Synthesis, *In Vitro* and *In Silico* Molecular Docking Studies of Novel Phthalimide–Pyrimidine Hybrid Analogues to Thalidomide as Potent Antitubercular Agents

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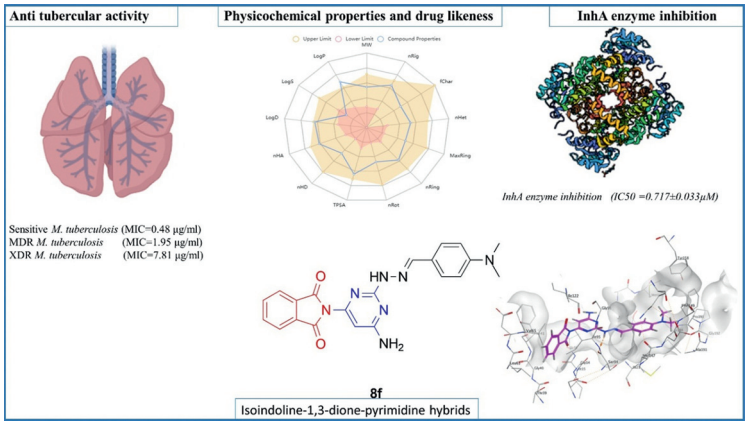

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Abstract Condensation reaction of aromatic aldehydes with 2-(6-amino-2-chloropyrimidin-4-yl)isoindoline-1,3-dione and 2-(6-amino-2-hydrazineylpyrimidin-4-yl)isoindoline-1,3-dione afforded 2-(2-chloro-6-((3 alkylbenzylidene)amino) pyrimidin-4-yl)isoindoline-1,3-dione derivatives **6a–f** and 2-(6-amino-2-(2-(arylidene)hydrazinyl)pyrimidin-4-yl)isoindoline-1,3-dione derivatives **8a–f**, respectively, as phthalimide–aminopyrimidine hybrids. The compounds showed a wide range of antitubercular activities against sensitive MDR and XDR *M. tuberculosis* strains, with **8f** and **6a** showing the highest activity; these compounds inhibited sensitive *M. tuberculosis* with MIC of 0.48 and 0.98 µg/mL, respectively, comparable to isonizide (INH) (MIC = 0.12 µg/mL). Both **8f** and **6a** inhibited the MDR strain with MIC of 1.95 and 7.81 µg/mL, respectively, and XRD with MIC of 7.81 and 15.63 µg/mL, respectively. Both **8f** and **6a** inhibited mycobacterial InhA enzyme *in vitro* (IC_{50} = 0.717± 0.033 µM and 1.646± 0.069 µM, respectively). Molecular docking simulations revealed that **8f** and **6a** were also capable of interacting at the catalytic site of the InhA enzyme via binding with NAD^+ and Tyr158, in a manner similar to that of the native ligand. Compounds **6a** and **8f** exhibited physicochemical properties of oral bioavailable drug-like compounds with high gastrointestinal absorption. Predictions showed that the compounds are unlikely to have side effects on the CNS and no anticipated hepatotoxicity, mutagenicity, or acute oral toxicity was identified in models.

Key words *Mycobacterium tuberculosis*, ADMET studies, docking simulation, enzyme inhibition, phthalimide–pyrimidine hybrids

Introduction

Tuberculosis (TB) is a serious lower respiratory tract infection caused by the bacteria *Mycobacterium tuberculosis*. The infections can be accompanied by other extra-pulmonary infections in the skin, brain, and lymph nodes.[1] The standard four-drug regimen (first-line drugs) for drug-sensitive TB includes isoniazid, rifampicin, ethambutol, and pyrazinamide, which must be taken for at least six months.[2] However, due to the emergence of both multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) strains, tuberculosis is still regarded by the World Health Organization (WHO) as a dangerous re-emerging illness with a high fatality rate and remains a major cause of death for patients with autoimmune disease.[3] Discovering potent and efficient antitubercular medications that can eradicate mycobacteria, prevent treatment resistance, and minimise illness recurrence is essential.[4] Due to its ability to interfere with lipid biosynthesis, the unique lipid cell wall of *M. tuberculosis*—which is necessary for its survival—also renders it susceptible to several treatment drugs.[5] *M. tuberculosis* enolyl acyl carrier protein reductase is a key enzyme that antitubercular medications target because it is essential for the biosynthesis of type II fatty acids (FASII), which is only produced by bacteria and not by humans.[6,7] By reducing the *trans* double bond, InhA covalently links an intermediate to an acyl carrier protein through its carbonyl group. A number of antitubercular medications, such as isoniazid, target InhA (INH). The cre-

ation of novel therapeutic drugs continues to focus on targeting InhA.[8] Isoniazid (INH), a first-line anti-TB drug, targets inhA through its activated metabolites. Despite its activity, INH shows high toxicity and side effects due to its nitrogen-centred free radicals (hydrazine metabolites) that can generate reactive oxygen species that induce high levels of lipid peroxidation, with consequent cell death and hepatic necrosis. Numerous types of InhA inhibitors have been developed, including diphenyl ether,[9] pyrrolidine carboxamide,[10] hydroxyl pyridines,[11] tetrahydropyran,[12] and 1,3,4-oxadiazole derivatives.[13]

Recently, thiazolidin-4-one-thiazole hybrids have been shown to have potent antitubercular activity comparable to that of INH.[14] Phthalimide is considered a scaffold for antitubercular activity.[15] Compounds **I** and **II** (Figure 1) demonstrated antitubercular action against sensitive *M. tuberculosis* strains, with MICs in the micromolar range; these compounds might interact with the mycobacterial InhA active site.[16] Pyrimidine is also considered a core scaffold with antitubercular activity.[17,18] As shown in Figure 1, many pyrimidine-containing compounds are being investigated in clinical trials (GSK286, TBA7371). Ceritinib is a pyrimidine core with confirmed antimycobacterial activity. Iclaprim and trimethoprim are examples of antibacterial drugs that are being considered for the treatment of mycobacterial infections.[19] The hybrid compounds (phthalimide/naphthalimide and dihydropyrimidinone) that contain these two essential heterocyclic scaffolds may exhibit significant or influential therapeutic potential.[20–22]

Arylamides constitute a general class of InhA inhibitors with features that accommodate the structural requirements for interaction at the InhA binding site, as shown in Figure 2. Arylamides have a broad range of antitubercular activity, with MIC values in the micro- to nonamolar range. Structurally, polyamides are composed of ring **A**, with the amide linker, which plays a role in the interaction at the catalytic site, forming an H-bond interaction at Tyr158, and other amino acids together with an H-bond interaction with the 2′-OH of the NAD cofactor at the InhA catalytic site (Site I). Ring **B** plays a role in the hydrophobic interaction at the hydrophobic site of the enzyme (Site II), while ring **C** is important for interaction at the exposed site (Site III), being surrounded by polar and non-polar groups at the active site of the enzyme.[23] In the present investigation, structural modifications of arylamide InhA inhibitors were performed to provide phthalimide–pyrimidine hybrids in which the phthalimide takes the place of ring **A** together with amide interaction via H-bonding at the catalytic site (Site I), and pyrimidine directly attached to phthalimide takes the place of ring **C** to maintain suitable distances. Ring **C** can accommodate small groups at position 2, such as a nonpolar chlorine group, or at position 6, such as a polar amino group, to allow for interaction at the solvent-exposed site (Site II). Different substituted benzene rings were used on ring **B** to interact at the hydrophobic site (Site II); they were introduced at the pyrimidine core either at position 6 through the methylene amino linker or at position 2 through the methylenehydrazinyl linker to afford 2-(2-chloro-6-((3-alkylbenzylidene)amino)pyrimidin-4-yl)isoindoline-1,3-dione

