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RESEARCH ARTICLE

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Novel fatty acid-thiadiazole derivatives as potential antimycobacterial agents

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Abstract

The discovery of antibiotics around the middle twentieth century led to a decrease in the interest in antimycobacterial fatty acids. In order to re-establish the importance of naturally abundant fatty acid, a series of fatty acid-thiadiazole derivatives were designed and synthesized based on molecular hybridization approach. In vitro antimycobacterial potential was established by a screening of synthesized compounds against *Mycobacterium tuberculosis* H37Rv strain. Among them, compounds **5a**, **5d**, **5h**, and **5j** were the most active, with compound **5j** exhibiting minimum inhibitory concentration of 2.34 μg/ml against M.tb H37Rv. Additionally, the compounds were docked to determine the probable binding interactions and understand the mechanism of action of most active molecules on enoyl-acyl carrier protein reductases (InhA), which is involved in the mycobacterium fatty acid biosynthetic pathway.

KEYWORDS

docking, fatty acid, *Mycobacterium tuberculosis*, resazurin microtiter assay, thiadiazole

1 | INTRODUCTION

The emergence of multidrug-resistant and extensively drug-resistant strains of *Mycobacterium tuberculosis* (*Mtb*), together with various other challenges in the development of new antitubercular agents (Gagneux, 2018; Laurenzi, Ginsberg, & Spigelman, 2007), has flooded the drug discovery pipeline with several antituberculosis drugs. Despite this, tuberculosis infection is the tenth leading cause of morbidity and mortality ranking above HIV/AIDS making it major health problem worldwide with a large number of MDR/RR-TB incident cases being in India, China, and the Russian Federation. After more than 50 years, two new drugs bedaquiline and delamanid have recently been approved and released for drug-resistant TB (World Health Organization, 2018). This rising problem of resistance to antituberculosis agents and the current situation can be solved by designing of a new molecular scaffold with the novel mechanism of action and with the ease of tailoring to the medicinal chemist (Patil, Bagade, Bonde, Sharma, & Saraogi, 2018). The molecular hybridization strategy is a rational design of new ligands or prototypes with better efficacy and physicochemical properties (Viegas-Junior, Danuello, da Silva Bolzani, Barreiro, & Fraga, 2007).

The antitubercular effects of free fatty acids on mammalian tubercle bacilli and various species of mycobacteria have been determined historically (Dubos, 1950; Kondo & Kanai, 1972). Before the advent of antibiotics, cod liver (a mixture of saturated and unsaturated fatty acids), chaulmoogra (cyclopentyl alkanoic acids), palm kernel, and turtle oil (10-, 12-, and 14-carbon saturated fatty acids) were used as a remedy for the treatment of tuberculosis (Grad, 2004; Nieman, 1954). Kondo and Kanai in 1972 reported that among saturated fatty acids, lauric and myristic acids were highly active in killing both H37Ra and H37Rv of *Mtb* at 20 μg/ml (Kondo & Kanai, 1972). Later, H Saito *et al.* established the cytotoxicity of a series of fatty acids against seventy-one strains of rapidly growing mycobacteria.

Amidst saturated fatty acid, lauric acid (C12:0) showed the maximum MIC's 6.25–25 μg/ml and capric acid (C10:0) was the next best active with MIC's 50–100 μg/ml. The lethal effects of fatty acids were due to disorganization of the bacterial cell membrane culminating into the change in membrane permeability. Fatty acids are naturally abundant, renewable, biodegradable, biocompatible, and cost-effective (Carballeira, 2008; Muniyan & Jayaraman, 2016; Saito, Tomioka, & Yoneyama, 1984).

