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Research Article

Synthesis and Antitubercular Activity of 2-(substituted phenyl/benzyl-amino)-6-(4-chlorophenyl)-5-(methoxycarbonyl)-4-methyl-3,6-dihydropyrimidin-1-ium Chlorides

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A series of 2-(substituted phenyl/benzyl-amino)-6-(4-chlorophenyl)-5-(methoxycarbonyl)-4-methyl-3,6-dihydropyrimidin-1-ium chlorides 7–13 and 15 was synthesized in their hydrochloride salt form. The title compounds were characterized by FT-IR, NMR (^{1}H and ^{13}C) and elemental analysis. They were evaluated for their *in vitro* antitubercular activity against *Mycobacterium tuberculosis* H37Rv, multidrug resistance tuberculosis and extensively drug resistance tuberculosis by agar diffusion method and tested for the cytotoxic action on peripheral blood mononuclear cells by MTT assay. Among all the tested compounds in the series, compounds 7 and 11 emerged as promising antitubercular agents at 16 μg/mL against multidrug resistance tuberculosis and over 64 μg/mL against extensively drug resistance tuberculosis. The conformational features and supramolecular assembly of the promising compounds 7 and 11 were determined by single crystal X-ray study.

Key words: anti-TB activity, cytotoxicity, dihydropyrimidine, single crystal X-ray crystallography
Abbreviations: Et-OH, ethanol; MDR-TB, multidrug resistance tuberculosis; MIC, minimum inhibitory concentration; XDR-TB, extensively drug resistance tuberculosis.

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Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*. It affects lungs and also other parts of the body and has lead to death of 1.5 million HIV-positive people from tuberculosis in 2010[a]. HIV-infected individuals are up to 37 times more susceptible to develop TB[b]. The emergence of multidrug-resistant tuberculosis (MDR-TB), extensively drug-resistant tuberculosis (XDR-TB) (1), totally drug-resistant tuberculosis (2) and co-infections with acquired immuno deficiency virus (AIDS) (3) has been responsible for this serious situation. Furthermore, current drugs used for the treatment of TB are cytotoxic and showing decreasing efficacy due to the development of resistant organisms (4). Therefore, new and improved anti-TB drugs with new targets and mechanism of action are urgently needed to gain sustained successful treatment of MDR-TB, XDR-TB and totally drug-resistant tuberculosis-infected patients (5).

In 1893, the synthesis of multifunctionalized 3,4-dihydropyrimidine-2(1*H*)-ones was reported by Pietro Biginelli (6). Among pharmacologically important heterocyclic compounds, dihydropyrimidine analogues have been reported to have several pharmacological activities (7) such as, antiviral (8), anticancer (9), antihypertensive (10), antitubercular (11), antimicrobial (12), anti-inflammatory (13) and calcium channel blocking action (14). Dihydropyrimidines are potential inhibitors of dihydrofolate reductase, which interrupts the supply of thymidine in the folate cycle. This inhibits DNA biosynthesis and influences cell proliferation (15) which may be a mechanism to inhibit MDR or XDR-TB.

Keeping all these observations in mind and as a part of our research work on the development of heterocyclic compounds for pharmacological activity (16–18), we synthesized and characterized 3,6-dihydropyrimidine analogues **7–13** and **15** in their hydrochloride salt form (Scheme 1) following Lipinski's rule of five (19). These analogues were then investigated for their *in vitro* activity against *M. tuberculosis* reference strain H37RV, MDR-TB strain and a well-characterized XDR-TB strain. To understand the safety of the molecules, the title compounds were tested for their cytotoxic potential against peripheral blood mononuclear cells (PBMC) by evaluating their viabil-

