JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

DESIGN AND SYNTHESIS OF NOVEL PYRIMIDINE ANALOGS AS ANTI-TUBERCULAR AGENTS TARGETING THYMIDINE KINASE DOMAIN

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https://doi.org/10.15414/jmbfs.1744

ARTICLE INFO

Received 30. 5. 2019
Revised 23. 4. 2021
Accepted 23. 4. 2021
Published 1. 10. 2021

Regular article

OPEN ACCESS

ABSTRACT

The inhibition of the enzyme TMP kinase (TMPKmt), is hypothesized as a significant therapy for tuberculosis. A series of designed pyrimidines were synthesized to inhibit the enzyme TMPKmt and evaluated for their enzyme-ligand interactions, antitubercular, physiochemical and ADMET properties. The pyrimidines were synthesized from chalcones and guanidine as the cyclizing agent. The molecular interactions were studied by Autodock 4.0 and physicochemical, druglikeness and ADMET properties were analysed by Molinspiration, Chemsketch program and admetSAR prediction tools. The confirmation of the synthesized titled compound's structures was by spectral analysis. Also, they were screened for their antitubercular activity. *In silico* studies reports that their physicochemical, ADMET and druglikeness properties were found to be in standard limit, which infers that, these compounds may not have problems with oral bioavailability. Molecular docking studies showed that the pyrimidines have better enzyme inhibitory activity onTMPKmt.

Keywords: Pyrimidines, Molecular docking, Thymidine kinase, Antitubercular activity

INTRODUCTION

Tuberculosis is one of the deadliest diseases, which are caused by the bacteria, *Mycobacterium tuberculosis* **(Dye *et al.*, 1998; Dye *et al.*, 2002)**. Globally, there were 9.2 million new cases and 1.7 million TB deaths in 2006, 0.7 million of which were in HIV-positive people and 0.2 million deaths **(B. Greenwood et al., 2008)**. There are several flaws in existing drugs, the most prominent of which is the rise of drug resistance. For more than 20 years, no new medicines for tuberculosis have been discovered. Owing to the growing resistance to today's leading anti tubercular drugs, new therapies are urgently needed, which stimulated the pursuit of novel targets for the disease tuberculosis. The application of computational tools in the identification of drug targets, especially for Mtb, can very quickly produce a list of reliable targets. The inactivation of suitable novel targets, which are responsible for bacterial metabolism, growth and viability, would lead to bacterial death, thus eliminating the drug-resistant strains and shortening the duration of the therapy **(Zhang 2005; Mdluli *et al.*, 2006)**.

In the development of antitubercular drugs, thymidine monophosphate kinase of *Mycobacterium tuberculosis* (TMPKmt) **(Gasse *et al.*, 2008)** is an obvious target. TMPK is a vital enzyme in the metabolism of *Mycobacterium tuberculosis*, and also, it is the precise enzyme in dTTP synthesis. It is unlike human enzyme analogues (22% homology). It catalyses the conversion of deoxythymidine monophosphate (dTMP) to deoxythymidine diphosphate (dTDP) using ATP as a phosphoryl donor **(Lavie *et al.*, 1998; Ostermann *et al.*, 2001)**. Due to its prospects as antimetabolites, TMPK inhibitors have gained considerable interest. Even, by inhibiting DNA synthesis in *M. tuberculosis*, these molecules can be promising leads in treating tuberculosis. Very few studies of lead generation and optimisation have been explored for TMPKmt inhibitors **(Pochet *et al.*, 2003; Pochet *et al.*, 2010)**.

Pyrimidines have been investigated extensively among the plethora of nitrogen-containing heterocycles. This chemical moiety is of paramount importance due to its activities such as antitubercular **(Breda *et al.*, 2012)**, anticancer **(El-Deeb *et al.*, 2010)**, **(Tan *et al.*, 2014)**, antimalarial **(Singh *et al.*, 2013)**, antitumor **(El-Nassan *et al.*, 2011)**, analgesic, anti-convulsant **(Deng *et al.*,2011)** etc. Since only limited reports are available about pyrimidines as anti-tubercular agents, hence, the design of novel pyrimidines with its target is a feasible choice **(Singh *et al.*, 2011)**.