These observations rekindled our interest in exploring fatty acids in tuberculosis infection. Fatty acids have high log P, and Rodrigues and Biava et al. demonstrated its importance that high values of log P represent an increase in drug permeability through the lipid-rich mycobacterial cell wall leading to increased antimycobacterial potency (Biava et al., 2006; Rodrigues et al., 2013). However, fatty acids are a less potent surface-active agent as they ionize into anionic form at physiological pH. Various authors have demonstrated the antitubercular effect of various fatty acids alone or in combination with the heterocyclic ring (Figure 1; Chatzipanagiotou et al., 2005; D'Oca et al., 2010; Menendez et al., 2012; Morbidoni et al., 2006; Rodrigues et al., 2013; Venepally & Reddy Jala, 2017). The physicochemical and pharmacokinetic properties of fatty acids can be improved by merging it with heterocycles or any molecule with relatively lower lipophilicity. Azaheterocycle derivatives have been widely explored as antimycobacterial agents (Danac & Mangalagiu, 2014; Mantu, Luca, Moldoveanu, Zbancioc, & Mangalagiu, 2010; Olaru, Vasilache, Danac, & Mangalagiu, 2017). Out of them 1, 3, 4-thiadiazoles are well-known privileged structures with remarkable pharmacological activities such as antimicrobial, antituberculosis (Figure 1), antioxidant, anti-inflammatory, anticonvulsants, antidepressant, anxiolytic, antihypertensive, anticancer, and antifungal activity. The biological activity of thiadiazoles is attributed to their meso-ionic nature and liposolubility which makes them capable of crossing the cellular membranes (Haider, Alam, & Hamid, 2015; Hu, Li, Wang, Yang, & Zhu, 2014; Jain, Sharma, Vaidya, Ravichandran, & Agrawal, 2013; Krátký & Vinsova, 2016). Keeping in perspective the promising antimycobacterial activity of 1, 3, 4-thiadiazoles and fatty acids, we designed novel series of mycobacterium inhibitors by merging medium (lauric acid) and long-chain fatty acids (myristic acid) with 1, 3, 4-thiadiazole through molecular hybridization technique and their antitubercular activity was evaluated against *Mycobacterium tuberculosis* H37Rv strain using resazurin microtiter plate assay (REMA; Figure 2). We speculated that fatty acid-thiadiazole derivatives might inhibit fatty acid biosynthesis, as mycolic acid, the major component of the cell wall of mycobacteria, is made from FabH (β-ketoacyl carrier protein synthase III) that catalyzes the extension of fatty acids such as lauroyl, myristoyl, and palmitoyl groups (Morbidoni et al., 2006). Based on similarities in the structure, we docked these molecules on InhA (enoyl-acyl carrier protein enzyme) to get insights into their interaction profiles and mechanism of action.

2 | METHODS AND MATERIALS

All the chemicals were purchased from Sigma-Aldrich and S. D. Fine Chemicals, India. ^{1}H NMR was recorded on 400 MHz and ^{13}C NMR at 101 MHz on Agilent Technology MR400 spectrometer. Merck silica gel 60 F-254 aluminum

