A Nano-MgO and Ionic Liquid-Catalyzed ‘Green’ Synthesis Protocol for the Development of Adamantyl-Imidazolo-Thiadiazoles as Anti-Tuberculosis Agents Targeting Sterol 14α-Demethylase (CYP51)

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Abstract

In this work, we describe the ‘green’ synthesis of novel 6-(adamantan-1-yl)-2-substituted-imidazo[2,1-b][1,3,4]thiadiazoles (AITs) by ring formation reactions using 1-(adamantan-1-yl)-2-bromoethanone and 5-alkyl/aryl-2-amino1,3,4-thiadiazoles on a nano material base in ionic liquid media. Given the established activity of imidazothiadiazoles against \textit{M. tuberculosis}, we next examined the anti-TB activity of AITs against the H37Rv strain using Alamar blue assay. Among the tested compounds 6-(adamantan-1-yl)-2-(4-methoxyphenyl)imidazo[2,1-b][1,3,4]thiadiazole (3f) showed potent inhibitory activity towards \textit{M. tuberculosis} with an MIC value of 8.5 μM. The inhibitory effect of this molecule against \textit{M. tuberculosis} was comparable to the standard drugs such as Pyrazinamide, Streptomycin, and Ciprofloxacin drugs. Mechanistically, an \textit{in silico} analysis predicted sterol 14α-demethylase (CYP51) as the likely target and experimental activity of 3f in this system corroborated the \textit{in silico} target prediction. In summary, we herein report the synthesis and biological evaluation of novel AITs against \textit{M. tuberculosis} that likely target CYP51 to induce their antimycobacterial activity.
Introduction

Tuberculosis (TB) is one of the leading contagious and airborne disease caused by *Mycobacterium tuberculosis* [1, 2] and according to 2013 report of World Health Organization, TB stands second in terms of global mortality from a single infectious agent with 1.5 million death in 2013 worldwide. The conventional TB treatment comprises a cocktail of first-line drugs, namely isoniazid, pyrazinamide, ethambutol and rifampicin which are associated with lowered efficacy due to resistance development and severe adverse effects [3, 4]. The subsequent use of second-line drugs were also reported to suffer from similar problems [5–7]. Gradual increase of multidrug and extensively drug resistant (XDR-TB) mycobacterial strains demands the need of new therapeutic agents which can effectively target TB. The presence of lipid-rich cell surface of mycobacterium provides an effective therapeutic target to design anti-TB agents [8]. Researchers have rightly called adamantanyl ring as ‘lipophilic bullet’ which effectively targets mycobacterium. Evidently, hybrid obtained from the coupling of adamantylacetamide ring with 1,2,3-triazoles resulted in development of potent inhibitors against *M. tuberculosis* [9]. SQ109, an adamantane based small molecule which is in phase-II clinical trials for the treatment of pulmonary TB [10–12]. On the other hand, Delamanid, an imidazo-oxazole based anti-tuberculosis drug was approved for the treatment of multidrug-resistant tuberculosis [13]. Thiadiazoles and imidazothiadiazoles were reported to have antitubercular activity against *M. tuberculosis* H37Rv strains [14–16]. Based on these reports, we attempted to tether the imidazo-thiadiazole nuclei to adamantyl ring in order to enhance the bioactivity profile of the newer drug-seeds. We previously developed several heterocycle based small molecules and explored the various pharmacological properties [17–25]. In the present report, we synthesized a series of novel adamantanyl-tethered imidazo-thiadiazoles for the first-time and evaluated for their inhibitory activity towards *M. tuberculosis*, and a subsequent mode-of-action analysis identified that they likely achieve this activity by targeting sterol 14α-demethylase (CYP51) (S1 Data).