With this data, a study has been focused on screening some compounds containing pyrimidine moiety by using computational tools. And the compounds with good binding energy will be synthesized, and further will proceed for their antitubercular activity screening.

METHODOLOGY

Materials and methods used

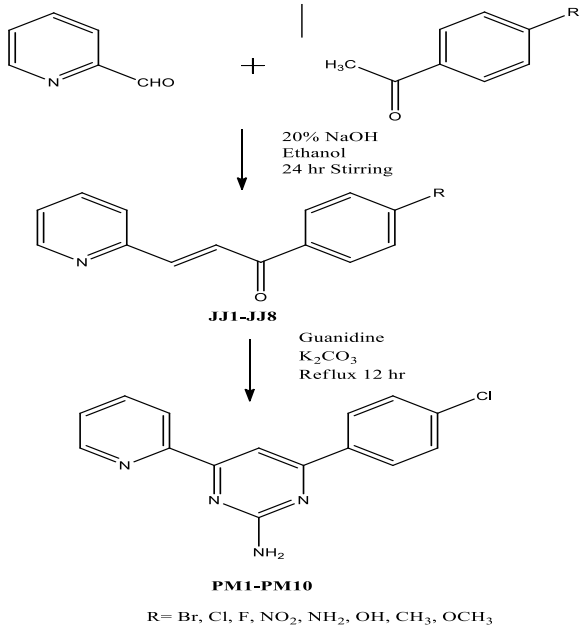
All the reagents were commercially obtained from Sigma Aldrich and utilized without further purification. Melting points were determined by capillary method and uncorrected. Shimadzu Perkin Ekmer 8201 Pc IR spectrometer (KBr Pellets), Bruker Avance II 400 NMR, JEOL SX- 102/DA-6000 FAB Mass spectrometer were used for recording IR, NMR and Mass spectra.

Synthetic Methods

Synthesis of 6-(pyridin-2-yl)-4-(aryl substituted) pyrimidin-2-amine (PM1-PM10):

0.1 mole of chalcones, 0.01 mole of guanidine, and 20 ml of potassium carbonate as catalyst were taken and were refluxed for 12 hrs. Then, it was cooled, poured to ice and dilute acetic acid was added for acidification. Ethanol was used for recrystallisation **(Singh *et al.*, 2011).** TLC medium was Ethylacetate:Chloroform(8:2), which was used for analysing purity periodically (Scheme1).

Scheme-1 Synthesis of Pyrimidines



Spectral data:

PM1: 6-(pyridin-2-yl)-4-(4-bromophenyl) pyrimidin-2-amine

IR (cm-1): 3415.8, 3347.7 (aromatic NH2 str), 3096 (aromatic CH str), 1626 (C=C str), 1652 (C=N str), 683 (C-Br).

^{1}H NMR (δ ppm): 6.98 (s, 2H, NH2), 7.30-8.15 (m, 9H, Ar- H).

Mass (m/z): (M^+) 327.

PM2: 6-(pyridin-2-yl)-4-(4-chlorophenyl) pyrimidin-2-amine

IR (cm-1): 3421.5, 3343.9 (aromatic NH2 str), 3084 (aromatic CH str), 1680 (C=C str), 1671 (C=N str), 857 (C-Cl).

^{1}H NMR (δ ppm): 6.81 (s, 2H, NH2), 7.39-8.71 (m, 9H, Ar- H).

Mass (m/z): (M^+) 282.

PM3: 6-(pyridin-2-yl)- 4-(4-fluorophenyl) pyrimidin-2-amine

IR (cm-1): 3402.1, 3392.9 (aromatic NH2 str), 3121.4 (aromatic CH str), 1671.7 (C=C str), 1664.3 (C=N str), 1401.3 (C-F).

^{1}H NMR (δ ppm): 6.46 (s, 2H, NH2), 7.12-8.91 (m, 9H, Ar- H).