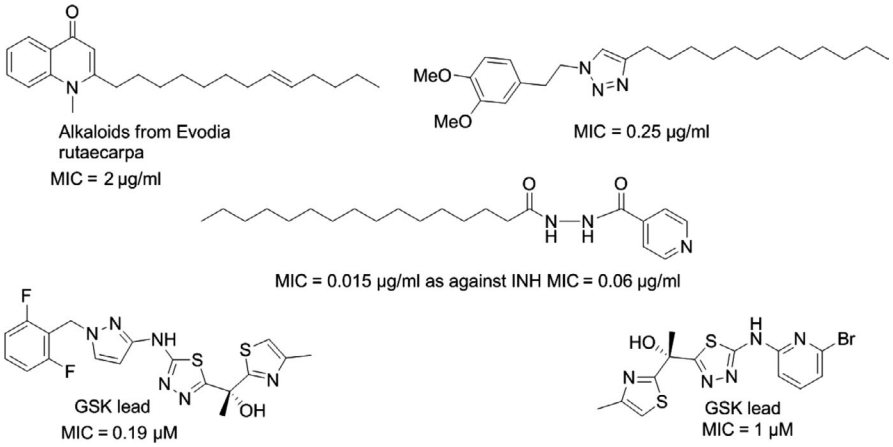


FIGURE 1 Fatty acid and thiadiazole derivatives with antimycobacterial activity

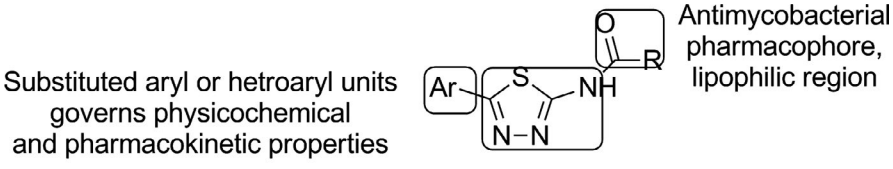


FIGURE 2 Pictorial representation of designed fatty acid derivatives

sheets were used for analytical thin-layer chromatography (TLC), and silica gel 60–120 mesh was used for column chromatography. Mass spectra were recorded by Shimadzu instrument in electrospray ionization in both positive and negative modes.

2.1 | General procedure for the synthesis of compounds 2a-k

A mixture of appropriate carboxylic acid (1.0 g, 0.1 mol), thiosemicarbazide (0.1 mol), and $POCl_3$ (0.3 mol) was heated to 75°C for 0.5 hr. The reaction mixture was cooled to room temperature, and then, water (9 ml) was added to it, and the mixture was refluxed for a further 4 hr. The mixture was cooled and basified to pH 8 with 50% NaOH solution. The mixture was filtered, and the residue was recrystallized from ethanol to afford the corresponding compounds characterized by melting point similar to reported compounds (Guan et al., 2014).

2.2 | General procedure for the synthesis of compound 1k

To a solution of 4-fluorobenzoic acid (5 g, 0.04 moles) in ethanol (50 ml), concentrated sulfuric acid (5 ml) was added, and the reaction mixture was refluxed for 16 hr. The reaction was monitored by TLC. After the consumption of starting material, the solvent was evaporated under reduced pressure. Saturated $NaHCO_3$ was added, and the resultant solution was extracted with ethyl acetate. The organic layer was washed with water and then with brine solution. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to obtain the desired product (ii) as yellow oil matched with the previously reported results (90%). A reaction mixture of ethyl 4-fluorobenzoate (1.00 g, 5.95 mmol), phenol (1.11 g, 6.55 mmol), and dry K_2CO_3 (1.64 g, 11.9 mmol) in DMSO (8 ml) was heated at 110°C for 18 hr. The mixture was poured into water and extracted with EtOAc several times. The organic layer was washed with water and brine and dried over anhyd·Na_2SO_4. The solvent was evaporated in vacuo, and the residue was purified by silica gel column chromatography (*n*-hexane/AcOEt) to give 1.47 g of iii (78% yields), to obtain colorless oil in 78% yield (boiling point = 150–152°C; Li et al., 2015; Palmer et al., 2015).

Ethyl 4-phenoxybenzoate (iii) (1.85 mmol), sodium hydroxide (80 mg, 2.0 mmol) in ethanol (4 ml), and water (4 ml) were stirred under reflux for 3 hr. The reaction mixture was allowed to cool to ambient temperature and concentrated in vacuo. The residue was partitioned between ethyl acetate (3 × 10 ml) and water (20 ml). The aqueous layer was separated and made acidic with 2 M HCl and then extracted with dichloromethane (3 × 10 ml). The organic layer was washed with brine (20 ml), dried over Na_2SO_4, and then evaporated under reduced pressure to give compound **1k** (yield 75%) as a white solid with melting point 159–161°C matched with literature was used directly for the next step without further purification (Yang et al., 2012).

2.3 | General procedure for the synthesis of compounds 4a and 4b

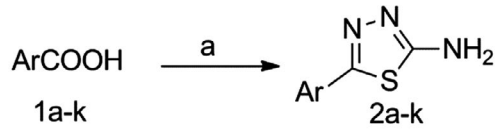
Dodecanoic (**3a**)/tetradecanoic acid (**3b**) (1g, 1.0 eq.) was dissolved in dry chloroform (20 ml/g), and a catalytic amount of DMF was added followed by slow addition of thionyl chloride (1.2 eq.). The reaction mixture was heated under reflux for 4 hr. The solvent was removed evaporated in vacuo to get the crude acid chlorides. The crude acid chlorides (colorless liquids) were used directly for next step without further purification (García-Barrantes et al., 2013).