Results and Discussion

Chemistry

The reaction between 1-(adamantan-1-yl)-2-bromoethanone and 5-substituted-2-amino-1,3,4-thiadiazoles yielded 6-(adamantan-1-yl)-2-substituted-imidazo[2,1-b][1,3,4]thiadiazoles (‘AITs’) with varying yields under different base and solvent conditions (Fig 1A, Scheme 1). Use of solvents such as ethanol, 1-butanol, N,N-dimethyl formamide resulted in poor yields. In order to overcome yield limitation, we next used various ionic liquids (ILs) in combination with a nano-catalyst, nano-MgO (Table 1) [26, 27]. ILs are molten salts, which can dramatically accelerate the rate of reactions, and have often been found to be a suitable substitute for low-boiling organic solvents in terms of toxicity, volatility and flammability [28]. In addition, ILs have more favorable ‘green’ properties, since they are reusable. In the current work, replacement of organic solvents with ILs significantly improved yields of the product to greater than 90%. In particular, [BMIM][BF4] and [BMPy][PF6] were found to be the better ILs and we have chosen [BMIM][BF4] for the preparation of compounds due to its solubility in water. Additionally, this method was found to be green protocol for the preparation of alkyl or aryl substitution on thiadiazole ring (Table 2). All the isolated products of the reaction were fully characterized by 1H NMR, LC-MS and elemental analysis. Finally, we prepared the single crystal of one of the AITs, namely 3b, via slow evaporation technique. The single crystal X-ray diffraction studies of 3b confirmed formation of the title compounds (Fig 1B).
**Scheme 1**

A \[
\text{Br}\text{C}_{\text{C}}\text{C} + \text{N}-\text{N}\text{S}-\text{NH}_{2} \xrightarrow{[\text{BMIM}][\text{BF}_4]} \text{R}\text{N}\text{S}\text{N}\text{C} \xrightarrow{\text{nano-MgO}} \text{R}\text{N}\text{S}\text{N}\text{C}\text{C} \]

B

**Figure 1**

**Figure 1**

**Figure 1**

**Figure 1**
Anti-TB activity of novel AITs

The *in vitro* Alamar Blue assay was employed to determine the Anti-TB activity of AITs against the *M. tuberculosis* H37Rv strain as described earlier [29]. Various concentrations of AITs were added to the culture of *M. tuberculosis* and minimum inhibitory concentrations (MIC) of AITs were measured and the results are tabulated in Table 2. Most AITs showed inhibitory activity towards the *M. tuberculosis* H37Rv strain, suggesting that AITs possess significant anti-TB activity. Notably, Compound 3a, 3f, and 3i displayed relatively low MIC values of 10.5, 8.5 and 12.5 μM respectively when compared to the other structurally related compounds. Compounds with electron-donating phenyl, 4-methoxy phenyl, and methyl substituents attached to the imidazo-thiadiazole scaffold were favorable for activity against *M. tuberculosis*.

**In silico** molecular interactions of AITs towards sterol 14α-demethylase

As sterol 14α-demethylase (CYP51) is known to process a variety of sterols and as a drug target in *M. tuberculosis* [30, 31], we attempted to rationalize the anti-TB activity of the AITs synthesized in this work on a structural basis. Therefore, we docked all AITs to the X-ray structure of *M. tuberculosis* CYP51 in complex with a small molecule inhibitor (PDB: 2CIB) [32] using MOE default settings (Fig 2A) [33] and visualized predicted protein-ligand interactions with Pymol [34]. It was found that the imidazo-thiadiazole scaffold of 3f likely interacts with the heme cofactor of CYP51 (see Fig 2B). Furthermore, the hydrophobic moieties are positioned in similar positions to the ring centers found in the co-crystallized ligand. Based on this analysis, CYP51 appeared to be a plausible target for AITs on a structure-based level. Further, in order to analyze the similarities in binding mode between AITs, we superposed the ligand in the co-crystal used for docking with compound 3a using MOE’s flexible alignment module and default settings [33]. We found an almost perfect shape overlap of the lowest energy conformations of AITs with all hydrophobic centers coinciding with the co-crystallized ligand (see Fig 2C). Therefore, the AITs...
presented in this work could be considered a continuation of the 1,3,4-thiadiazole series presented earlier by Oruc et al. [16] including an isosteric replacement of the core ring fragment.

**In vitro** anti-microbial activity of AITs against fungal strains that express 14\(^\alpha\)-demethylase (CYP51)

Our *in vitro* and *in silico* studies revealed that AITs showed good anti-TB activity at the low micro molar concentrations, and by plausibly targeting sterol 14\(^\alpha\)-demethylase (CYP51). In order to find further experimental support for the mode-of-action analysis of the AITs presented here, the CM237 and akuB strains of *A. fumigatus* that express CYP51 were selected for the next step. We further investigated the effect of AITs at various concentrations against both the fungal strains by broth microdilution method as reported previously [35]. MICs were determined visually in duplicate and recorded after 48 h. Results are summarized in Table 3. Interestingly, the most active compounds against *M. tuberculosis*, namely 3a, 3f, and 3i also exhibited significant inhibitory effect against the tested *A. fumigates* strains. These results lend further support--though not definite proof to their plausible mode-of-action by targeting sterol 14\(^\alpha\)-demethylase (CYP51).

