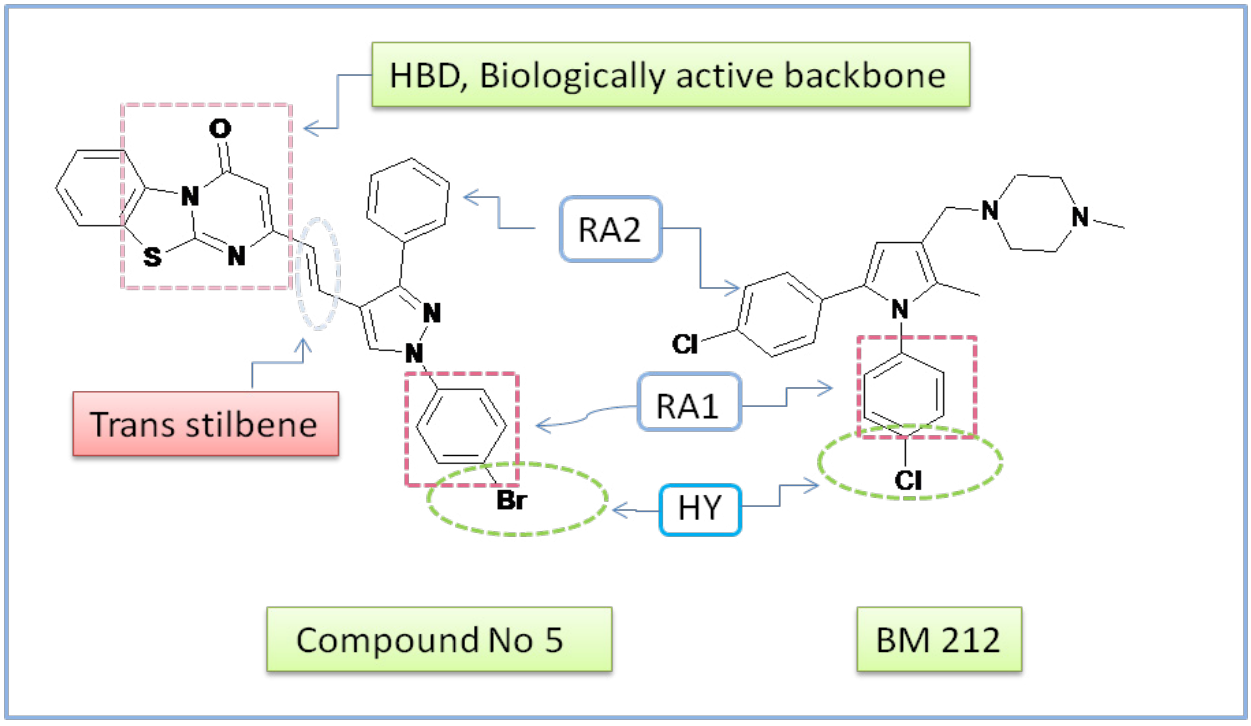

Figure 1: Phramacophoric model for molecules 1-12.

Table 1. Substitution strategy for compounds1-**12.**

ID	R1	R2	R3	R4
1	H	H	H	H
2	H	H	H	F
3	H	H	H	OMe
4	H	H	H	Me
5	H	H	H	Br
6	Me	H	H	H
7	H	Me	H	H
8	H	H	Me	H
9	OMe	H	H	H
10	H	OMe	H	H
11	Me	H	OMe	H
12	H	H	H	Br

Table 2. Anti-tubercular activity of 1-12 against *M. smegmatis* mc^2 155 and their toxicity index towards human monocyte derived macrophages.

ID	*M. smegmatis* mc^2 155		IC_{50}[c] µg/mL
	MIC (µg/mL)[a]	REF ± SD[b]	
1	42	1.3±0.09*	170
2	30	0.13±0.01	371
3	600	0.68±0.03	ND
4	520	0.78±0.01	ND
5	29	0.42±0.01	378
6	410	0.86±0.09	ND
7	525	0.79±0.081	ND
8	310	0.85±0.05	ND
9	239	2.19±0.02	ND
10	271	8.3±0.08*	ND
11	281	1.9±0.03*	ND
12	321	0.61±0.05	ND
Rifampicin	32	1.67±0.09*	400

Table 3. Screening of anti-tubercular activity of selected compounds against *M. tuberculosis* H37Rv.

ID	*M. tuberculosis* H37Rv	
	MIC (µg/mL)	REF ± SD
2	2.9	2.13 ± 0.10*
5	2.6	2.45± 0.03*
Rifampicin	2.8	1.35 ± 0.04*

MIC was determined by microdilution; REF (Relative final fluorescence) based on accumulation of EtBr at 0.5 µg/mL; the results are presented as the average of three independent assays plus standard deviation (± SD). The results were considered significant when *P<0.05 and highly significant when **P<0.01 and ***P<0.001; [c]Due to the reduced solubility of the compounds it was not possible to test at higher concentrations.

Table 4. Synergistic effect and modulation factor (MF) for compound 2 and 5 with first- and second- line drugs against *M. tuberculosis* H37Rv.

ID	Test Conc.	MIC (µg/mL) against *M. tuberculosis* H37Rv			
		INH	RIF	OFX	AMK
Standalone MIC		1.2	1.7	2.4	2.9
2	9.60	0.41	0.11	0.069 (35)	0.045 (65)
	4.80	0.51	0.19	0.83	0.13
	2.40	1.2	0.38	1.3	1.11
	1.20	1.2	0.81	2.4	2.9
	0.60	1.2	1.7	2.4	2.9
	0.30	1.2	1.7	2.4	2.9
5	9.60	0.022 (54)	0.038 (45)	0.05 (48)	0.09 (32)
	4.80	0.089 (14)	0.10 (12)	0.25	2.1
	2.40	1.2	1.7	2.4	2.9
	1.20	1.2	1.7	2.4	2.9
	0.60	1.2	1.7	2.4	2.9
	0.30	1.2	1.7	2.4	2.9

Test Conc:- Concentration at which the compound was tested (in µg/mL); INH:-Isoniazid; RIF:-Rifampin; OFX:-Ofloxacin; AMK:-Amikacin; Modulation factor (MF) = Ratio of standalone MIC of antibiotic to the MIC after combination.