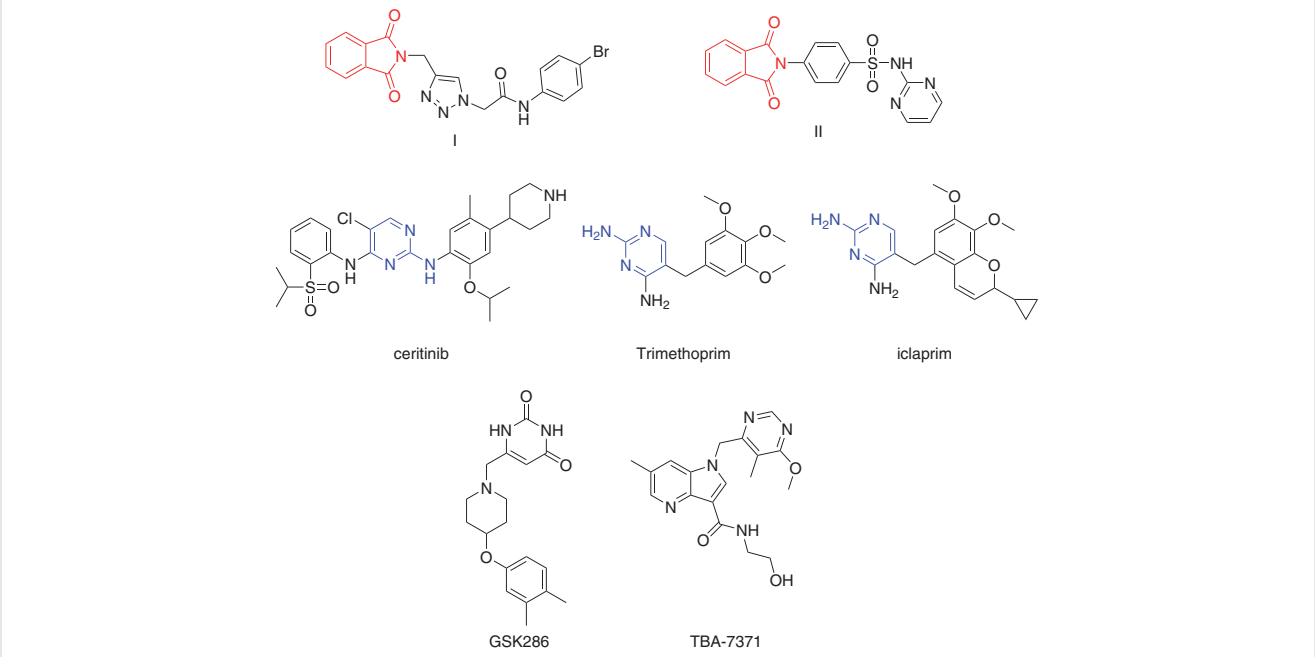


Figure 1 Reported antitubercular compounds

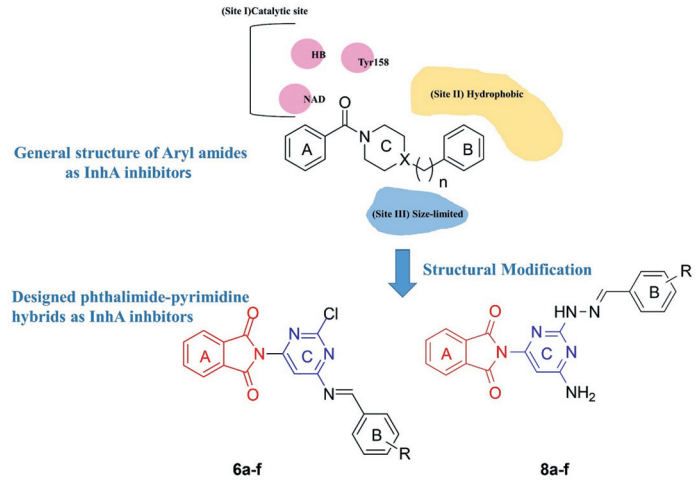


Figure 2 Designed phthalimide–pyrimidine hybrids

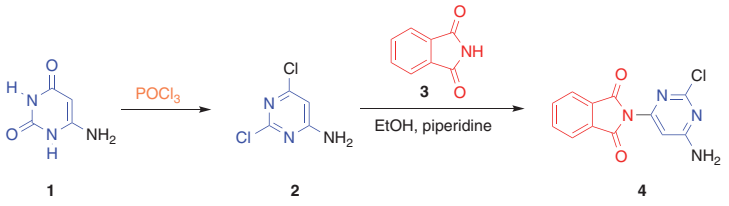
derivatives **6a–f** and 2-(6-amino-2-(2-(arylidene)hydrazinyl)pyrimidin-4-yl)isoindoline-1,3-dione derivatives **8a–f**, respectively, as shown in Figure 2. The designed compounds were screened for their antitubercular activity against sensitive, multi-drug-resistant (MDR), and extra-drug-resistant (XDR) *M. tuberculosis* strains compared to INH. Compounds were also screened for their *in-vitro* inhibition of mycobacterial InhA enzyme compared to IHN. Molecular docking simulations were used to study the binding mode of synthesised compounds at the binding site of InhA enzyme.

Results and Discussion

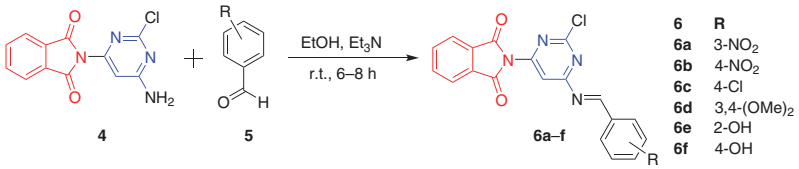
Chemistry

In a continuation of our research interest concerning the synthesis of nitrogen heterocycles and their potential application as anticancer agents[24–26] the phthalimide–pyrimidine hybrids **6a–f** and **8a–f** were prepared from 2-(6-amino-2-chloropyrimidin-4-yl)isoindoline-1,3-dione (**4**). The pyrimidinylisoindolinedione **4** was obtained from readily available 6-aminouracil (**1**), according to our recent study.[27,28] Initially, chlorination of **1** with phosphorus oxychloride ($POCl_3$) yielded 6-amino-2,4-dichlorouracil (**2**), which, on refluxing with phthalimide (**3**) in piperidine as a basic medium, produced **4** (Scheme 1). The structure of **4** was established by ^{1}H NMR spectroscopic analysis, which revealed singlet signals at δ = 5.51 and 6.39 ppm, assigned to the CH-5 and NH_2-6 units of the pyrimidine ring, respectively.

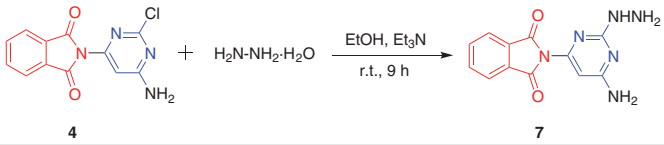
The base-mediated condensation of aminopyrimidine derivative **4** with different aromatic aldehydes **5** (namely, 3-, 4-nitro-, 4-chloro-, 3,4-dimethoxy-, 2-, and 4-hydroxybenzaldehydes) in ethanol at room temperature for 6–8 h, led to formation of the corresponding Schiff bases **6a–f** in good to excellent yields (Scheme 2). The chemical structures of **6a–f** were confirmed by IR, ^{1}H NMR, and ^{13}C NMR spectroscopy and mass spectrometry. IR spectra of **6a–f** showed no stretching frequencies for NH_2, while absorption bands were observed at 2849–2991 cm^{-1} assigned to N=CH aliphatic moieties. In addition, the ^{1}H NMR spectra (DMSO-d_6) of **6a–f** showed no signals for NH_2 but exhibited a singlet signal at δ = 8.24–9.89 ppm characteristic of an azomethine proton N=CH. The geometric isomerism of Schiff bases is dependent on the imine structure (C=N bond). The *E*-isomer (*trans*) is often more stable than the *Z*-isomer (*cis*) for Schiff bases that have a double bond. Steric effects, which lead to the bulky substituents being positioned farther apart in the *E*-isomer to reduce repulsion, are the primary cause of this difference.