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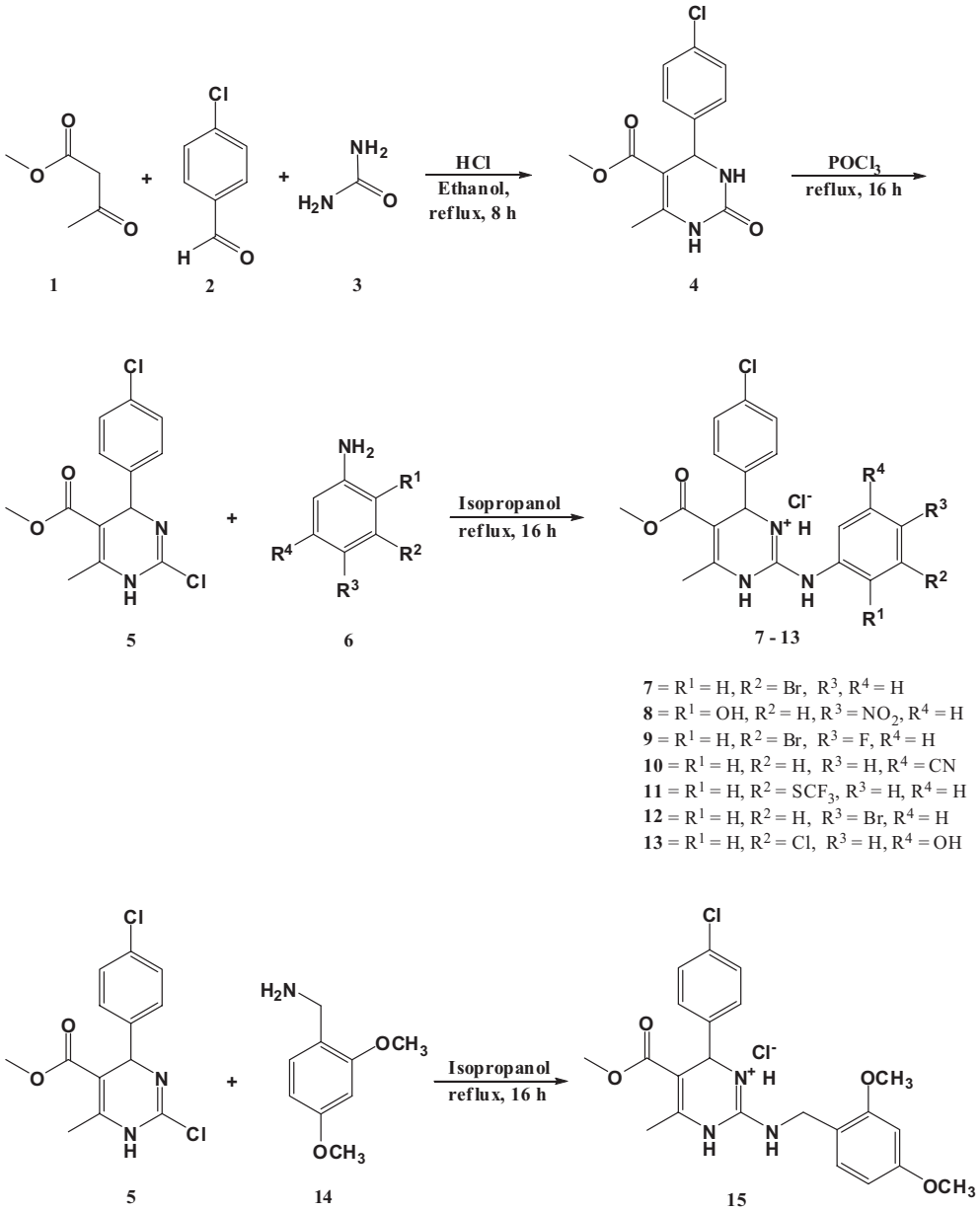

Scheme 1: Synthesis of 3,6-dihydropyrimidine salts (7–13 and 15).

ity after exposure to the compounds using the MTT assay (20). In addition, we report herein the single crystal X-ray studies of two of the active molecules **7** and **11** from the series to understand their conformational features and supramolecular assembly.

Methods and Materials

All the chemicals were obtained from Sigma Aldrich, South Africa and Merck, South Africa chemical company. Reactions were monitored by thin layer chromatography (TLC). Thin layer chromatography was performed on Merck 60 F-254 silica gel plates with ethyl acetate and *n*-hexane (6:4) as solvent system and visualization with UV-light. Melting points were determined on a Büchi Melting Point B-545 apparatus. The IR spectra were recorded on a Nicolet 6700 FT-IR spectrometry. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AVANCE III 400 MHz instruments in DMSO as a solvent. Chemical shifts (δ) were indicated in parts per million downfield from tetramethylsilane and the coupling constants (J) are recorded in Hertz. Splitting pattern is abbreviated as follows; s, singlet; d, doublet; m, multiplet. Mass spectra were recorded using LC-MS-Agilent 1100 series with MSD (Ion trap) using 0.1% aqueous trifluoroacetic acid in acetonitrile system on C18-BDS column. Elemental analysis was performed on Thermo Finningan FLASH EA 1112 CHN analyzer, Italy.

Synthesis of methyl 4-(4-chlorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4)

A solution of methyl acetoacetate (0.12 mol), 4-chlorobenzaldehyde (0.1 mol) and urea (0.1 mol) was refluxed in the presence of concentrated hydrochloric acid (0.05 mol) for 8 h in 5 mL of ethanol (Et-OH; Scheme 1). Completion of reaction was monitored on TLC. The reaction mixture was then cooled to room temperature, and the pure precipitate was collected by filtration. The solid obtained was washed with cold Et-OH, dried and recrystallized using Et-OH solvent. Appearance: yellow solid; yield 66%. mp 205–206 °C. IR (KBr) ν/cm 3240, 3110, 1724, 1704, 1649, 1490, 1461, 781; ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ 2.30 (s, 3H), 3.92 (s, 3H), 5.43 (s, 1H), 7.13 (d, 2H, J = 9.05 Hz), 7.51 (s, 1H), 7.86 (d, 2H, J = 9.05 Hz), 9.01 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d$_6$) δ 18.66, 52.05, 54.35, 109.58, 113.20, 128.23, 136.26, 148.25, 153.40, 159.18, 167.76. MS: m/z 281 [M + H]$^+$.

Synthesis of methyl 2-chloro-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (5)

A solution of compound **4** (1 mol) in $POCl_3$ (5 mol) was refluxed for 16 h (Scheme 1). The reaction was monitored by TLC. Unreacted $POCl_3$ was evaporated completely and the remaining residue was taken in ethyl acetate and washed with 10% sodium bicarbonate solution followed by water and finally brine solution. The ethyl acetate layer was dried over sodium sulphate and evaporated to obtain a solid which was recrystallized using Et-OH solvent. Appearance: brown solid; yield 72%. mp 218–219 °C. IR (KBr) ν/cm 3244, 3108, 1708, 1641, 1491, 1466, 786. ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ 2.32 (s, 3H), 3.91 (s, 3H), 5.44 (s, 1H), 7.13–7.86 (m, 4H), 9.02 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d$_6$) δ 18.72, 53.67, 54.35, 105.41, 113.20, 128.54, 135.29, 141.20, 145,67, 158.39, 167.76. MS m/z 299 [M + H]$^+$.