Mass (m/z): (M^+) 266.

PM4: 6-(pyridin-2-yl) -4-(4-nitrophenyl) pyrimidin-2-amine

IR (cm-1): 3393.2, 3376.9 (aromatic NH2 str), 3222.8 (aromatic CH str), 1689.2 (C=C str), 1634.6 (C=N str), 1543.3 (C-NO2).

^{1}H NMR (δ ppm): 6.55 (s, 2H, NH2), 8.01-8.99 (m, 9H, Ar- H).

Mass (m/z): (M^+) 293.

PM5: 6-(pyridin-2-yl) -(4-hydroxyphenyl) pyrimidin-2-amine

IR (cm-1): 3433.9, 3391.3 (aromatic NH2 str), 3451.3 (aromatic CH str), 1672.8 (C=C str), 1672.7 (C=N str), 1423.7 (C-OH).

^{1}H NMR (δ ppm): 6.63 (s, 2H, NH2), 7.09-8.87 (m, 9H, Ar- H), 5.63 (s, 1H, OH).

Mass (m/z): (M^+) 264.

Antitubercular activity

The method adopted for the antitubercular study was microplate Alamar blue assay **(Collins *et al*.,1997)** against *M.tuberculosis*. The diluted serial compounds (0.2 to 100.0 µM) and Middlebrook 7H9 broth (100 µl) containing *Mycobacterium tuberculosis* was poured to 96 well plates. The standard used was Isoniazid. After the sealing off the plates, incubation was done for seven days at 37ºC. After, the addition of the dye, it was re-incubated for 24h. The changes in the colour were noted, which was interpreted for pink as the growth of bacteria and blue as vice versa.

Software used

The software used for molecular docking is Autodock 4.0 **(Goodsell *et al*., 1996)** and the procedure followed for performing protein and ligand preparation is described elsewhere **(Vanommeslaeghe *et al*., 2010; Morris *et al*., 1998; Chennu *et al*., 2015)**. Ligands were designed with the basic pharmacophore (pyridine) using pyrimidine as the primary nucleus with different cyclizing agents like guanidine and thiourea, substitutions like fluoro, chloro, bromo, nitro, methyl, methoxy, hydroxyl, and amino groups at 4 and 3 positions. About 12 compounds were designed for TMPKmt inhibition by incorporating these functional groups. Structure of the ligands was drawn using Chemsketch and SMILES notation was developed for the designed ligands. Molecular docking studies have carried out for compounds that have passed the ADMET **(Feixiong *et al*., 2012)** and Lipinski's rule of 5 **(Lipinski, 2004).**

Docking studies

Target and Ligand Preparation

TMPKmt crystal structure was downloaded from the Protein Data Bank with PDB ID as 4UNR **(Naik *et al*. 2015)**. The repair commands module of AutoDock added the missing atoms. Before the docking process, the water molecules were removed, and H-atoms were supplemented to the targets, to for tautomerism and ionise amino acids. The modified structure was then applied for semi-flexible dockings. The energy minimization was performed by Discovery Studio (Version 4.0, Accelrys Software Inc 2007, with the CHARMM force field **(Vanommeslaeghe *et al*.,2010)**.

Semi-flexible docking

The energy scoring grid box was customized to 126, 126 and 126 Å (x, y, and z) centered at X = 0.041; Y = -0.068 and Z = 0.128 with 0.375 angstroms grid points spacing assigned with default atomic salvation parameters. Three-dimensional grid boxes enclose the active site of the enzyme TMPKmt, which locates the ligand active binding site at the centre. Lamarckian Genetic Algorithm (LGA) **(Morris *et al*., 1998)** was the docking engine used in this study. After each LGA run, Autodock reports the best docking orientation, and the results were based on cluster analysis. The energy docking mode was found from a total of 10 docking modes. The lowest energy docking pose was chosen, from a total of 10 docking poses in each docking simulation.