2.4 | General procedure for the synthesis of compounds 5a-k and 6a-k

Thiadiazole amines (1 equiv) were dissolved in pyridine (20 ml/g) and stirred at 0°C under an inert atmosphere. After 15 min, 1.2 equiv of dodecanoyl/tetradecanoyl chloride was added to the above stirred solution. The mixture was stirred at room temperature for 4 hr, and completion of the reaction was monitored by TLC. After completion of the reaction, the reaction mixture was diluted with water and ethyl acetate followed by the addition of 2 N HCl to neutralize the mixture. The precipitate obtained was then washed with water and stirred in saturated bicarbonate solution to remove acid impurities. (Wherever the residue was soluble in the organic layer, the organic layer was washed with water and saturated bicarbonate solution sequentially. The organic layer was dried over anhydrous Na_2SO_4, and then evaporated under reduced pressure to afford corresponding amides.) The crude compounds were purified either by column chromatography or by recrystallization from chloroform and methanol. The compounds were characterized by melting point, ^{1}H NMR, ^{13}C NMR, and mass spectrometer (Figures S3–S53).

2.5 | In vitro antitubercular activity

Resazurin microtiter assay protocol (Palomino et al., 2002) was used to determine the minimum inhibitory concentration (MIC) of all the synthesized *N*-(5-aryl-1, 3, 4-thiadiazole-2-yl) alkanamide derivatives **(5a–5k** & **6a–6k)**. The synthesized compounds were screened against *Mtb* H37Rv 25177 using serial dilution technique in Middlebrook 7H9-S broth medium. *Mtb* H37Rv was grown in media till the cells reached mid log phase. Each compound (2 mg) was dissolved in 1 ml of DMSO. The serial dilution of each compound was prepared using 96-well microtiter plate and



SCHEME 1 Synthesis of unsubstituted and substituted 5-aryl-1, 3, 4-thiadiazol-2-amine derivatives (**2a–k**)

1a, Ar= C_6H_5 ; 1b,Ar= 4-FC_6H_4;
1c, Ar= 3-$NO_2C_6H_4$; 1d,Ar= 3,5$(NO_2)_2C_6H_3$;
1e, Ar= 3,4-$(OCH_3)_2C_6H_3$; 1f,Ar= 4-$CH_3C_6H_4$;
1g, Ar= 3-Br,4-$OCH_3C_6H_3$;1h,Ar= C_6H_5CH=CH;
1i, Ar= 2-ClC_6H_4 ; 1j,Ar= C_5H_4N
1k, Ar= $C_6H_5OC_6H_4$

Reagents (a) Thiosemicarbazide, $POCl_3$, 75°C, 0.5h then H_2O, reflux 4h

100 µl of *Mtb* H37Rv cell suspension in nutrient media was added to each well. The pellicle was vortexed with glass beads in sealed tube in Middlebrook 7H9broth to obtain homogeneous suspension. The supernatant formed after standing the suspension for 15 min was taken in fresh tube, and optical density at 540 nm was adjusted to 0.1 (~10^7 cells/ml). 100 µl of *Mtb* H37Rv culture suspension was seeded in 96-well microtiter plates at a density of 10^4 cells per well and serially diluted with compounds to a final volume of 200 µl. Equivalent amount of DMSO was to the controls instead of compounds. The plates were incubated at 37°C for 7 days, after that 30 µl of the resazurin dye (0.02% w/v dissolved in distilled water) was added to all the wells and, the plates were again sealed and re-incubated for 48 hr. The MIC values were calculated by visual inspection for each well displaying change in color of the resazurin from blue to pink. Isoniazid (INH) was used as the standard drug.