General procedure for the synthesis of 7–13 and 15

A solution of compound **5** (1 mmol) and aromatic substituted amine (1 mmol) in isopropanol (10 mL) was refluxed for 16 h (Scheme 1). The reaction completion was monitored by TLC. The reaction medium was cooled to room temperature, and the product obtained was filtered, washed with cold isopropanol and dried to get the pure product. The product obtained was purified by column chromatography using ethyl acetate and *n*-hexane (6:4) as eluent (60–120 silica gel). Compounds **7–13** and **15** were achieved as hydrochloride salts.

2-(3-Bromophenylamino)-6-(4-chlorophenyl)-5-(methoxycarbonyl)-4-methyl-3,6-dihydropyrimidin-1-ium chloride (7)

Appearance: white solid; yield 65%. mp 217–218 °C. IR (KBr) ν/cm 3199, 3068, 1707, 1671, 1582, 1475, 787, 676. ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ 2.41 (s, 3H), 3.62 (s, 3H), 5.43 (s, 1H), 7.19–7.51 (m, 8H), 10.20 (s, 1H), 11.14 (s, 2H). ^{13}C-NMR (400 MHz, DMSO-d$_6$) δ 17.53, 51.68, 103.31, 122.17, 123.19, 126.87, 128.46, 128.93, 129.70, 131.67, 132.98, 136.24, 140.00, 144.90, 148.71, 164.61. Anal. calcd for $C_{19}H_{18}BrCl_2N_3O_2$ (471.2): C, 48.43; H, 3.85; N, 8.92%; found C, 48.39; H, 3.84; N, 8.93%.

6-(4-Chlorophenyl)-2-(2-hydroxy-4-nitrophenylamino)-5-(methoxycarbonyl)-4-methyl-3,6-dihydropyrimidin-1-ium chloride (8)

Appearance: yellow solid; yield 67%. mp 172–173 °C. IR (KBr) ν/cm 3356, 3193, 3067, 1707, 1674, 1588, 1492, 769. ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ 2.40 (s, 3H), 3.61 (s, 3H), 5.39 (s, 1H), 7.17 (d, J = 9.00 Hz, 1H), 7.34 (d, J = 8.48 Hz, 2H), 7.45 (d, J = 8.44 Hz, 2H), 8.10–8.15 (m, 2H), 9.75 (s, 1H), 10.21 (s, 1H), 11.01 (s, 1H), 11.93 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d$_6$) δ 19.87, 50.77, 52.68, 99.77, 116.06, 116.92, 120.21, 128.20, 128.42, 129.57, 131.99, 138.23, 143.60, 148.79, 151.05, 157.11, 163.69, 165.94. Anal. calcd for $C_{19}H_{18}Cl_2N_4O_5$ (453.3): C, 50.35; H, 4.00; N, 12.36%; found: C, 50.36; H, 4.04; N, 12.35%.

2-(3-Bromo-4-fluorophenylamino)-6-(4-chlorophenyl)-5-(methoxycarbonyl)-4-methyl-3,6-dihydropyrimidin-1-ium chloride (9)

Appearance: white solid; yield 71%. mp 236–236 °C. IR (KBr) ν/cm 3191, 3032, 1718, 1676, 1583, 1487, 769, 699. ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ 2.41 (s, 3H), 3.59 (s, 3H), 5.35 (s, 1H), 7.32–7.77 (m, 7H), 9.63 (s, 1H), 10.68 (s, 1H), 11.25 (s,1H). ^{13}C-NMR (400 MHz, DMSO-d$_6$) δ 17.57, 51.56, 51.97, 102.85, 116.22, 116.44, 120.50, 120.76, 122.59, 122.69, 128.70, 128.79, 129.38, 131.35, 131.44, 132.88, 140.16, 144.58, 149.52, 160.10, 162.58, 164.60. Anal. calcd for $C_{19}H_{17}BrCl_2FN_3O_2$ (489.2): C, 46.65; H, 3.50; N, 8.59%; found C, 46.65; H, 3.51; N, 8.58%.

6-(4-Chlorophenyl)-2-(4-cyanophenylamino)-5-(methoxycarbonyl)-4-methyl-3,6-dihydropyrimidin-1-ium chloride (10)

Appearance: yellow solid; yield 63%. mp 116–117 °C. IR (KBr) ν/cm 3194, 3063, 2226, 1673, 1562, 1489, 770. ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ 2.38 (s, 3H), 3.60 (s, 3H), 5.41 (s, 1H), 7.35 (t, J = 8.62 Hz, 4H), 7.44 (d, J = 8.48 Hz, 2H), 7.80 (d, J = 8.60 Hz, 2H), 9.88 (s, 1H), 10.11 (s, 1H), 10.58 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d$_6$) δ 18.22, 51.37, 52.13, 102.30, 106.66, 118.81, 122.48, 128.10, 128.37, 128.80, 132.60, 133.70, 141.23, 147.73, 165.05. Anal. calcd for $C_{20}H_{18}Cl_2N_4O_2$ (417.3): C, 57.57; H, 4.35; N, 13.43%; found C, 57.58; H, 4.35; N, 13.44%.