RESULTS AND DISCUSSION

Chemistry

Chalcone and guanidine in equimolar quantities with anhydrous potassium carbonate as the catalyst resulted in pyrimidines in better yields (Table 1). The IR spectra of compound PM1 shows an aromatic peak at 3415.8 and 3347.7 cm^{-1} which corresponds to the amino group and C=N stretching at 1652 cm^{-1}. In addition, the ^{1}H NMR spectra predicted the presence of two hydrogens at 6.98 (s, 2H), which relates to aromatic NH_2 group. Further, this compound got support from the mass spectrum, which depicted its molecular ion peak at 327, which corresponds to its molecular weight.

Table 1 Physical data of synthesized pyrimidines

Comp. Code:	Ar	Molecular Formula	Physical State (crystals)	Mol. Wt	% Yield	M.P (°C)	R Value f
PM1	4-Br	$C_{15}H_{11}BrN_4$	Brown	327	68	196-198	0.73
PM 2	4-Cl	$C_{15}H_{11}ClN_4$	Red	282.73	67	182-184	0.75
PM 3	4-F	$C_{15}H_{11}FN_4$	Red	266.28	62	174-176	0.75
PM 4	4-NO_2	$C_{15}H_{11}N_5O_2$	Red	293.29	64	186-188	0.67
PM 5	4-OH	$C_{15}H_{12}N_4O$	Brown	264.29	50	174-176	0.74
PM 6	4-NH_2	$C_{15}H_{13}N_5$	Brown	263.3	48	184-186	0.64
PM 7	4-CH_3	$C_{16}H_{14}N_4$	Brown	262.32	39	176-179	0.76
PM 8	4-OCH_3	$C_{16}H_{14}N_4O$	Red	278.31	45	186-188	0.79
PM9	3-Br	$C_{15}H_{11}BrN_4$	Red	327	57	186-188	0.73
PM10	3-Cl	$C_{15}H_{11}ClN_4$	Red	282.73	56	172-174	0.72

Antitubercular activity

The titled compounds were screened for their antitubercular activity by *in vitro* methods. All the compounds were found to have good antitubercular activity, and results are tabulated in Table 2. Compounds PM2 and PM4 were found to be active as standard, and their activity is due to the substitution of chloro and nitro in the 4-position of benzyl group of PM2 and PM4 respectively and the presence of the core groups pyridine and pyrimidine must have also contributed equally, with MIC value of 0.2 µM. With various other changes, an extensive structure-activity relationship could be derived in the future.

Table 2 Antitubercular activity of synthesized pyrimidines

Comp.Code	Ar	MIC in µM
PM1	4-Br	0.8
PM2	4-Cl	0.2
PM3	4-F	0.8
PM4	4-NO_2	0.2
PM5	4-OH	0.8
PM6	4-NH_2	3.125
PM7	4-CH_3	12.5
PM8	4-OCH_3	12.5
PM9	3-Br	3.125
PM10	3-Cl	3.125
Standard	Isoniazid	0.2

***In silico* studies**

The predictions of drug-likeness and pharmacokinetic properties (ADMET) were performed by online tools molinspiration and admetSAR. The analogues owned desired physicochemical properties by satisfying all the Lipinski's RO5 properties with no violations from the standard limits (Table 3). The partition coefficient of all the compounds was found to be good values (1-3), which is essential for the absorption and distribution of drugs. ADMET properties were predicted, and it was found that all the parameters were within the acceptable range when compared with the standard (Table 4). Thus, all the hits were predicted with excellent physicochemical and druggable properties, rendering them good oral bioavailability.