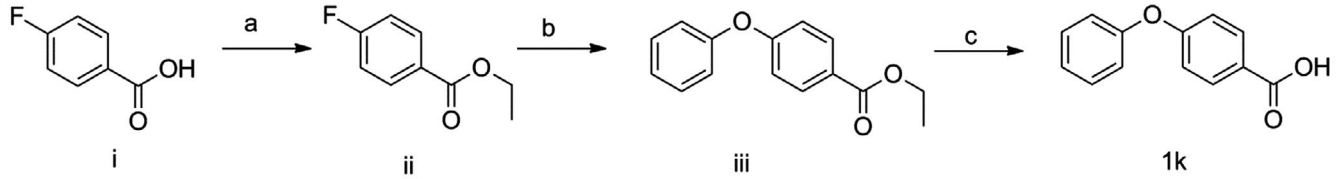
2.6 | Docking studies and in silico ADME prediction

The docking studies were performed using Glide module (version 7.1, Schrödinger, LLC, NY) installed on Linux workstation, and in silico ADME was predicted by Qikprop module as described in the Supplementary Information.

3 | RESULTS AND DISCUSSION

3.1 | Chemistry

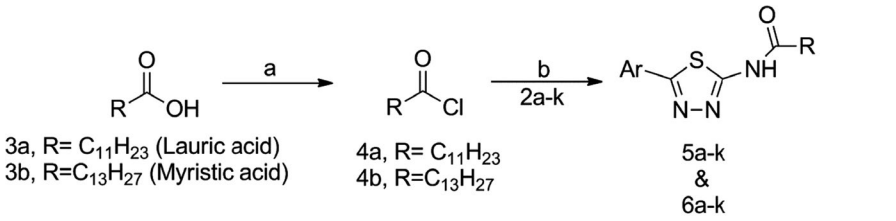
The novel series of *N*-(5-aryl-1, 3, 4-thiadiazole-2-yl) alkanamide derivatives were synthesized according to Schemes 1, 2 and 3. The intermediates, 5-sub and unsub aryl-1, 3, 4-thiadiazol-2-amine, were synthesized as depicted in Scheme 1. Acylthiosemicarbazides were prepared in situ by heating the carboxylic acid and thiosemicarbazide in the acidic medium and cyclized subsequently. Various aryl and heteroaryl acid were reacted with thiosemicarbazide in the presence of $POCl_3$ to form in situ 2-acyl thiosemicarbazides which later underwent cyclodehydration to afford corresponding amine intermediates. **1k** was synthesized by esterification of 4-flourobenzoic acid followed by nucleophilic aromatic substitution of ethyl-4-flourobenzoate with phenol in the presence of K_2CO_3 as a base in DMSO at 110°C; then, subsequent hydrolysis of the ester with aqueous NaOH gave the 4-phenoxybenzoic acid (**1k**) as depicted in Scheme 2. As described in Scheme 3, the lauric and myristic acid was treated with thionyl chloride to obtain the corresponding acyl chloride and was reacted with various amines, **2a–k** in the presence of pyridine to afford the target compounds, **5a–k**, and **6a–k** (Scheme 3). The structures of **5a–k** and **6a–k** were characterized by ^{1}H NMR, ^{13}C NMR, and mass spectroscopic analysis. In ^{1}H NMR spectra, the aromatic protons resonated at δ



Reagents (a) EtOH, conc.H_2SO_4 (cat.), 80°C, 16h; (b) Phenol, K_2CO_3, DMSO, 110°C, 18h; (c) NaOH, EtOH/H_2O, reflux 3h

SCHEME 2 Synthesis of 4-phenoxybenzoic acid (**1k**)

SCHEME 3 Synthesis of *N*-(5-aryl-1, 3, 4-thiadiazole-2-yl) alkanamide derivatives (**5a–k**) and (**6a–k**)



Reagents (a) $SOCl_2$, dry $CHCl_3$, reflux 4h; (b) amines 2a-k, pyridine at 0°C to r.t, 4h

TABLE 1 Antitubercular activity of *N*-(5-aryl/heteroaryl)-1, 3, 4-thiadiazol-2-yl) dodecanamide derivatives