6-(4-Chlorophenyl)-5-(methoxycarbonyl)-4-methyl-2-(3-(trifluoromethylthio) phenylamino)-3,6-dihydropyrimidin-1-ium chloride (11)

Appearance: pale yellow solid; yield 69%. mp 220–221 °C. IR (KBr) ν/cm 3191, 3043, 1714, 1671, 1585, 1472, 1088, 766. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 2.42 (s, 3H), 3.62 (s, 3H), 5.44 (s, 1H), 7.36 (d, J = 8.48 Hz, 2H), 7.42–7.63 (m, 6H), 10.29 (s, 1H), 11.09 (s, 2H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ 17.56, 25.44, 51.61, 51.69, 103.32, 124.31, 124.33, 126.67, 127.85, 128.43, 128.91, 130.80, 131.28, 132.97, 133.78, 136.35, 140.03, 148.62, 164.65. Anal. calcd for $C_{20}H_{18}Cl_2F_3N_3O_2S$ (492.3): C, 48.79; H, 3.69; N, 8.53%; found C, 48.80; H, 3.68; N, 8.54%.

2-(4-Bromophenylamino)-6-(4-chlorophenyl)-5-(methoxycarbonyl)-4-methyl-3,6-dihydropyrimidin-1-ium chloride (12)

Appearance: pale yellow solid; yield 66%. mp 227–228 °C. IR (KBr) ν/cm 3184, 3042, 1707, 1671, 1571, 1488, 771, 688. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 2.41 (s, 3H), 3.61 (s, 3H), 5.40 (s, 1H), 7.16 (d, J = 8.64 Hz, 2H), 7.34 (d, J = 8.48 Hz, 2H), 7.47 (d, J = 8.44 Hz, 2H), 7.64 (d, J = 8.64 Hz, 2H), 9.93 (s, 1H), 10.76 (s, 1H), 11.13 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ 19.49, 50.84, 52.39, 99.86, 114.98, 123.88, 128.25, 128.54, 131.88, 131.99, 140.90, 143.41, 148.00, 150.94, 165.80. Anal. calcd for $C_{19}H_{18}BrCl_2N_3O_2$ (471.2): C, 48.43; H, 3.85; N, 8.92%; found C, 48.45; H, 3.86; N, 8.94%.

2-(3-Chloro-5-hydroxyphenylamino)-6-(4-chlorophenyl)-5-(methoxycarbonyl)-4-methyl-3,6-dihydropyrimidin-1-ium chloride (13)

Appearance: white solid; yield 67%. mp 198–199 °C. IR (KBr) ν/cm 3646, 3198, 3062, 1719, 1677, 1581, 1481, 787. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 2.40 (s, 3H), 3.58 (s, 3H), 5.32 (s, 1H), 6.77–7.32 (m, 5H), 7.43 (d, J = 8.48 Hz, 2H), 9.41 (s, 1H), 10.30 (s, 2H), 10.80 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ 17.68, 51.46, 52.03, 102.35, 115.15, 116.31, 118.94, 128.63, 128.67, 130.67, 132.72, 140.60, 145.33, 148.79, 157.38, 164.78. Anal. calcd for $C_{19}H_{18}Cl_3N_3O_3$ (442.7): C, 51.55; H, 4.10; N, 9.49%; found C, 51.57; H, 4.08; N, 9.50%.

6-(4-Chlorophenyl)-2-(2,4-dimethoxybenzylamino)-5-(methoxycarbonyl)-4-methyl-3,6-dihydropyrimidin-1-ium chloride (15)

Appearance: pale yellow solid; yield 71%. mp 221–222 °C. IR (KBr) ν/cm 3222, 3029, 1714, 1680, 1585, 1468, 762. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 2.37 (s, 3H), 3.60 (s, 3H), 3.74 (s, 6H), 4.30 (d, J = 14.72 Hz, 1H), 4.45 (d, J = 14.32 Hz, 1H), 5.43 (s, 1H), 6.41 (d, J = 7.68 Hz, 1H), 6.54 (s, 1H), 7.17 (d, J = 8.36 Hz, 1H), 7.30 (d, J = 8.48 Hz, 2H), 7.41 (d, J = 8.40 Hz, 2H), 8.50 (s, 1H), 10.26 (s, 1H), 11.11 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ 17.69, 51.29, 51.46, 55.23, 55.42, 98.47, 104.28, 115.86, 128.42, 128.70, 129.60, 132.71, 140.53, 149.67, 158.01, 160.64, 164.78. Anal. calcd for $C_{22}H_{25}Cl_2N_3O_4$ (466.3): C, 56.66; H, 5.40; N, 9.01%; found C, 56.68; H, 5.36; N, 9.07%.