Scheme 1 Synthesis of 2-(6-amino-2-chloropyrimidin-4-yl)isoindoline-1,3-dione (**4**)



Scheme 2 Synthesis of 2-(2-chloro-6-((3-alkylbenzylidene)amino)pyrimidin-4-yl)isoindoline-1,3-dione derivatives **6a–f**



Scheme 3 Synthesis of 2-(6-amino-2-hydrazineylpyrimidin-4-yl)isoindoline-1,3-dione **7**

On the other hand, chloropyrimidine derivative **4** undergoes a nucleophilic substitution reaction by hydrazine hydrate in ethyl alcohol in the presence of a catalytic amount of triethylamine at room temperature for 9 h, giving 2-hydrazino derivative **7** in good yield, as shown in Scheme 3. IR spectrum of **7** showed three absorption peaks at 3320, 3290, 3165 cm^{-1} for NH_2/NH. The chemical structure of **7** was further confirmed by ^1H NMR spectroscopy, which showed a singlet signal at δ = 5.49 ppm characteristic for the -CH of the pyrimidine ring, two singlets at δ = 7.88 and 8.08 ppm for 2 × NH_2, and a broad signal at δ = 10.51 ppm for the NH proton.

Finally, the base-catalysed condensation of the 2-hydrazinylpyrimidine **7** with aromatic aldehydes in ethanol at room temperature afforded the respective hydrazones **8a–f** in good to excellent yield, as shown in Scheme 4. IR spectra of **8a–f** showed absorption bands at 2918–2922 cm^{-1} assigned to C–H aliphatic groups, with the other expected absorption peaks for NH_2, NH, and imide carbonyl. In addition, the ^1H NMR spectra of **8a–f** showed a singlet signal at δ = 7.40–8.77 ppm assigned to the CH hydrazone proton, while no hydrazinylamino proton signal was observed.

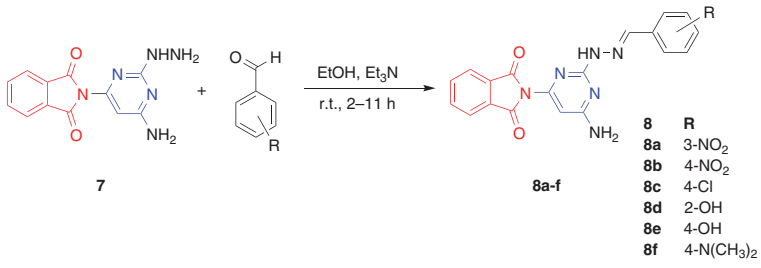
Biological Evaluation

Antitubercular Activity

All newly synthesised compounds were tested for antitubercular activity against *M. tuberculosis* (ATCC 25177 H37Ra), a drug-sensitive strain, using the microplate Alamar blue assay (MABA) and INH as the reference drug. The minimum inhibitory concentration (MIC) anti-mycobacterial activity of **6a–f** and **8a–f** are displayed in Table 1. The compounds showed a wide range of activity, with compounds **8d** and **8e** exhibiting mild antimycobacterial activity against sensitive strains with MIC 125 μg/mL. Compounds **6c**, **6d**, **8b**, and **8c** showed moderate activity with MIC values of 15.63–62.5 μg/mL. Compounds **6b**, **6e**, **6f**, and **8a** were found to be potent antitubercular agents with MIC values in the range of 3.9–7.81 μg/mL. The highest activities against sensitive TB strains were exhibited by **8f** (MIC=0.48 μg/mL) and **6a** (MIC = 0.98 μg/mL).

Antitubercular Activity towards Isoniazid, Cycloserine, Kanamycin, and Rifampin-Resistant *M. Tuberculosis*(ATCC 35822)

Compounds **6a–f** and **8a–f** were further tested for antitubercular activity against the multi-drug-resistance strain (MDR) *M. tuberculosis* ATCC 35822 (isoniazid, cycloserine,



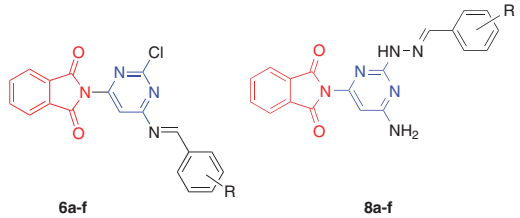
Scheme 4 Synthesis of 2-(6-amino-2-(2-(arylidene)hydrazineyl)pyrimidin-4-yl)isoindoline-1,3-dione derivatives **8a–f**

kanamycin, and rifampin-resistant strain) using the microplate Alamar blue assay. Table 1 displays the MIC antimycobacterial activities of **6a–f** and **8a–f**. Compounds demonstrated a variety of activity from no activity to high potency; compounds **6c**, **8c**, **8d**, and **8e** were inactive, compounds **6b**, **8a**, and **8b** showed mild activity (MIC 31.25–62.5 μg/mL), and compounds **6a**, **6e**, and **6f** were found to be potent with MIC values of 7.81 μg/mL. Compound **8f** showed the highest antimycobacterial activity against the MDR strain, with a MIC of 1.95 μg/mL.

Antitubercular Activity towards Isoniazid, Rifampicin, Ethambutol, Pyrazinamide, Ethionamide, and Moxifloxacin-Resistant *M. Tuberculosis* (RCMB 2674)

The antitubercular activities of the compounds were tested against *M. tuberculosis* (RCMB 2674), an extensively drug-resistant strain (XDR), using a microplate Alamar blue assay with isoniazid, rifampicin, ethambutol, pyrazinamide, ethionamide, and moxifloxacin. Table 1 shows the antimycobacterial activity of **6a–f** and **8a–f** expressed as MIC values. Compounds **6b–d**, **8a**, and **8c–e** were inactive, compounds **6a**, **6e**, **6f**, and **8b** showed moderate activity (MIC 15.63–31.25 μg/mL), and compound **8f** exhibited potent antitubercular action against XDR strain with a MIC of 7.81 μg/mL.

Table 1 Antitubercular Activity of **6a–f** and **8a–f**



6a-f 8a-f

Compd	R	*M. tuberculosis* MIC (μg/mL)[a]		
		Sensitive	MDR	XDR
6a	3-NO_2	0.98	7.81	15.63
6b	NO_2	7.81	31.25	NA
6c	Cl	31.25	NA	NA
6d	3,4-$(OMe)_2$	31.25	125	NA
6e	OH	3.9	7.81	31.25
6f	OH	3.9	7.81	31.25
8a	NO_2	7.81	31.25	NA
8b	NO_2	15.63	62.5	31.25
8c	Cl	62.5	NA	NA
8d	OH	125	NA	NA
8e	OH	125	NA	NA
8f	4-NMe_2	0.48	1.95	7.81
Isoniazid		0.12	IA	NA

[a] MIC = minimal drug concentration required to stop the growth of *M. tuberculosis*. NA= No activity (MIC>125 μg/mL).