Compound	Ar	QPlogPo/w[a]	Mol. weight	MIC in µg/ml (µM)
5a	Ph	5.393	359.529	4.69 (13.04)
5b	4-F-Ph	5.633	377.519	150 (397.32)
5c	3-NO_2-Ph	4.686	404.526	9.38 (23.18)
5d	3,5-diNO_2-Ph	3.987	449.524	4.69 (10.43)
5e	3,4-diOMePh	5.52	419.581	18.75 (44.67)
5f	4-MePh	5.749	373.555	18.75 (50.1)
5g	3-Br-4-OMePh	6.094	468.451	9.38 (20.02)
5h	C_6H_5-CH=CH-	6.043	385.566	4.69 (12.16)
5i	2-Cl-Ph	5.836	393.974	75 (190.37)
5j	4-pyridinyl	4.427	360.516	2.34 (6.49)
5k	Ph-O-Ph	7.018	451.626	37.5 (83.03)

[a]Determined by QikProp analysis.

TABLE 2 Antitubercular activity of *N*-(5-aryl/heteroaryl)-1, 3, 4-thiadiazol-2-yl) tetradecanamide derivatives

Compound	Ar	QPlogPo/w[a]	Mol. weight	MIC in µg/ml (µM)
6a	Ph	6.172	387.582	9.38 (24.20)
6b	4-F-Ph	6.409	405.573	>150 (>369.84)
6c	3-NO_2-Ph	5.456	432.58	18.75 (43.34)
6d	3,5-diNO_2-Ph	4.744	477.577	9.38 (19.64)
6e	3,4-diOMePh	6.307	447.635	37.5 (83.77)
6f	4-MePh	5.749	401.609	37.5 (93.37)
6g	3-Br-4-OMePh	6.852	496.504	9.38 (18.89)
6h	C_6H_5-CH=CH-	6.815	413.62	18.75 (45.33)
6i	2-Cl-Ph	6.615	422.027	>150 (>355.43)
6j	4-pyridinyl	5.185	388.57	18.75 (48.25)
6k	Ph-O-Ph	7.686	479.679	75 (156.34)
INH	—	—		0.4 (2.91)
Rifampicin	—	—		0.60 (0.729)

[a]Determined by QikProp analysis.

7.10–9.50 ppm. Resonance signals of the aliphatic regions were seen in the range of δ 0.88–2.37 ppm. The terminal methyl group was seen at 0.85–0.89 ppm. Broad singlet between δ 12.0 and 13.5 ppm confirmed the presence of proton of -CONH group. In ^{13}C NMR spectra, aromatic carbon resonated between δ 111 and 160 ppm with many overlaps in these regions, while the C=O carbon resonated around δ171–173 ppm. The C-5 of the 1, 3, 4-thiadiazole nucleus appeared downfield in the NMR spectra in comparison to C-2 of the ring. Further structural confirmation was done using mass spectrometric data analysis (Supplementary Information).

3.2 | Biological Screening

All 22 novel, *N*-(5-aryl-1, 3, 4-thiadiazole-2-yl) alkanamide derivatives were evaluated in a whole-cell assay against *Mtb* strain H37Rv using REMA (Palomino et al., 2002). The