Single crystal X-ray crystallographic study

A particular size of single crystals **7** and **11** was determined by X-ray diffraction study using Bruker KAPPA APEX II DUO diffractometer using graphite-monochromator, Mo-Kα radiation (λ = 0.71073 Å; Table 1). Single crystals of compounds **7** and **11** were grown from acetone by slow evaporation method at room temperature for X-ray study. Data collection was carried out at 100 (2) K temperature using liquid Nitrogen (N_2) cryo-system attached with Oxford Cryostat. Cell refinement and data reduction were performed using the program SAINT[C]. The crystal structure solution was worked out by full matrix least-squares method using SHELXL97 and absorption correction performed using SADABS (21). All the non-hydrogen atoms and some of hydrogen atoms were located in difference Fourier maps and were refined anisotropically and the remaining hydrogen atoms were fixed geometrically and refined isotropically. Graphical presentations were drawn using Ortep-3 and Mercury (22,23). The crystal structure solution

Table 1: Crystal data and measurement details for compounds **7** and **11**

Crystal data	Compound **7**	Compound **11**
Formula	$C_{19}H_{18}BrCl_2N_3O_2$	$C_{20}H_{18}Cl_2F_3N_3O_2S$
CCDC Number	884242	884243
Formula weight	471.2	492.33
Crystal morphology	Needle	Needle
Crystal size(mm)	0.18 × 0.07 × 0.05	0.17 × 0.08 × 0.04
Temperature/K	100 (2)	100 (2)
Radiation	Mo-K_α	Mo-K_α
Wavelength (Å)	0.71073	0.71073
Crystal system	Monoclinic	Monoclinic
Space group	*P* $2_1/c$	*C* 2/*c*
a (Å)	7.7294(9)	28.174(3)
b (Å)	23.290(3)	7.7682(7)
c (Å)	11.0671(1)	21.941(2)
α (°)	90	90
β (°)	97.608(3)	112.101(2)
γ (°)	90	90
Volume (Å^3)	1974.7(1)	4449.2(7)
Z	4	8
Density (gm/cm^3)	1.58	1.47
μ (1/mm)	2.372	0.433
F (000)	951.8	2015.7
θ (min, max)	1.8, 27.2	2.0, 26.4
Total number of refl^n	14372	14087
No. Unique refl^n	4366	4559
No. of parameters	258	294
R_ obs, wR_2_obs	0.042, 0.085	0.044, 0.086
$\Delta\rho_{min}$(eÅ^{-3}), $\Delta\rho_{max}$(eÅ^{-3})	0.910,−0.758	0.263, −0.295
GooF	1.012	0.989

was worked out by full matrix least-squares method using SHELXL97 and absorption correction performed using SADABS[d]. All the non-hydrogen atoms and some hydrogen atoms were located in difference Fourier maps and were refined anisotropically, and other hydrogen atoms were fixed geometrically and refined isotropically.

Antitubercular activity

All the mycobacterial culture preparations were performed in a Level II Biosafety Laboratory using personal protective equipment. H37Rv (ATCC No. 25177), a MDR-TB strain and XDR-TB strains were clinical isolates (Department of Microbiology, Inkosi Albert Luthuli Central Hospital, National Health Laboratory Services, Durban) tested for antimycobacterial activity in Middlebrook 7H10 agar enriched with OADC (0.005%, v/v, oleic acid; 0.5%, 171 w/v, BSA; 0.2%, w/v, glucose; 0.02%, v/v, catalase and 0.085%, w/v, NaCl) and incubated at 37 °C for 3 weeks. Rifampcin, isoniazid, ofloxacin and kannamycin were used as controls. Title compounds **7–13** and **15** were dissolved in distilled water to yield concentration ranging from 64 to 4 μg/mL and these were sterilized using a 0.22 μ polycarbonate filter. Tests were conducted in triplicate.

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was performed according to the agar dilution method as previously described (24) with slight modifications. *Mycobacterium tuberculosis* reference strains H37Rv, MDR and XDR were cultured in Middlebrook 7H10 medium (25). Cultures with confluent growth were vortexed thoroughly in a sterile tube containing 4.5 mL phosphate buffer, 0.05% tween 80 with glass beads (5 mm diameter) to remove clumps. After settling for 45 min, the clear bacterial supernatant was standardized to a McFarland Number 1 with sterile water. This gave a bacterial concentration of approximately 1×10^7 cfu/mL. The bacterial suspension was diluted 10-fold with sterile water and 100 μL of the appropriate dilution was spotted onto Middlebrook 7H10 agar plates containing 4–64 μg/mL of the drug compounds. The plates were read after a 3-week incubation period at 37 °C and the MIC was defined as the minimum drug concentration to inhibit growth of the organism when compared to the drug-free controls.

Cytotoxicity evaluation

The cytotoxic effect of the compounds was tested using the standard protocol (20). Stock solution of 1 mg/mL of title compounds **7–13** and **15** was dissolved in water and further twofold dilutions were made to obtain concentrations of 15.625–1000 μg/mL. Ficoll separated PBMC (1×10^6 cells/mL) were seeded in 96-well plates containing 180 μL of media and 20 μL of each sample was added to each well and this was incubated at 37 °C for 24 h in a 5% CO_2 humidified incubator. After 24 h, 10 μL of MTT salt (5 mg/mL) was added to each well and incubated for a further 4 h in a 37 °C humidified incubator. The purple formazan precipitate formed was dissolved in 50 μL of 2% (v/v) dimethyl sulphoxide/water and incubated for another 30 min at room temperature. The absorbance was read at 570 nm with a reference wavelength of 650 nm in an ELISA microplate reader. The results were evaluated against untreated cells. All the experiments were conducted in triplicate.