**Materials and Methods**

All solvents used were of analytical grade and reagents used were purchased from Sigma-Aldrich chemicals. All IR spectra were obtained in a KBr disc on a Shimadzu FT-IR 157 Spectrometer. \(^1\)H and \(^{13}\)C NMR spectra were recorded on a Bruker WH-200 (400 MHz) in CDCl\(_3\).
or DMSO-d$_6$ as solvent, using tetramethylsilane (TMS) as an internal standard and chemical shifts are expressed as ppm. Mass spectra were determined on a Shimanzu LC-MS. The elemental analyses were carried out using an Elemental Vario Cube CHNS rapid Analyzer. The progress of the reaction was monitored by TLC pre-coated silica gel G plates.

### Typical procedure for the synthesis of AITs

A mixture of 5-alkylaryl-2-amino1,3,4-thiadiazole (0.01 mol), 1-adamantyl bromomethylketone (0.01 mol) and nano magnesium oxide (0.001 mol) in 2 ml of 1-Butyl-3-methylimidazoliun tetrafluoroborate [BMIM]$_2$[BF$_4$]$_2$ was stirred at 60°C for the appropriate time (Table 2). After completion of the reaction, as determined by TLC, the reaction mixture was cooled down and then quenched into ice water. The product was extracted from the water layer by 3×5 mL diethyl ether, dried with magnesium sulfate, filtered, and concentrated in vacuo. The crude product was purified by chromatography employing a column of 30 mm diameter using 60–120 silica gel and hexane/ethyl acetate (80:20) as mobile phase. All new compounds exhibited spectral properties consistent with the assigned structures (S2 Data).

#### 6-(Adamantan-1-yl)-2-phenylimidazo[2,1-b][1,3,4]thiadiazole (3a):  
$^1$H NMR (400 MHz, CDCl$_3$) δ: 8.0 (s, 1H), 7.8 (m, 2H), 7.6 (m, 2H), 7.4 (m, 1H), 2.1 (m, 6H), 1.9 (m, 3H), 1.7 (m, 6H); 13C NMR (CDCl$_3$): 175.73, 134.92, 133.26, 132.17, 129.39, 128.09, 127.56, 123.43, 45.59, 42.75, 38.19, 28.48; LCMS (MM:ES+APCI) 336.3 (M+H)$^+$; Anal. Calcd for C$_{20}$H$_{21}$N$_3$S: C 71.61; H 6.31; N 12.53. Found: C, 71.43; H, 6.47; N, 12.66.

#### 6-(Adamantan-1-yl)-2-benzylimidazo[2,1-b][1,3,4]thiadiazole (3b):  
$^1$H NMR (400 MHz, CDCl$_3$) δ: 8.0 (s, 1H), 7.4 (m, 2H), 7.3 (m, 3H), 4.4 (s, 2H), 1.7–2.2 (m, 15H); 13C NMR (CDCl$_3$): 161.09, 135.82, 134.73, 129.04, 128.23, 125.56, 123.65, 45.56, 42.78, 38.65, 38.32, 28.66; LCMS (MM:ES+APCI) 350.3 (M+H)$^+$; Anal. Calcd for C$_{21}$H$_{23}$N$_3$: C, 72.17; H, 6.63; N, 12.02. Found: C, 71.98; H, 6.78; N, 12.24.

#### 6-(Adamantan-1-yl)-2-(4-nitrophenyl)imidazo[2,1-b][1,3,4]thiadiazole (3c):  
$^1$H NMR (400 MHz, CDCl$_3$) δ: 8.1 (d, 2H), 8.0 (s, 1H), 7.9 (d, 2H), 2.1 (m, 6H), 1.9 (m, 3H), 1.7 (m, 6H); 13C NMR (CDCl$_3$): 175.54, 148.97, 139.92, 135.33, 134.83, 128.65, 123.34, 121.78, 45.56, 42.78, 38.12, 28.55; LCMS (MM:ES+APCI) 381.2 (M+H)$^+$; Anal. Calcd for C$_{20}$H$_{20}$N$_4$O$_2$S: C, 63.14; H, 5.30; N, 14.73. Found: C, 63.18; H, 5.88; N, 13.89.