***M. Tuberculosis* InhA Enzyme Inhibition Activity**

The InhA enzyme from Mycobacterium TB is necessary for cell-wall metabolism and mycolic acid production. Compounds **8f** and **6a** demonstrated the best antitubercular effectiveness with the least bacterial resistance. To compare **8f** and **6a** to isoniazid (INH) at various doses, their *in-vitro* inhibitory activity against InhA was assessed using a previously described methodology.[29] Table 2 displays the 50% inhibitory concentration (IC_{50}), which is the concentration necessary to inhibit 50% of InhA. Compounds **8f** and **6a** inhibited inhA with IC_{50} values of 0.717 ± 0.033 μM and 1.646 ± 0.069 μM, which were equivalent to the IC_{50} of isoniazid (0.323 ± 0.014 μM).

Table 2 *In-vitro M. tuberculosis* Enoyl-Acyl Carrier Protein Reductase Inhibitory Activity of **8f**, **6a**, and Isoniazid

Compound	InhA IC_{50} (mean ± SD) (μM)
6a	1.646 ± 0.069
8f	0.717 ± 0.033
Isoniazid	0.323 ± 0.014

Molecular Docking Simulation

Several antitubercular medicines, notably the activated version of INH, target the enoyl-acyl carrier protein reductase of *M. tuberculosis*.[30] The binding pocket of the InhA enzyme consists of three main sites: the catalytic site (Site I), including key amino acids Tyr158, 2'-OH of the nicotinamide ribose of the nicotinamide adenine dinucleotide NAD+, and other amino acids; and the hydrophobic region (Site II), which accommodates the substrate binding loop in InhA. It is composed of a number of amino acids, including Met103, Glu104, Phe149, Ala157, Ala198, Met199, Ile202, Ile215, and Leu218; and a size-limiting solvent-exposed site (Site III). It has been discovered that Site I and parts of Site II are occupied by InhA inhibitors. According to the literature,[31] Tyr158 stabilizes substrates during the catalytic reaction of the enzyme, which is important for InhA activity. It was also stated that the interaction of NAD+ with inhibitors is important for activity. Tyr158 exists in two conformations: the IN-conformation and the OUT-conformation. Tyr158 in its IN-form has the ability to bind to inhibitors; the aromatic groups of the inhibitors occupy the hydrophobic site (Site II) together with π-π stacking against Phe149, while the hydroxyl group of the inhibitors is directed towards the inhibitors in the catalytic site (Site I).[30,32] The X-ray crystal of the InhA enzyme has been solved with natural ligand 2-(2,4-dichlorophenoxy)-5-(pyridin-2-ylmethyl)phenol (DCPP) (PDB ID: 3FNE).[33] The native ligand DCPP at the InhA binding site showed H-bond interaction with the key amino acids Tyr158 and NAD^+ at Site I that was occupied by the phenolic ring, the 2,4-dichlorophenyl ring

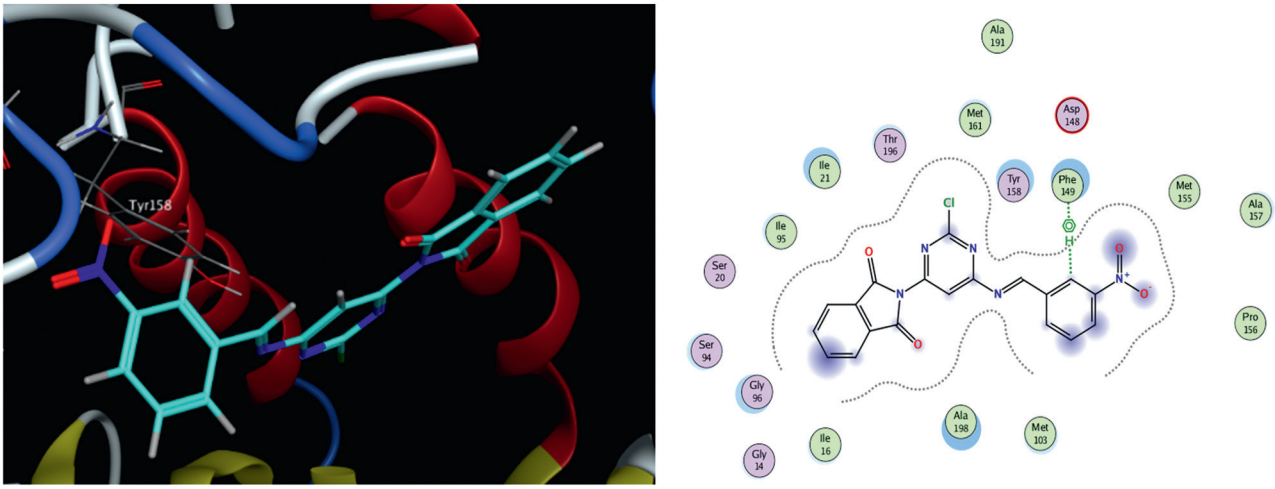


Figure 3 2D and 3D representation of **6a** at the binding site of Mycobacterial InhA (3FNE); **6a** in blue, DCPP in yellow, and NAD in green

occupied Site II and formed hydrophobic interactions at Met199, Ala198, Gly96, and Met161, while the pyridine ring was accommodated at Site III.

MOE 2022.20 was used for docking. To verify the docking procedure, the co-crystallised ligand was re-docked into the InhA enzyme. Between the co-crystallised ligand and docking posture, the computed root means square differences (RMSD) value was less than 2Å. The newly synthesised compounds were docked and docking poses with higher energy scores and a lower RMSD between the docked compound geometry and the co-crystallised native ligand were studied.

Compound **6a** acquired a different orientation to that of **8f**, where the interaction at Tyr158 was afforded by ring **C** (pyrimidine) through N3, while interaction at NAD was afforded via phthalimide carbonyl at Ring A. The hydrophobic interaction at Ala198, Met199 was afforded by ring **C**. Ring B (3-nitrobenze) was oriented towards Site III, as shown in Figure 3.

Compound **8f** showed a mode of interaction similar to that of InhA enzyme inhibitors, where ring A occupied Site II and the carbonyl group of phthalimide formed H-bond interactions at Tyr158 and NAD. Furthermore, the benzene ring of the phthalimide was stacked against Phe 149. Ring B (dimethyl aminobenzene) occupied Site II, forming hydrophobic interactions at Ala198, Met199, Phe97, Gly96, and Met161. Ring B showed stacking against Phe97. Ring C (pyrimidine) occupied Site III (Figure 4).

Drug-Likeness and ADMET Properties *8f 6a*

It is critical to study the physicochemical properties as well as the absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties of drug candidates. Ad-

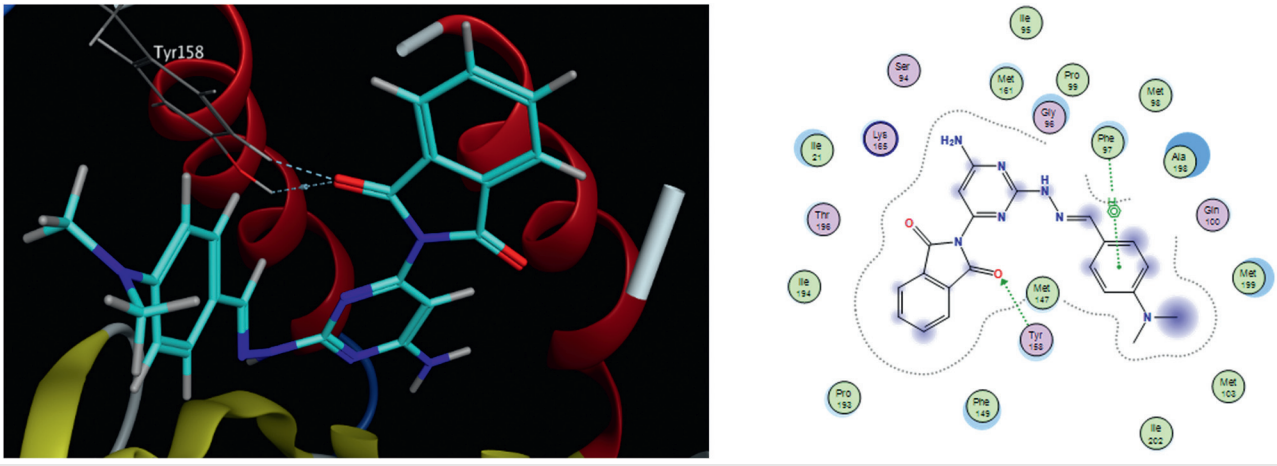


Figure 4 2D and 3D representation of **8f** at the binding site of Mycobacterial InhA (3FNE); **8f** in blue, DCPP in yellow, and NAD in green