Results and Discussion

Chemistry

The 3,6-dihydropyrimidine analogues **7–13** and **15** were synthesized as outlined in Scheme 1 starting from methyl 4-(4-chlorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate **4** which has been synthesized by Bigi-

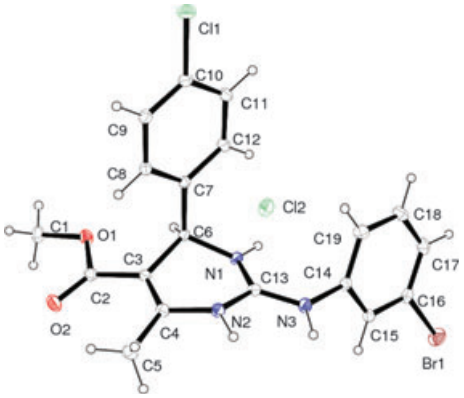

Figure 1: ORTEP diagram with atom labelling of **7** with displacement ellipsoids drawn at the 50% probability level except hydrogen atoms as arbitary circle. Quaternary chloride salt of N1 (pyrimidine nitrogen) is shown as proton transfer.

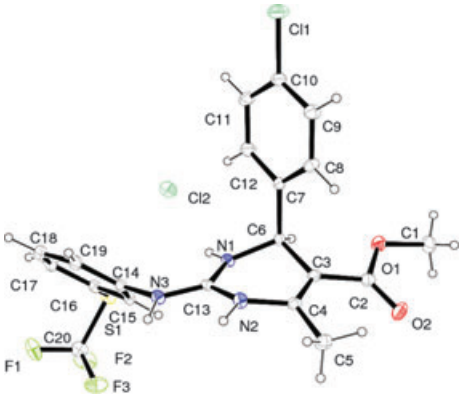

Figure 2: ORTEP diagram with atom lebelling of **11** with displacement ellipsoids drawn at the 50% probability level except hydrogen atoms as arbitary circle. Quaternary chloride salt of N1 (pyrimidine nitrogen) is shown as proton transfer.

nelli reaction by employing Lewis acid according to the procedure described in our previous study (26). The purification of the product was accomplished using Et-OH by recrystallization method and the yield of the product was 66%. The formation of methyl 4-(4-chlorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate **4** was confirmed by its ^{1}H-NMR spectrum, in which the appearance of methyl singlet peak at δ = 2.31 ppm indicated the presence of methyl group on heterocyclic ring due to less deshielding effect and methoxy singlet peak in ester group at δ = 3.92 ppm due to more deshielding effect. In ^{13}C-NMR, δ = 159.18 ppm was accounted for the less deshielded carbonyl carbon on heterocyclic ring and δ = 167.76 ppm was accounted for more deshielded carbonyl carbon in ester group. On LC-MS molecular mass of the compound was in good agreement with the molecular weight of compound **4**. Synthesis of methyl 2-chloro-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate **5** was achieved by treating compound **4** with phosphorous oxychloride for 16 h. The yield of compound **5** was 72% after recrystallization using Et-OH. Formation of methyl 2-chloro-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate **5** was confirmed by proton NMR spectrum, where the disappearance of a proton from the nitrogen atom that is adjacent to *para* chlorophenyl ring. This can be attributed in ^{13}C-NMR spectrum in which missing of less deshielded carbonyl carbon is observed that is due to the transformation of heterocyclic carbonyl carbon to heterocyclic carbon chlorine single bond. Its formation is further confirmed by LC-MS spectral data in which molecular ion peak is observed at m/z 299 due to M + H peak. Title compounds **7–13** and **15** were achieved as hydrochloride salt by refluxing equimolar proportion of compound **5** and mono/disubstituted aromatic amines in isopropanol medium. The yield of the compounds was found to be in the range of 63–71% after purification by column chromatography. The structures of novel 3,6-dihydropyrimidine analogues **7–13** and **15** were characterized by IR, NMR (^{1}H and ^{13}C) and elemental analysis. In infrared spectra of **7–13** and **15**, the secondary amino group and ester carbonyl group are observed in the range 3184–

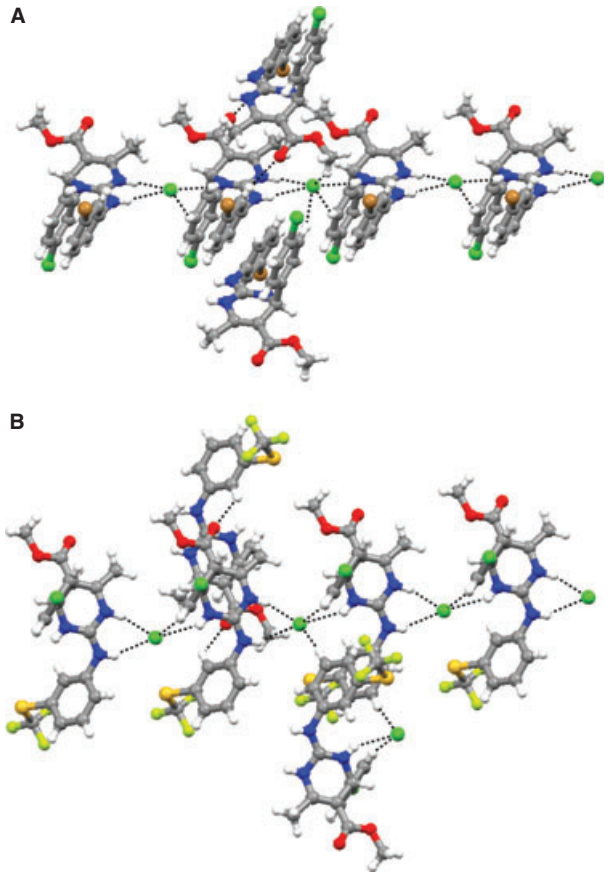

Figure 3: N-H···Cl hydrogen bonds form infinite molecular chain, and C-H···O and C-H···Cl weak hydrogen bonds lead dimer formation for **7** (A) and **11** (B). Interactions are shown as black dotted lines.