Table 3 *In silico* physicochemical properties of synthesized pyrimidines

Comp. Code:	Mol. Wt	Log P	nNHAcc	nHDon	tPSA	nrobs	Molar volume cm^3
PM1	327	3.04	4	2	64.7	2	243.57
PM 2	282.73	2.91	4	2	64.7	2	213.9
PM 3	266.28	2.4	4	2	64.7	2	206.1
PM 4	293.29	2.19	7	2	110.52	3	213.7
PM 5	264.29	1.75	5	3	84.93	2	200.3
PM 6	263.3	1.31	5	4	80.72	2	204.2
PM 7	262.32	2.68	4	2	64.70	2	218.2
PM 8	278.31	2.29	5	2	73.93	3	225.9
PM 9	280.36	2.22	4	2	64.70	2	243.34
PM 10	281.34	2.67	4	1	58.90	2	240.07
Isoniazid	137.14	-0.92	4	3	68.01	1	122.56

Molecular docking

The designed compounds were found to have excellent binding affinity to the enzyme. The ten compounds were docked with TMPKmt domain protein to calculate its binding energy and understand its molecular interactions, which is in charge of target inhibition. Table 5, depicts the docking score of the docked compounds with the enzyme TMPKmt, within the range of -8.4 to -9.3Kcal/mol. Compounds PM3, PM7, PM8 obtained the best binding score (-9.3 Kcal/mol). This may be due to the great involvement of van der Waals and pi-pi interactions with the amino acids of the target. Figure 1a, describes the TMPKmt domain inhibited complex formation with the compound PM3 of maximum binding energy (-9.3 Kcal/mol), by forming pi-pi interaction with the residue Y103. Also showed van der Waals interactions with the residues L52; F70; S104; S99; Y103; R107. Figure 1b explains the TMPKmt domain inhibited complex formation with the compound PM4 of binding energy (-9 Kcal/mol), by forming two pi-pi interaction with the residue Y103. The complex also displayed two hydrogen bond of distance of 2.046 and 2.134 A and van der Waals relations with the amino acid residues Y103; R74; R95; N100; F70; S104; Y103; R107. The obtained interactions were found to be similar for the other docked pyrimidines, with the active amino acid residues, such as, Y103; R74; R95; N100; F70; S104; Y103; R107 and further, showed pi-pi interactions with the residues Y103.

Table 4 *In silico* ADMET studies of pyrimidines

Comp. Code	BBB	HIA	Caco-2 permeability	AMES toxicity	Carcinogenicity
PM1	0.9209	1	0.7407	Non-AMES toxic	Non-carcinogens
PM 2	0.9368	0.9965	0.7801	Non-AMES toxic	Non-carcinogens
PM 3	0.9401	0.9965	0.7375	Non-AMES toxic	Non-carcinogens
PM 4	0.9012	1.0000	0.6035	AMES toxic	Non-carcinogens
PM 5	0.84	0.9930	0.6	AMES toxic	Non-carcinogens
PM 6	0.8707	0.9933	0.7535	Non-AMES toxic	Non-carcinogens
PM 7	0.8993	1.0000	0.7718	Non-AMES toxic	Non-carcinogens
PM 8	0.93	1.0000	0.66	AMES toxic	Non-carcinogens
PM 9	0.8928	0.8997	0.6291	Non-AMES toxic	Non-carcinogens
PM 10	0.8480	0.9529	0.5000	Non-AMES toxic	Non-carcinogens
Isoniazid	0.9832	0.9905	0.6274	AMES toxic	Non-carcinogens

Table 5 Docking results of the pyrimidines with TMPKmt receptor domain

Comp. Code:	Docking energy (Kcal/mol)	Hydrogen bonds	Pi-Pi interactions	Van der waals interactions
PM1	-8.8	1(2.248)	Y103	R107,L171, L62,N100, Y103,S99, P37, S104, F70, R74,
PM 2	-9.2	0	Y103	L52, R95, N100, R107 Y103, S99, P37, S104, F70, R74
PM 3	-9.3	0	Y103	L52, Y103, S99, N100, P37, S104, F70, R107
PM 4	-9.0	2(2.134, 2.046) R95:HH21	Y103	R95, Y103, R107, N100, S104, F70, R74
PM 5	-9.0	0	Y103	R107, N100, L52,Y103, S99, P37, S104, F70, R74
PM 6	-8.8	0	Y103	R107, L52, N100,Y103, S99, P37, S104, F70, R74
PM 7	-9.3	0	Y103	R107,R95 N100,Y103, S99, P37, S104, F70, R74
PM 8	-9.3	1	Y103	R95,L52, Y103,N100, S99, P37, S104, F70, R74
PM 9	-8.4	0	Y103, F70	L171, R95, R107, Y103, S99, P37, S104, F70, R74
PM 10	-8.7	1 R74:HH12	Y103, F70	Y103,S99, S104, F70, R74

