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Graphical Abstract

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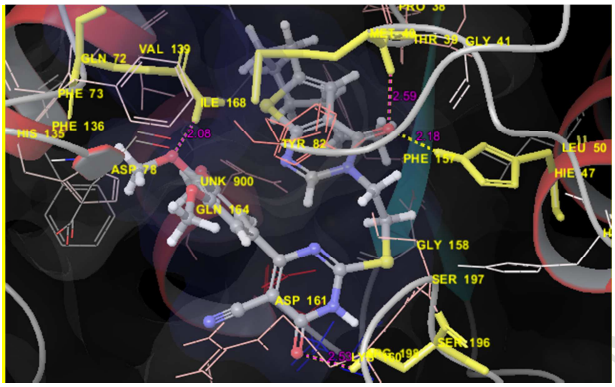

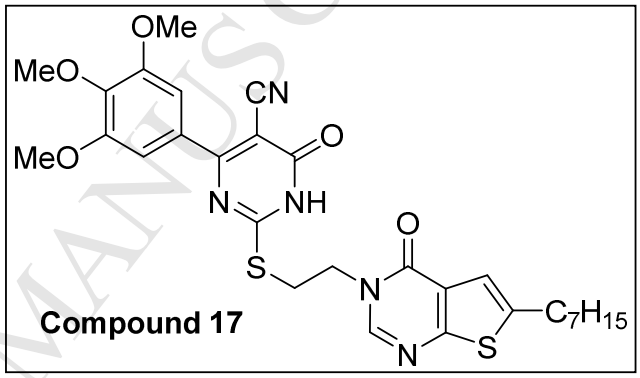

Hybrids of thienopyrimidinones and thiouracils as anti-tubercular agents: SAR and docking studies

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Abstract

A number of hybrid molecules containing thienopyrimidinones and thiouracil moieties were designed, synthesized and tested against *Mycobacterium tuberculosis* H37Ra wherein it was observed that the compounds **11**-~~15~~**14** exhibited antitubercular activity *in vitro* (MIC 7.6-19.1 µg/ml, 12-35 µM) against dormant stage while compound **15** exhibited antitubercular activity *in vitro* against dormant (MIC 23.4 µg/ml, 41 µM) as well as active (MIC 25.4 µg/ml, 45 µM) stage. Structural modifications of compound **15** were carried out to study the structure-activity relationship and it was observed that compound **18** exhibited antitubercular activity comparable to compound **15**. Cytotoxicity studies revealed that these molecules were non-toxic. The docking study of compound **15** showed that there ~~is~~ was binding with the active site of mycobacterial pantothenate synthetase. Further docking studies led to the synthesis of the compounds **16** and **17** and the antitubercular activity screening results showed that these compounds have significant antitubercular activity. The compounds **15**-**18** (MIC 11-29 µg/ml, 19-51 µM) can be used as starting points for further optimization. The synthetic strategies used in the present work have potential to prepare a large number of compounds for further refinement of structures and the present results will be very useful in the development of a new class of antimycobacterial agents.

Key words: Tuberculosis; Antitubercular activity; Thienopyrimidinone; Thiouracil; Docking.