metlab 2.0 is an online tool that allows systematic evaluation of ADMET properties, as well as some physicochemical properties. Admetlab 2.0 was used to study the physicochemical properties, drug likeness, and ADMET properties of compounds **8f** and **6a** compared to INH. The generated radar chart for basic physicochemical properties and drug likeness of **8f**, **6a** and INH is shown in the Supporting Information (File S2; Figure 1).

***INH* Studied Parameters for Drug Likeness and Physicochemical Properties**

Table 1 in the Supporting Information (File S2) shows the predicted physicochemical properties of oral bioavailable drug-like compounds 6a and 8f compared to INH. Table 2 in the Supporting Information (File S2) shows the predicted ADMET properties of compounds 6a and 8f compared to INH. Both compounds **6a** and **8f** demonstrated good oral bioavailability, together with good gastrointestinal absorption. Based on the analysis, it is likely that neither compound will exhibit hepatotoxicity or CNS toxicity. Compounds showed no mutagenicity as predicted by the Ames test and no acute oral toxicity in the animal model used in Admet Lab 2.0.

Conclusion

The phthalimide–pyrimidine hybrids demonstrated antitubercular efficacy against MDR, XDR, and sensitive strains of tuberculosis. All compounds showed activity against sensitive *M. tuberculosis* strain with an MIC range of 0.48–62.5 μg/mL. Compounds **6a**, **6b**, **6e**, **6f**, **8a**, **8b**, and **8f** showed antitubercular activity against MDR *M. tuberculosis* strains with an MIC range of 1.95–62.5 μg/mL. Compounds **6a**, **6e**, **6f**, **8b**, and **8f** showed activity against XDR *M. tuberculosis* strains with an MIC range of 7.8–31.25 μg/mL. Compounds **8f** and **6a** had the most potent antitubercular activity against sensitive (MIC 0.48 and 0.98 μg/mL), MDR (MIC 1.95 and 7.81 μg/mL), and XDR (MIC 7.81 and 15.63 μg/mL) *M. tuberculosis* strains. Both **8f** and **6a** inhibited the mycobacterial InhA enzyme with IC_{50}=0.717±0.033 μM and 1.646±0.069 μM, respectively. Molecular docking simulations revealed that **8f** and **6a** interacted with the InhA enzyme in an inhibitory mode, forming H-bond interactions at Tyr158 and NAD^+ at the catalytic site, in agreement with the co-crystallized ligand and other reported inhibitors. Compounds **6a** and **8f** showed physicochemical properties of oral bioavailable drug-like compounds with gastrointestinal absorption. Predictions showed that compounds will have no side effects on the CNS and no anticipated hepatotoxicity. Predictions showed no mutagenicity or acute oral toxicity in models.

All melting points (°C) were measured with a Stuart melting-point apparatus (SMP 30) and are uncorrected. All the reactions were monitored by TLC using plastic sheets pre-coated with silica gel (Merck 60 F254) and spots were visualized by irradiation with UV light (254 nm); the solvent system was chloroform/MeOH (9:1) or EtOAc/MeOH (10:1). IR spectra were recorded with a Pye-Uniearn using the KEr wafer technique and with Beckman spectrophotometers in Zagazig. ^{1}H NMR spectra were recorded with a Bruker 400 MHz spectrometer and ^{13}C NMR spectra were run at 125 MHz in dimethyl sulfoxide (DMSO-d_6) and TMS as an internal standard, at the Applied Nucleic Acid Research Center, Zagazig University, Egypt. Mass spectra were recorded using a Direct Inlet to the mass analyzer with a Thermo Scientific GCMS model ISQ at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Nasr City, Cairo. Microanalysis was carried out at the Microanalytical Center, Mansoura University; the results were within ±0.4% of the theoretical values. All the chemicals and reagents used were purchased from Aldrich Chemicals Co, USA, and other commercial sources.

6-Aminouracil (l)

Compound **1** was purchased from Aldrich Chemical Company Inc. and was also synthesised in our lab. As described previously, ethyl cyanoacetate and urea were heated under reflux in sodium ethoxide and ethanol.[24–26,34]

4-Amino-2,6-dichloropyrimidine (2)

Compound **2** was purchased from Aldrich Chemical Company Inc. and was also synthesised in our laboratory by refluxing 6-aminouracil (**1**) with phosphorous oxychloride, as reported previously.[27,28]

2-(6-Amino-2-chloropyrimidin-4-yl)isoindoline-1,3-dione (4)

A mixture of **2** (1.63 g, 0.01 mol) and phthalimide[35] (**3**) (1.47 g, 0.01 mol) in absolute EtOH (35 mL) in the presence of few drops of piperidine was heated at reflux for 6 h. Once the reaction was finished (TLC), the reaction mixture was cooled and poured onto ice-cold water (50 mL). The resulting solid was filtrated, dried, and recrystallised from methanol.

Yield: 1.60 g (58.3%); yellow solid; mp 117–120 °C.

IR (KBr): 3465, 3211 (NH_2), 3102 (CH aromatic), 1716 (C=O) cm^{-1}.

^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.82 (s, 4 H, Ar-H), 6.39 (s, 2 H, NH_2, exchangeable in D_2O), 5.51 (s, 1 H, CH).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 171.45, 164.26, 155.21, 151.02, 134.34, 132.65, 122.94, 74.08.

MS: *m*/*z* (%) = 276 (2) [M^+ + 2], 274 (14) [M^+], 239 (17), 213 (21), 197 (13), 185 (100).

Anal. Calcd. for $C_{12}H_7ClN_4O_2$: C, 52.48; H, 2.57; N, 20.40. Found: C, 52.58; H, 2.59; N, 20.45.

2-(2-Chloro-6-((3-alkylbenzylidene)amino)pyrimidin-4-yl)isoindoline-1,3-diones 6a–f; General Procedure

Compound **4** (2.74 g, 0.01 mol) and the appropriate aromatic aldehyde (0.01 mol) were combined with abs. EtOH (50 mL) and Et_3N (two drops), and the mixture was stirred at r.t. for 6–8 h. When the reaction (TLC) was complete, the mixture was cooled to r.t. and the solid product was filtered, dried, and recrystallised from ethanol.

)*E*)-2-(2-Chloro-6-((3-nitrobenzylidene)amino)pyrimidin-4-yl)isoindoline-1,3-dione (6a)

The mixture was stirred for 8 h.

Yield: 3.06 g (75%); light-yellow solid; mp 200–202 °C.

IR (KBr): 3079 (CH aromatic), 2922 (CH aliphatic), 1705 (C=O), 1635 (C=N), 1401, 1337 (NO_2) cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 8.68 (s, 1 H, $CH_{azomethine}$), 8.53–8.51 (d, *J* = 7.2 Hz, 1 H, Ar-H), 8.33–8.32 (d, *J* = 7.2 Hz, 1 H, Ar-H), 8.03–8.01 (d, *J* = 7.2 Hz, 1 H, Ar-H), 7.86 (s, 1 H, CH), 7.82 (s, 4 H, Ar-$H_{phthalimide}$), 7.63–7.60 (t, *J* = 8.4 Hz, 1 H, Ar-H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 191.83, 172.83, 169.25, 163.74, 158.08, 147.82, 142.62, 140.95, 137.03, 134.33, 132.61, 130.96, 128.55, 122.94, 99.13.