Table 2: Intermolecular interactions of compounds **7** and **11**

	D-X···A(Å)	D-X (Å)	X···A (Å)	D···A (Å)	∠D-X···A (°)	Symmetry code
Compound **7**	N1-H1···Cl2	0.77 (3)	2.39 (3)	3.100 (3)	154 (3)	1–*x*,–*y*,1–*z*
	N2-H2···Cl2	0.86 (4)	2.24 (4)	3.070 (3)	164 (3)	2–*x*,–*y*,1–*z*
	N3-H3···Cl2	0.76 (4)	2.57 (4)	3.268 (3)	152 (3)	2–*x*,–*y*,1–*z*
	C11-H11···Cl2	0.93	2.81	3.678 (3)	156	*x*,*y*,1 + *z*
	C15-H15···O2	0.93	2.45	3.290 (4)	151	2–*x*,–*y*,1–*z*
	Cg1···Cg2	–	3.675 (2)	–	–	2–*x*,–*y*,2–*z*
Compound **11**	N1-H1···Cl2	0.80 (2)	2.44 (2)	3.148 (2)	149 (2)	1/2–*x*,3/2–*y*,–*z*
	N2-H2···Cl2	0.86 (3)	2.28 (3)	3.101 (2)	162 (3)	1/2–*x*,1/2–*y*,–*z*
	N3-H3···Cl2	0.81 (2)	2.45 (2)	3.198 (2)	155 (3)	1/2–*x*,1/2–*y*,–*z*
	C1-H1C···Cl2	0.98	2.68	3.628 (3)	163	–1/2 + *x*,1/2 + *y*,*z*
	C15-H15···O2	0.95	2.59	3.298 (4)	132	–*x*,1–*y*,–*z*
	C18-H18···Cl2	0.95	2.73	3.649 (3)	163	*x*,*y*,*z*
	C8-H8···F3	0.95	2.54	3.391 (3)	148	*x*,1–*y*,1/2 + *z*
	C9-H9···Cg1	0.95	2.97	3.898 (3)	167	x,1–*y*,1/2 + *z*
	Cg1···Cg1	–	3.996 (2)	–	–	1/2–*x*,3/2–*y*,–*z*

Cg1 = Centroid of six-member ring C14–C19; Cg2 = Centroid of six-member ring C7–C12.

3222 and 1705–1719 per cm, respectively. The nitrile stretching is observed at 2226 per cm for compound **10**. The ^{1}H-NMR spectrum of **7–13** and **15** indicated the chemical shift of the methyl group on heterocyclic ring and ester methoxy group in the range of δ = 2.33–2.42 ppm and δ = 3.54–3.62 ppm, respectively. Heterocyclic methine proton is observed in the range of δ = 5.26–5.44 ppm. Salt form of the title compounds were confirmed by proton NMR in which proton on quaternary nitrogen that was involved in the formation of chloride salt was observed in the range of δ = 10.18–10.98 ppm and also supported by single crystal X-ray studies. In ^{13}C-NMR, ester carbonyl carbon is observed in the range of δ = 164.60–165.94 ppm. Elemental analysis results were within ±0.04% of the calculated values of the proposed title compounds **7–13** and **15**. The IR, NMR (^{1}H & ^{13}C) and elemental analysis data are discussed in detail under experimental section.

Single crystal X-ray studies

Crystallographic details are listed in Table 1. Figures 1 and 2 provide the thermal ellipsoid plot with atom labelling of **7** and **11** drawn at the 50% probability level, respectively, and which evidence the hydrochloride salt formation with protonation of N1 (pyrimidine nitrogen) atom and Cl2 (chloride ion) in the crystal structure.

The molecular packing of **7** is mainly based on all the three N-H groups that participate in hydrogen bond with the chloride ion (Table 2), additional weak interactions of C-H···Cl, C-H···O and $\pi\cdots\pi$ enhance the stability of three-dimensional molecular assembly (Figure 3A, Table 2). Similarly, in case of **11**, the N-H···Cl hydrogen bonds form infinite molecular chain along with weak C-H···O hydrogen bonds that form dimer (Figure 3B, Table 2).

Further, additional weak intermolecular interactions of C-H···F (27), C-H···π (28,29) along with $\pi\cdots\pi$ (30) (Table 2) short contacts in **11** structure do not alter much in basic three-dimensional structure in comparison with **7** structure. However, the molecular assembly is preferred as zig-zag arrangement with all the intermolecular interactions that are shown along *a*-axis and *b*-axis of the unit cell for the **7** and **11,** respectively (Figure 4A,B).