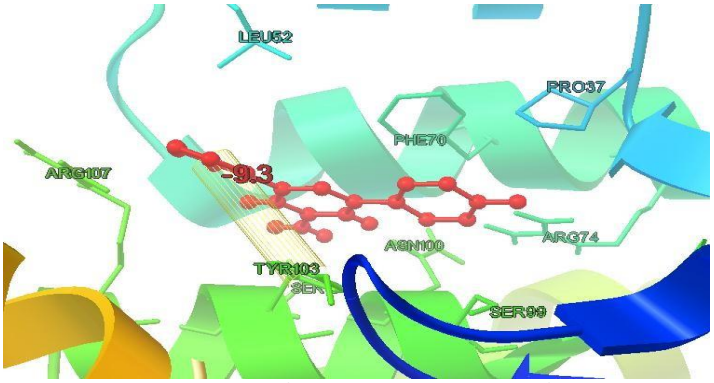


Figure 1a) 3D representation of the molecular interactions of compound PM3, within the binding pocket of protein and forming a pi-pi interactions with Y103 residue

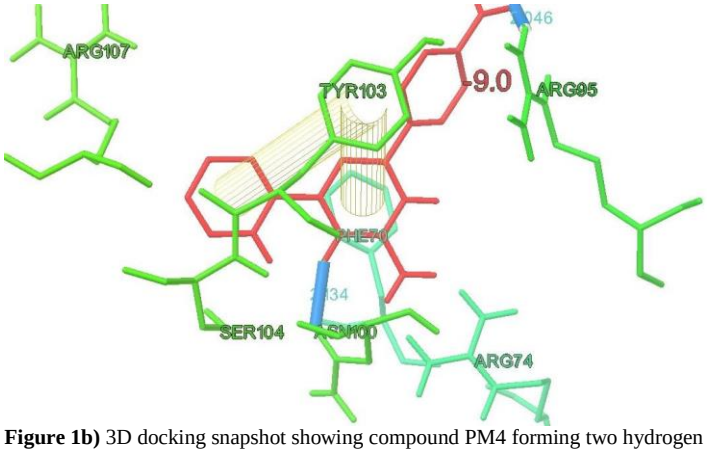


Figure 1b) 3D docking snapshot showing compound PM4 forming two hydrogen bonds shown in blue, two pi-pi interactions with Y103 residue

CONCLUSION

In the present investigation, different pyrimidine derivatives were designed, synthesized and screened for their *in silico* properties such as physicochemical, druglikeness, and ADMET and the toxic-less compounds were docked and aligned to the active pockets of the TMPKmt enzyme. The results thus obtained revealed that the amino pyrimidines may have a considerable impact on the enzyme inhibitory activity. From these computational data, the molecular interactions between the ligand pyrimidines and the enzyme TMPKmt have been obtained. Thus, docking studies have proved that these pyrimidine derivatives, as ligands have a strong affinity for the enzyme TMPKmt, which might be a reason for its antitubercular activity. In conclusion, the investigated pyrimidines are found to be suitable lead structures with better inhibitory action towards the target TMPKmt.

Acknowledgements: This study was funded by the Nitte University Research Grant (NURG/STF/01/7-2015). And the authors acknowledge NITTE (Deemed to be University), Mangaluru, for financial assistance, and NGSM Institute of Pharmaceutical Sciences, Mangaluru for providing the necessary facilities for the studies. Thanks also to Maratha Mandal Dental College, Belgaum for antitubercular tests, Director-SAIF, IISc, Bangaluru, and Director-CSRI, Lucknow, India for NMR and mass spectral analysis.

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