MS: *m*/*z* (%) = 409 (24) [M^+ + 2], 407 (36) [M^+], 373 (28), 353 (99), 322 (22), 246 (25), 173 (23), 147 (84), 107 (11), 79 (19), 43 (100).

Anal. Calcd. for $C_{19}H_{10}ClN_5O_4$: C, 55.97; H, 2.47; N, 17.18. Found: C, 55.99; H, 2.50, N, 17.25.

(*E*)-2-(2-Chloro-6-((4-nitrobenzylidene)amino)pyrimidin-4-yl)isoindoline-1,3-dione (6b)

The mixture was stirred for 6 h.

Yield: 3.48 g (85.4%); light-yellow solid; mp 180–182 °C.

IR (KBr): 3078 (CH aromatic), 2869 (CH aliphatic), 1679 (C=O), 1624 (C=N), 1513, 1345 (NO_2) cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 9.46 (s, 1 H, $CH_{azomethine}$), 8.43–8.40 (d, *J* = 8.8 Hz, 1 H, Ar-H), 8.24–8.22 (d, *J* = 8.8 Hz, 1 H, Ar-H), 8.17–8.15 (d, *J* = 8.8 Hz, 1 H, Ar-H), 7.82 (s, 4 H, Ar-$H_{phthalimide}$), 7.53–7.51 (d, *J* = 8.8 Hz, 1 H, Ar-H), 5.45 (s, 1 H, $CH_{pyrimidine}$).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 192.36, 169.26, 168.26, 164.73, 155.08, 147.82, 142.84, 134.34, 132.62, 130.66, 124.29, 122.95, 91.14.

MS: *m*/*z* (%) = 409 (17) [M^+ + 2], 407 (28) [M^+], 372 (41), 325 (7), 297 (14), 280 (100), 259 (98), 117 (96).

Anal. Calcd. for $C_{19}H_{10}ClN_5O_4$: C, 55.97; H, 2.47; N, 17.18. Found: C, 55.98; H, 2.48; N, 17.23.

(*E*)-2-(2-Chloro-6-((4-chlorobenzylidene)amino)pyrimidin-4-yl)isoindoline-1,3-dione (6c)

The mixture was stirred for 6 h.

Yield: 2.51 g (63.3%); yellow solid; mp 175–177 °C.

IR (KBr): 3090 (CH aromatic), 2849 (CH aliphatic), 1752, 1704 (2 × C=O), 1676 (C=$N_{azomethine}$), 1631 (C=N).

^1H NMR (400 MHz, DMSO-d_6): δ = 8.24 (s, 1 H, $CH_{azomethine}$), 8.09–8.06 (d, *J* = 8.8 Hz, 1 H, Ar-H), 7.82 (s, 4 H, Ar-$H_{phthalimide}$), 7.53–7.51 (d, *J* = 8.8 Hz, 1 H, Ar-H), 7.43–7.41 (d, *J* = 8.8 Hz, 1 H, Ar-H), 7.22–7.20 (d, *J* = 8.8 Hz, 1 H, Ar-H), 5.23 (s, 1 H, $CH_{pyrimidine}$).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 192.18, 169.53, 169.30, 161.64, 153.06, 136.79, 134.73, 134.37, 132.64, 131.62, 128.14, 122.98, 99.47.

MS: *m*/*z* (%) = 400 (9) [M^+ + 4], 398 (9) [M^+ + 2], 396 (27) [M^+], 363 (25), 324 (48), 296 (8), 280 (21), 249 (100).

Anal. Calcd. for $C_{19}H_{10}Cl_2N_4O_2$: C, 57.45; H, 2.54; N, 14.11. Found: C, 57.50; H, 2.56; N, 14.18.

(*E*)-2-(2-Chloro-6-((3,4-dimethoxybenzylidene)amino)pyrimidin-4-yl)isoindoline-1,3-dione (6d)

The mixture was stirred for 8 h.

Yield: 3.88 g (92%); yellow solid; mp 208–210 °C.

IR (KBr): 3061 (CH aromatic), 2924 (CH aliphatic), 1709 (C=O), 1631 (C=N) cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 9.29 (s, 1 H, $CH_{azomethine}$), 7.81 (s, 4 H, Ar-$H_{phthalimide}$), 7.54–7.52 (d, *J* = 8.4 Hz, 2 H, Ar-H), 7.14–7.12 (d, *J* = 8.4 Hz, 1 H, Ar-H), 5.43 (s, 1 H, $CH_{pyrimidine}$), 3.16 (s, 6 H, $2OCH_3$).

MS: *m*/*z* (%) = 424 (19) [M^+ + 2], 422 (19) [M^+], 387 (43), 243 (86), 224 (100).

Anal. Calcd. for $C_{21}H_{15}ClN_4O_4$: C, 59.65; H, 3.58; N, 13.25. Found: C, 59.70; H, 3.60; N, 13.30.

(*E*)-2-(2-Chloro-6-((2-hydroxybenzylidene)amino)pyrimidin-4-yl)isoindoline-1,3-dione (6e)

The mixture was stirred for 6 h.

Yield: 3.28 g (86.8%); yellow solid; mp 188–191 °C.

IR (KBr): 3656 (OH), 3061 (CH aromatic), 2925 (CH aliphatic), 1710 (C=O), 1632 (C=N) cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 11.32 (s, 1 H, OH), 9.25 (s, 1 H, $CH_{azomethine}$), 7.81 (s, 4 H, Ar-H), 7.55–7.53 (d, *J* = 7.6 Hz, 1 H, Ar-H), 7.33–7.29 (t, *J* = 7.6 Hz, 1 H, Ar-H), 7.24–7.21 (t, *J* = 7.6 Hz, 1 H, Ar-H), 7.09–7.07 (d, *J* = 7.6 Hz, 1 H, Ar-H), 6.32 (s, 1 H, $CH_{pyrimidine}$).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 191.83, 169.27, 168.47, 162.84, 156.18, 134.34, 133.86, 132.63, 130.56, 125.96, 123.31, 122.95, 119.47, 99.05.

MS: *m*/*z* (%) = 380 (40) [M^+ + 2], 378 (29) [M^+], 306 (49), 174 (49), 146 (30), 121 (100).

Anal. Calcd. for $C_{19}H_{11}ClN_4O_3$: C, 60.25; H, 2.93; N, 14.79. Found: C, 60.30; H, 2.95; N, 14.89.

(*E*)-2-(2-Chloro-6-((4-hydroxybenzylidene)amino)pyrimidin-4-yl)isoindoline-1,3-dione (6f)

The mixture was stirred for 6 h.

Yield: 3.12 g (82.6%); yellow solid; mp 198–200 °C.

IR (KBr): 3631 (OH), 3063 (CH aromatic), 2991 (CH aliphatic), 1710 (C=O), 1633 (C=N) cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 11.32 (s, 1 H, OH), 9.89 (s, 1 H, $CH_{azomethine}$), 7.82 (s, 4 H, Ar-H), 7.41–7.39 (d, *J* = 8.4 Hz, 2 H, Ar-H), 7.09–7.07 (d, *J* = 8.4 Hz, 2 H, Ar-H), 6.33 (s, 1 H, $CH_{pyrimidine}$).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 192.18, 175.24, 169.24, 166.53, 166.30, 158.08, 134.33, 132.61, 129.64, 126.94, 122.94, 119.24, 99.22.