synthesized compounds exhibited interesting activity with MIC ranging from 2.34 to 150 μg/ml against *Mtb* H37Rv, as seen in Tables 1 and 2. INH was used as a standard drug. The results of in vitro antimycobacterial evaluation revealed that substitution at the *para* position with electron withdrawing group such as fluoro was deleterious for the activity (**5b**, **6b**). Introduction of the weaker electron-releasing group such as methyl at the *para* position of phenyl ring (**5f**) displayed a decrease in the potency as compared to the unsubstituted phenyl ring in dodecanamide series (**5a**). Similarly, in tetradecanamide series, methyl substituted ring (**6f**) was least active as compared to its unsubstituted counterparts (**6a**). The presence of bulkier groups at *para* position resulted in compounds with reduced antitubercular activity in both the series (**5k**, **6k**) may be due to high steric hindrance. Halogen substitution at *ortho* position was unfavorable in both dodecanamide and tetradecanamide series (**5i, 6i**). In general, halogen substitution was detrimental for the activity. Substitution at *meta* position with nitro was favorable for activity (**5c**, **6c**). Disubstitution at 3, 5 positions with electron withdrawing group such as nitro showed an improved biological profile as compared to monosubstituted nitro at 3-position indicating that the presence of both nitro groups in dodecanamide series is requisite for the high efficacies (**5d**, **6d**). Disubstitution at 3, 4 positions with one electron withdrawing and one electron donating have shown equal potency in dodecanamide (**5g**) and tetradecanamide (**6g**) series and showed better activity as compared to electron donating groups. (**5e**, **6e**) Disubstitution was favorable over monosubstitution. Styryl group in case of dodecanamide (**5h**) series exhibited good antitubercular activity as compared to tetradecanamide series (**6h**). Replacement of phenyl ring with heteroaryl ring such as 4-pyridinyl resulted in compounds with the highest antimycobacterial activity in dodecanamide series (**5j**, **6j**). The above finding revealed that the potency decreased with the increase in the carbon chain. Overall, dodecanamide series displayed greater antitubercular potency as compared to tetradecanamide series.

3.3 | Molecular docking studies

The mycobacterial cell wall biosynthetic pathway (FAS II) is distinct from mammalian (FAS-I) multienzyme complex. So inhibition of InhA enzyme, involved in mycobacterium cell wall biosynthesis, is an attractive target. As per previous reports on the antitubercular activity of some alkanamide and thiadiazole derivatives (Martínez-Hoyos et al., 2016; Saha, Alam, & Akhter, 2015; Shirude et al., 2013; Šink et al., 2015), molecular docking was carried out on the basis of the similarity of the structures with the bound ligand in the target. All the molecules were docked into the active site of the crystal structure of enoyl-ACP reductase (5JFO) target enzymes to determine the possible mode of action and binding orientations, as seen in Figures S1 and S2.

3.4 | In Silico ADME prediction

Fatty acids being lipophilic imparted distinct lipophilicity to the designed molecules and hence imparted distinct MIC's. QikProp analysis data reveal that three compounds follow the Lipinski rule of five, and others have varying Log *P* values which range from 3.987 to 7.686 (Table S1), as increase in the lipophilicity could ease the entrance of these molecules through the lipid-enriched bacterial membrane (Biava et al., 2008; Navarrete-Vázquez et al., 2007).

4 | CONCLUSION

In summary, a new series of *N*-(5-aryl-1, 3, 4-thiadiazole-2-yl) alkanamides were designed successfully through molecular hybridization approach based on the inherent antitubercular activity of abundant fatty acids. The synthesized derivatives exhibited good to moderate in vitro inhibitory activity against *Mtb* H37Rv strain (ATCC 25177). These fatty acid-thiadiazole derivatives were synthesized from readily accessible reactants and reagents by simple and efficient synthetic protocols. Out of 22 fatty acid-thiadiazole derivatives, compounds **5a**, **5d**, and **5h** showed in vitro inhibitory activity with MIC 4.69 μg/ml and **5j** with 4-pyridinyl moiety displayed maximum *Mtb* inhibitory activity with MIC 2.34 μg/ml. The structure–activity relationship revealed that electron withdrawing group such as nitro on phenyl ring (mono- and disubstitution) enhanced the inhibitory activity of the compounds as compared to electron donating groups. Lauric acid derivatives showed higher activity as compared to myristic acid derivatives. SAR revealed that the increase in log *P* did not improve the inhibitory activity toward mycobacteria. Additionally, docking and MM-GBSA studies on the enzyme InhA were carried out with the most active molecules to examine the putative interactions responsible for biological activity. Docking studies revealed that compounds **5a** and **5j** bind to the enzyme InhA. However, the biological target for compounds **5d** and **5h** needs to be defined. Further studies are underway to improve antitubercular potency and to elucidate the precise mechanism of action.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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