#### 6-(Adamantan-1-yl)-2-(4-methoxybenzyl)imidazo[2,1-b][1,3,4]thiadiazole (3d):  
$^1$H NMR (400 MHz, CDCl$_3$) δ: 8.0 (s, 1H), 7.9 (d, 2H), 7.6 (d, 2H), 4.3 (s, 3H), 4.0 (s, 2H), 2.1 (m, 6H), 1.9 (m, 3H), 1.7 (m, 6H); 13C NMR (CDCl$_3$): 161.06, 159.43, 135.92, 134.83, 130.33, 128.23, 123.45, 114.45, 55.89, 45.56, 42.78, 38.22, 28.51; LCMS (MM:ES+APCI) 380.2 (M+H)$^+$; Anal. Calcd for C$_{22}$H$_{25}$N$_3$OS: C, 69.62; H, 6.64; N, 11.07. Found: C, 69.48; H, 7.03; N, 11.86.

#### 6-(Adamantan-1-yl)-2-(furan-2-yl)imidazo[2,1-b][1,3,4]thiadiazole (3e):  
$^1$H NMR (400 MHz, CDCl$_3$) δ: 8.1 (d, 1H), 8.00 (s, 1H), 7.4 (d, 2H), 6.8 (t, 1H), 2.1 (m, 6H), 1.9 (m, 3H), 1.7 (m, 6H); 13C NMR (CDCl$_3$): 175.82, 148.97, 139.92, 135.33, 134.83, 128.65, 123.34, 121.78, 45.56, 42.78, 38.12, 28.55; LCMS (MM:ES+APCI) 381.2 (M+H)$^+$; Anal. Calcd for C$_{20}$H$_{20}$N$_4$O$_2$S: C, 63.14; H, 5.30; N, 14.73. Found: C, 63.18; H, 5.88; N, 13.89.

### Table 3. MIC values obtained from the lead AIT compounds against A. fumigates, which expresses CYP51.

Given the activity of compounds in this system this finding corroborates CYP51 as a plausible target of the AIT series.

| Aspergillus fumigatus strain | Compound 3a MIC (μg/ml) | Compound 3f MIC (μg/ml) | Compound 3i MIC (μg/ml) |
|-----------------------------|-------------------------|-------------------------|-------------------------|
| WT                          | 237                     | 16                      | 16                      |
| WT akuB                     | 8                       | 8                       | 8                       |

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X-ray crystal structure determination of (3b)

A single crystal of compound 3b with dimensions of $0.30 \times 0.25 \times 0.20 \text{ mm}$ was chosen for X-ray diffraction studies. The data were collected on a Bruker SMART APEX II X-ray diffractometer with Cu Kα radiation. Raw data was processed and reduced by using APEX2 and SAINT [36, 37]. The crystal structure was solved by direct methods using SHELXS-97 [38]. All non-hydrogen atoms were revealed in the first Fourier map itself. Anisotropic refinement of non-hydrogen atoms was started at this stage. Subsequent refinements were carried out with anisotropic thermal parameters for non-hydrogen atoms and isotropic temperature factors for the hydrogen atoms which were placed at chemically acceptable positions. Full-matrix least squares refinement was carried out using SHELXL-97 [39] with a final residual value of $R1 = 0.079$. The thermal ellipsoid plot [40] of the molecule at 50% probability is represented in Fig 1B. The details of crystallographic information have been deposited at the CCDC with number 1056579.

The crystal structure analysis showed that the compound 3b crystallizes in a triclinic system under the space group P-1, with cell parameters $a = 6.3060(5) \text{ Å}$, $b = 10.4279(7) \text{ Å}$, $c = 14.1099(10) \text{ Å}$, $\alpha = 81.101(2)^\circ$, $\beta = 79.845(2)^\circ$, $\gamma = 82.918(2)^\circ$ and $Z = 2$. The benzyl imidazothiadiazole moiety adopts a chair conformation with puckering parameters $Q = 0.622(4) \text{ Å}$ and $\varphi = 201(19)^\circ$ [31] and the maximum deviation found on the puckered atom at C14 is $0.255(3) \text{ Å}$. The benzyl imidazothiadiazole moiety and the phenyl ring are bridged by the carbon atom (C6) with a dihedral angle of 69.73(5)$^\circ$. The structure does not contain any classical hydrogen bonds.
Anti-tubercular activity assay