MS: *m*/*z* (%) = 380 (24) [M^+ + 2], 378 (18) [M^+], 248 (33), 173 (52), 117 (3), 90 (100).

Anal. Calcd. for $C_{19}H_{11}ClN_4O_3$: C, 60.25; H, 2.93; N, 14.79. Found: C, 60.28; H, 2.98; N, 14.84.

2-(6-Amino-2-hydrazinylpyrimidin-4-yl)isoindoline-1,3-dione (7)

Compound **4** (2.74 g, 0.01 mol) and hydrazine hydrate (0.03 g, 0.03 mol) were combined with ethyl alcohol (20 mL) and Et_3N (two drops), and the mixture was stirred at r.t. for 9 h. The mixture was refrigerated once the reaction (TLC) was completed. The solid product was filtrated, dried, and recrystallised from ethanol.

Yield: 2.19 g (81%); yellowish-white solid; mp 290–292 °C.

IR (KBr): 3320, 3290, 3165 (NH_2 and NH), 3019 (CH aromatic), 1661 (C=O) cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 10.51 (bs, 1 H, NH), 8.08 (s, 2 H, $NH_{2\ pyrimidine}$), 8.07–8.03 (t, *J* = 8.8 Hz, 2 H, Ar-H), 7.88 (s, 2 H, NH_2), 7.86–7.84 (d, *J* = 8.8 Hz, 2 H, Ar-H), 5.49 (s, 1 H, $CH_{pyrimidine}$).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 168.71, 166.83, 164.59, 154.71, 132.62, 127.23, 125.18, 77.07.

MS: *m*/*z* (%) = 271 (42) [M⁺], 255 (33), 237 (19), 199 (29), 184 (100).

Anal. Calcd. for $C_{12}H_{10}N_6O_2$: C, 53.33; H, 3.73; N, 31.10. Found: C, 53.36; H, 3.75; N, 31.17.

2-(6-Amino-2-(2-(arylidene)hydrazinyl)pyrimidin-4-yl)isoindoline-1,3-diones 8a–f; General Procedure

Compound **7** (0.27 g, 0.001 mol) and aromatic aldehyde (0.001 mol) were combined with EtOH (50 mL) and two drops of triethylamine (Et_3N), and the mixture was stirred for 2–11 h at r.t.. Following the completion of the reaction (TLC), the solid product that had been separated from the mixture was filtered out, dried, and then recrystallised from ethanol.

2-(6-Amino-2-(2-(3-nitrobenzylidene)hydrazinyl)pyrimidin-4-yl)isoindoline-1,3-dione (8a)

The mixture was stirred for 6 h

Yield: 0.36 g (88.9%); yellow solid; mp 140–142 °C.

IR (KBr): 3509, 3453, 3401 (NH_2 and NH), 3081 (CH aromatic), 2922 (CH aliphatic), 1661 (C=O), 1626 (C=N), 1528, 1351 (NO_2) cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 11.50 (bs, 1 H, NH), 8.93 (s, 4 H, Ar-H), 8.72 (s, 1 H, $CH_{hydrazone}$), 8.41–8.39 (d, *J* = 8 Hz, 1 H, Ar-H), 8.39–8.36 (t, *J* = 2.4 Hz, 2 H, Ar-H), 7.85–7.81 (t, *J* = 8 Hz, 1 H, Ar-H), 7.22 (s, 2 H, NH_2), 4.13 (s, 1 H, $CH_{pyrimidine}$).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 169.15, 167.80, 166.43, 160.49, 148.23, 143.14, 134.43, 131.05, 130.67, 130.02, 125.84, 122.67, 121.35, 118.78, 73.07.

MS: *m*/*z* (%) = 404 (22) [M⁺], 388 (25), 242 (8), 165 (12), 163 (100).

Anal. Calcd. for $C_{19}H_{13}N_7O_4$: C, 56.58; H, 3.25; N, 24.31. Found: C, 56.60; H, 3.30; N, 24.40.

2-(6-Amino-2-(2-(4-nitrobenzylidene)hydrazinyl)pyrimidin-4-yl)isoindoline-1,3-dione (8b)

The mixture was stirred for 3 h.

Yield: 0.32 g (80%); yellow solid; mp 160–162 °C.

IR (KBr): 3452, 3424 (NH_2 and NH), 2922 (CH aliphatic), 1661 (C=O), 1629 (C=N), 1522, 1345 (NO_2) cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 11.50 (s, 1 H, NH), 8.17–8.15 (d, *J* = 8.8 Hz, 2 H, Ar-H), 8.07 (s, 1 H, $CH_{hydrazone}$), 7.87 (s, 4 H, Ar-$H_{phthalimide}$), 7.68–7.66 (d, *J* = 8 Hz, 2 H, Ar-H), 7.55 (s, 2 H, NH_2), 4.02 (s, 1 H, $CH_{pyrimidine}$).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 169.58, 167.93, 166.29, 159.20, 145.62, 143.49, 134.12, 132.61, 131.58, 129.59, 128.69, 125.29, 123.99, 71.14.

MS: *m*/*z* (%) = 403 (59) [M⁺⁺], 387 (26), 341 (46), 265 (22), 119 (70), 42 (100).

Anal. Calcd. for $C_{19}H_{13}N_7O_4$: C, 56.58; H, 3.25; N, 24.31. Found: C, 56.68; H, 3.29; N, 24.38.

2-(6-Amino-2-(2-(4-chlorobenzylidene)hydrazinyl)pyrimidin-4-yl)isoindoline-1,3-dione (8c)

The mixture was stirred for 4 h.

Yield: 0.35 g (88.9%); white solid; mp 200–202 °C.

IR (KBr): 3456, 3165, 3125 (NH_2 and NH), 3019 (CH aromatic), 2918 (CH aliphatic), 1660 (C=O), 1623 (C=N) cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 11.47 (s, 1 H, NH), 8.71 (s, 1 H, $CH_{hydrazone}$), 8.07 (s, 4 H, Ar-$H_{phthalimide}$), 7.88–7.58 (m, *J* = 8 Hz, 3 H, Ar-H), 7.56 (s, 2 H, NH_2), 7.37–7.35 (d, *J*=8 Hz, 1 H, Ar-H), 4.33 (s, H, $CH_{pyrimidine}$(.

^{13}C NMR (125 MHz, DMSO-d_6): δ = 170.08, 167.66, 164.64, 160.62, 136.05, 132.60, 130.05, 129.72, 129.11, 128.25, 127.17, 125.16, 76.07.

MS: *m*/*z* (100%) = 394 (5) [M⁺ + 2], 392 (12) [M⁺], 357 (15), 270 (6), 133 (13), 104 (100).

Anal. Calcd. for $C_{19}H_{13}ClN_6O_2$: C, 58.10; H, 3.34; N, 21.40. Found: C, 58.15; H, 3.36; N, 21.49.

2-(6-Amino-2-(2-(2-hydroxybenzylidene)hydrazinyl)-pyrimidin-4-yl)isoindoline-1,3-dione (8d)

The mixture was stirred for 6 h.

Yield: 0.36 g (95.5%); yellow solid; mp 176–178 °C.

IR (KBr): 3655 (OH), 3450, 3447, 3368 (NH_2 and NH), 3042 (CH aromatic), 2920 (CH aliphatic), 1688 (C=O), 1623 (C=N) cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 11.16 (bs, 2 H, NH and OH), 8.98 (s, 4 H, Ar-$H_{phthalimide}$), 8.77 (s, 1 H, $CH_{hydrazone}$), 7.69–7.67 (d, *J* = 7.2 Hz, 1 H, Ar-H), 7.42–7.38 (t, *J* = 7.2 Hz, 2 H, Ar-H), 6.98–6.96 (d, *J* = 7.2 Hz, 1 H, Ar-H), 6.95 (s, 2 H, NH_2), 3.16 (s, 1 H, $CH_{pyrimidine}$).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 174.42, 168.90, 163.34, 159.05, 154.23, 133.80, 132.64, 132.43, 131.86, 131.40, 120.16, 118.56, 116.99, 110.61, 72.83.