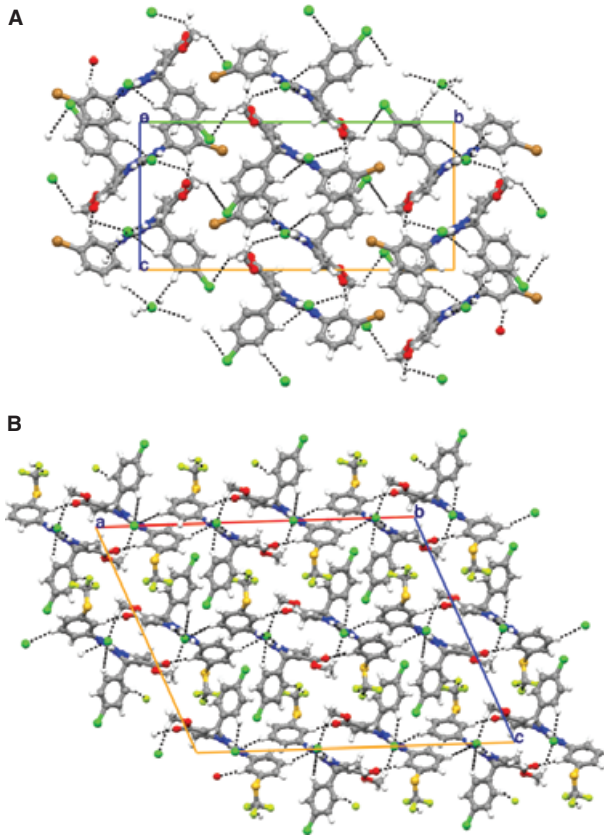

Figure 4: The hydrogen bonds and weak intermolecular interactions (as black dotted lines) lead to zig-zag molecular packing in the unit cell for 7 structure along *a*-axis (A) and for the 11 structure along *b*-axis (B).

Pharmacology

3,6-Dihydropyrimidine analogues **7–13** and **15** were screened for their *in vitro* antitubercular and cytotoxicity activity following standard protocol. The purity of the compounds was ascertained by HPLC and it was found to be more than 99% and their structures have been elucidated by spectral interpretation. To investigate the role of the substitution types and patterns on the aryl/benzyl ring which is attached to dihydropyrimidine ring through secondary amine group (-NH-) for *in vitro* antitubercular and cytotoxicity activity, various mono and disubstituted compounds were prepared. Compounds **7–13** and **15** were

Table 3: *In vitro* antitubercular activity of 3,6-dihydropyrimidine analogues **7–13** and **15**

	Minimum inhibitory concentration (μg/mL)		
Compounds	H37Rv(ATCC 25177)	MDR-TB	XDR-TB
7	4	16	>64
8	8	32	>64
9	8	32	>64
10	8	32	>64
11	4	16	>64
12	16	64	>64
13	32	32	>64
15	16	32	>64
Standards Isoniazid	2	8	8
Rifampicin	1	32	64
Ofloxacin	0.5	16	16
Kannamycin	2	32	32

MDR-TB, multidrug resistance tuberculosis; XDR-TB, extensively drug resistance tuberculosis.

evaluated for their *in vitro* antitubercular activity against H37Rv, MDR-TB and XDR-TB strains. The results of the antitubercular activity are tabulated in Table 3. Compounds **7** and **11** exhibited activity at 4 μg/mL against H37Rv and were emerged as promising molecules against MDR-TB at 16 μg/mL. Compound **7** having electron withdrawing bromine atom at third position of the aryl group that is connected to dihydropyrimidine nucleus through secondary amine bridge exhibited significant antitubercular activity at 16 μg/mL against MDR-TB. Compound **11** also revealed similar activity at 16 μg/mL having trifluoromethylthio group at third position of the aryl ring which is connected to dihydropyrimidine through secondary amine bridge. However, compound **12** having bromine atom at fourth position of the aryl ring exhibited moderate activity at 64 μg/mL against MDR-TB. Compound **10** having cyano group at fourth position exhibited activity similar to that of compound **12**. Analogues such as **8**, **9**, **13** and **15** that are having disubstituted phenyl amino group on dihydropyrimidine nucleus showed moderate activity (32 μg/mL) against MDR-TB, and none of the compounds significant activity against XDR-TB (MIC > 64 μg/mL). Cytotoxicity of the title compounds were evaluated by MTT assay and twofold dilutions were made to achieve a concentration ranges from 15.625 to 1000 μg/mL and no cytotoxicity is observed up to 1000 μg/mL concentration.

Conclusions and Future Directions

Herein, we describe the synthesis and characterization of new series of 3,6-dihydropyrimidine analogues **7–13** and **15**, which were evaluated for their *in vitro* antitubercular and cytotoxicity studies. Compounds **7** and **11** appeared as potential antitubercular agents against MDR-TB. Disubstituted analogues exhibited moderate activity when compared to mono substituted potent analogues such as **7** and **11**. Further single-crystal X-ray study for **7** and **11** was performed to understand the nature of conformation and molecular assembly. All the title compounds were non-toxic up to 1 mg/mL concentration by MTT assay. Additional investigations including computational studies with respect to targets, protein binding and synergistic properties of promising molecules **7** and **11** with first line and second line anti-TB therapy drugs are in progress.

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Competing interests

The authors declare that they have no competing interests.

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Notes

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Spectral details such as IR and NMR (^{1}H and ^{13}C) of compounds **7–13** and **15**, single-crystal X-ray data of compounds **7** and **11** (CCDC number 884242 and 884243, respectively).