All the novel AITs were screened for anti-tubercular activity against *M. tuberculosis* H₃₇Rv strain (ATCC 27294) using a microplate Alamar Blue assay (MABA) as described previously [29, 31]. Briefly, 200 μl of sterile deionized water was added to all outer-perimeter wells of sterile 96-well plates to minimize evaporation of the medium in the test wells during incubation. The wells in rows B to G in columns 3 to 11 received 100 μl of Middlebrook 7H9 broth. 100 μl of 2X drug solutions were added to the wells in rows B to G in columns 2. 100 μl was transferred from column 2 to column 3, and the contents of the wells were mixed well. Identical serial 1:2 dilutions were continued through column 10, and 100 μl of excess medium was discarded from the wells in column 10 in order to get the final concentration of 0.2 μg/ml. 100 μl of *M. tuberculosis* inoculum was added to the wells in rows B to G in columns 2 to 11 (yielding a final volume of 200 μl per well). Thus, the wells in column 11 served as drug-free control. The plate was sealed with parafilm and incubated at 37°C for 5 days. Thereafter, 50 μl of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% tween-80 was added to each well and incubated for 24 h. Appearance of blue color was interpreted as no bacterial growth, and pink color was used as indicator of bacterial growth. The inhibitory activity of AITs against *M. tuberculosis* was expressed as the minimum inhibitory concentration (MIC) in μM. MIC was defined as lowest drug concentration which prevented the color change from blue to pink. Pyrazinamide, streptomycin, and ciprofloxacin were used as a positive controls.

Molecular docking analysis

*In silico* molecular docking was performed based on the X-ray structure of *M. tuberculosis* CYP51 in complex with a small molecule inhibitor (PDB: 2CIB) [32]. The ligand structures were energy-minimized and protonated using the MOE platform [33]. The protein was prepared for docking using protonate3D [41]. Afterwards, we docked all AITs to the X-ray structure of using MOE’s default settings for flexible docking. This includes an initial placement using Triangle Matcher, primary scoring via London dG and a forcefield refinement of 30 poses followed by a re-scoring step using GBVI/WSA dG. We visualized predicted protein-ligand interactions with pymol [34].

Anti-microbial activity

The CM237 and akuB strains of *Aspergillus fumigatus*, that expresses CYP51, were used in this work. Fungal strains were grown in Potato Dextrose Agar (PDA, Becton Dickinson, Madrid, Spain) at 37°C. After three days of growth a suspension of spores was prepared by harvesting the surface of the culture with phosphate buffered saline (PBS) plus 0.01% Tween 20. Inoculum size was then adjusted using a hemocytometer chamber according to needs. Stock solutions of each compound were first dissolved in chloroform and subjected to serial dilution to obtain the concentrations of 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 μg/ml. Each compound was tested between 64 μg/ml and 0.125 μg/ml. Compounds susceptibility was determined by broth micro-dilution (BMD) using RPMI 2% glucose. MICs were determined visually and recorded after 48 h. Amphotericin B and chloroform were used as controls of the study.

Conclusion

In this work, we synthesized novel AITs using 1-adamantyl bromomethylketone and 5-alkyl/aryl-2-amino1,3,4-thiadiazoles employing nano-MgO in ionic liquid media. We experimentally confirmed the anti-TB activity of all AITs against *M. tuberculosis* H₃₇Rv strain with MICs in the low micromolar range. Subsequent docking studies revealed sterol 14α-demethylase
(CYP51) as a plausible target, and subsequent activity determination against fungal strains that express sterol 14α-demethylase (CYP51) corroborated this hypothesis. In summary, we herein report the synthesis and anti-TB activity of novel AITs that likely target sterol 14α-demethylase (CYP51) to induce their antimycobacterial activity.

Supporting Information
S1 Data. Graphical abstract which provides the overview of the present work.

S2 Data. Scanned spectral images and structural analysis of novel adamantyl-imidazolo-thiadiazole derivatives.

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Author Contributions
Conceived and designed the experiments: B KSR AB. Performed the experiments: SA BRCP SR SM C SP LM JEF B CDM. Analyzed the data: MM CDM JM AB B KSR. Contributed reagents/materials/analysis tools: AB B KSR MM. Wrote the paper: B AB KSR.

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