MS: *m*/*z* (%) = 374 (19) [M⁺], 280 (26), 206 (93), 176 (18), 150 (100).

Anal. Calcd. for $C_{19}H_{14}N_6O_3$: C, 60.96; H, 3.77; N, 22.45. Found: C, 60.98; H, 3.80; N, 22.50.

2-(6-Amino-2-(2-(4-hydroxybenzylidene)hydrazineyl)pyrimidin-4-yl) isoindoline-1,3-dione (8e)

The mixture was stirred for 2 h.

Yield: 0.34 g (90.91%); yellow solid; mp 210–212 °C.

IR (KBr): 3656 (OH), 3510, 3452, 3400 (NH_2 and NH), 3042 (CH aromatic), 2919 (CH aliphatic), 1688 (C=O), 1623 (C=N) cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 11.13 (s, 1 H, NH), 9.00 (s, 1 H, OH), 7.72–7.70 (d, *J* = 9.2 Hz, 2 H, Ar-H), 7.68–7.66 (d, *J* = 9.2 Hz, 2 H, Ar-H), 7.44–7.42 (d, *J* = 9.2 Hz, 1 H, Ar-H), 7.40 (s, 1 H, $CH_{hydrazone}$), 7.38–7.36 (d, *J* = 9.2 Hz, 1 H, Ar-H), 6.99–6.97 (d, *J* = 9.2, 2 H, Ar-H), 6.95 (s, 2 H, NH_2), 4.35 (s, 1 H, $CH_{pyrimidine}$).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 170.11, 162.83, 158.67, 153.66, 151.66, 140.10, 138.28, 133.28, 130.88, 119.65, 118.22, 116.57, 76.07.

MS: *m*/*z* (%) = 374 (25) [M⁺], 372 (22), 348 (100), 320 (12).

Anal. Calcd. for $C_{19}H_{14}N_6O_3$: C, 60.96; H, 3.77; N, 22.45. Found: C, 60.99; H, 3.79; N, 22.50.

2-(6-Amino-2-(2-(4-(dimethylamino)benzylidene)hydrazineyl)pyrimidin-4-yl)isoindoline-1,3-dione (8f)

The mixture was stirred for 11 h.

Yield: 0.37 g (92.9%); yellow solid; mp 242–244 °C.

IR (KBr): 3509, 3452, 3167 (NH_2 and NH), 3018 (CH aromatic), 2918 (CH aliphatic), 1661 (C=O), 1621 (C=N) cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 10.81 (s, 1 H, NH), 8.49 (s, 1 H, $CH_{hydrazone}$), 8.33 (s, 4 H, Ar-$H_{phthalimide}$), 7.65–7.63 (d, *J* = 7.2 Hz, 2 H, Ar-H), 7.33 (s, 2 H, NH_2), 6.77–6.75 (d, *J* = 7.2 Hz, 2 H, Ar-H), 4.79 (s, 1 H, $CH_{pyrimidine}$), 2.99 (s, 6 H, $2CH_3$).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 171.91, 169.18, 165.69, 160.44, 155.03, 141.13, 132.42, 130.07, 128.07, 126.45, 124.49, 112.15, 72.36, 48.97.

MS: *m*/*z* (%) = 401 (46) [M$^+$], 252 (54), 245 (100).

Anal. Calcd. for $C_{21}H_{19}N_7O_2$: C, 62.83; H, 4.77; N, 24.42. Found: C, 62.88; H, 4.79; N, 24.48.

Biological Activity

Antitubercular Activity

The American Type Culture Collection (ATCC), USA, provided *M. tuberculosis* (ATCC 25177/H37Ra) as a drug-sensitive strain (DS) and *M. tuberculosis* (ATCC 35822) as a multidrug-resistant strain (MDR). Apart from the moxifloxacin-resistant *M. tuberculosis* (RCMB 2674), pyrazinamide, ethionamide, ethambutol, isoniazid, and rifampicin-resistant strains, an extensively drug-resistant strain (XDR) was obtained from the Culture Collection Unit of the Regional Center for Mycology and Biotechnology (RCMB). Dubos medium mixed with 50 mM sodium nitrate was used to cultivate all strains of *M. tuberculosis*. The cultures were cultivated aerobically at 37 °C and 150 rpm until they reached log-phase optical density (OD595 = 1). Mycobacteria develop as aggregated clumps; thus, they were subjected to ultrasound irradiation and sonication for 2 min using a water bath (Ultrasonic, Freeport, IL, USA). Microplate Alamar blue test was used to assess the MIC values of drugs against DS, MDR, and XDR *M. tuberculosis* strains (MABA).[36,37] Rifampicin and isoniazid were used as positive controls. The chemical stock solutions and final testing concentrations ranged from 1000 to 0.003 μg/mL. In Difco Middlebrook 7H9 Broth (Seebio) supplemented with 0.2% (vol/vol) glycerol, 0.05% Tween 80, and 10% (vol/vol) albumin-dextrose-catalase, *M. tuberculosis* was raised to late log phase (70 to 100 Klett units) (7H9-ADC-TG). Compounds were produced as two-fold dilutions in 100 μL volumes in 7H9-ADC-TG clear-bottom microplates (BD). A final testing volume of 200 μL was obtained by adding *M. tuberculosis* (100 μL containing 2 × 105 CFU). The plates were incubated at 37 °C, and on the seventh day, each well received the addition of 20 μL of Alamar blue and 12.5 μL of 20% Tween 80. The fluorescence was measured at an excitation of 530 nm and an emission of 590 nm during an incubation period of 16–24 h at 37 °C. The MIC was identified as the concentration at which a 90% decrease in fluorescence was seen in comparison to duplicate bacterium-only controls.

*In-vitro M. Tuberculosis*InhA Enzyme Inhibition Activity

Enzymatic Assay

InhA activity was followed by a colorimetric assay that measured the oxidation of NADH at 340 nm in the presence of 2-*trans*-octanoyl-CoA in a buffer that contained 30 mM PIPES, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, and 100 nM InhA. This was preincubated for 10 min at r.t. with 0.25 mM NADH and varying concentrations of the compounds with 1% (v/v) DMSO in a 150 μL reaction volume. The reaction was started by the addition of 2-*trans*-octanoyl-CoA at a final concentration of 1.5 mM. The reactions were followed for 20 min using a plate reader (CLARIOstar, BMG LABTECH).[29]

Molecular Docking Simulation

Molecular docking simulations were performed in the Computational Chemistry and Molecular Modeling Lab, Pharmaceutical Organic Chemistry Department, Faculty of Pharmacy, Mansoura University using 'Molecular Operating Environment (MOE) 2022.20'. Docking was performed according to the literature.[31] The crystallographic structure of the InhA enzyme (PDB ID: 3FNE) was acquired from the PDB and prepared for molecular docking by removing ligands, introducing hydrogens, and decreasing energy with MOE 2022.20. The structure with the lowest energy was then employed as a docking receptor. MOE's site finder method was utilized to identify the catalytic site of InhA. Chem Bio Office was used to create the two-dimensional structures of the synthesized compounds, which were subsequently built from fragment libraries in MOE 2009 and reduced energy with the MMFF94x force field in MOE. To discover and analyze the interaction between ligands and the catalytic site of InhA, docking was done with chosen parameters (rescoring function 1 and rescoring function 2: London).

Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

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