1. Introduction

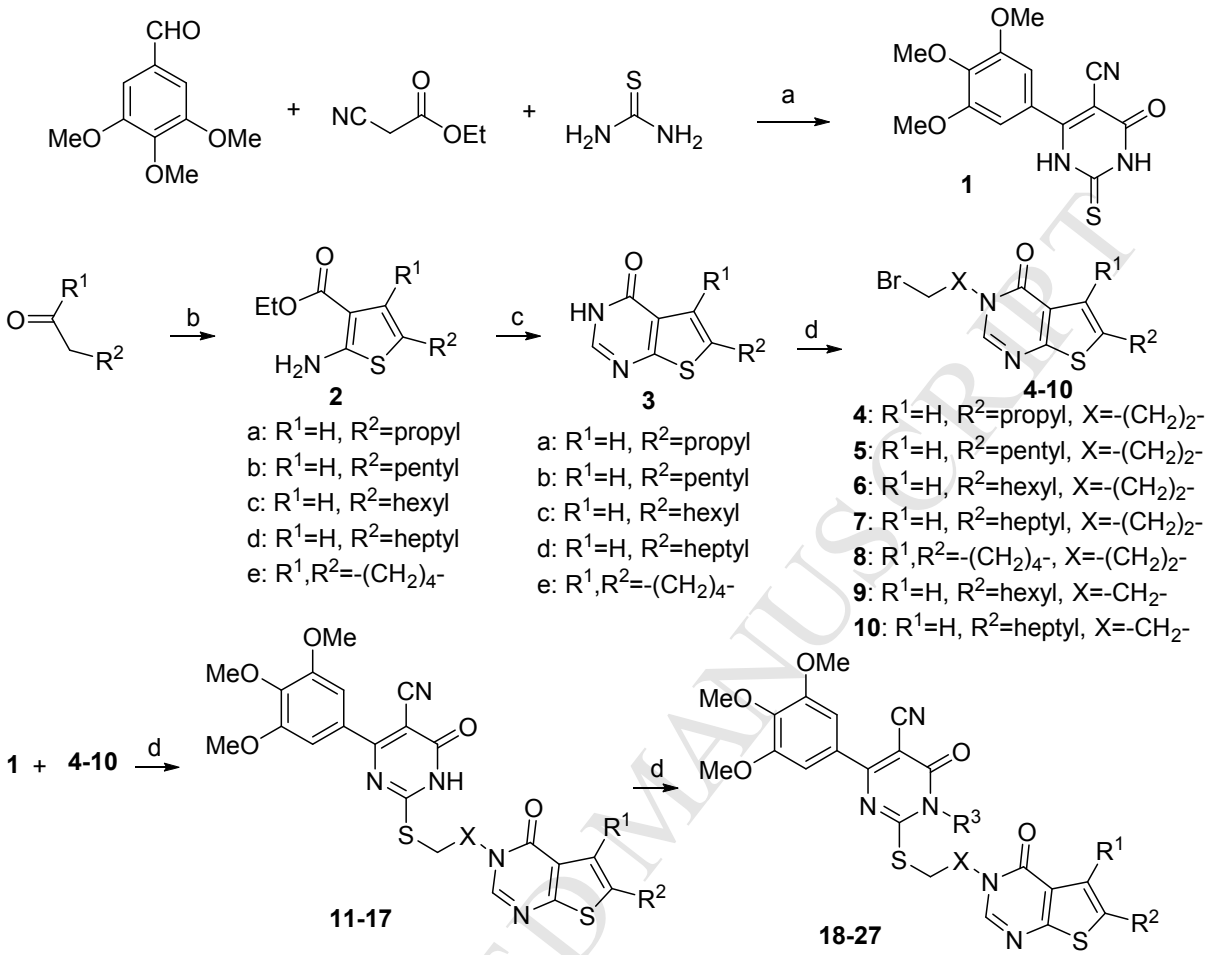
Tuberculosis is an infectious disease that claims a number of deaths paralleled only by those from HIV/AIDS [1]. Development of new antitubercular drugs is very important due to occurrence of multidrug resistant tuberculosis (MDR-TB) and emergence of extensively drug-resistant tuberculosis (XDR-TB). However, tuberculosis is one of the neglected tropical diseases (NTDs), a diverse group of communicable diseases that prevail in tropical and subtropical conditions which affect populations living in poverty, without adequate sanitation and in close contact with infectious vectors. The development of new antitubercular drugs is very slow due to lack of adequate funding. As a result, after a gap of 40 years, the U.S. Food and Drug Administration (FDA) approved bedaquiline [2,3] in December 2012 as part of combination therapy in adults to treat pulmonary MDR-TB, followed by the interim guidance on the use of delamanid [4] in 2014. New drug development is a continuous, lengthy process and it is necessary to synthesize and screen a large number of chemical entities as a slight modification in the structure can cause dramatic decrease/increase in the biological activity and the same is applicable to the development of antitubercular agents [5-8]. As a part of a program to develop new drugs, we have synthesized a number of new molecules containing thienopyrimidinone moiety and have found that some of them exhibited promising antitubercular activity [~~5,6~~9,10] while some exhibited antifungal activity [~~7,8~~11,12]. We wished to explore the potential of hybrid molecules containing thienopyrimidinones and thiouracils (which are known to exhibit various biological activities [~~9-14~~13-18] including anticancer, anti-inflammatory, antibacterial, antifungal *etc*) as the hybrid molecules are reported to have better activity in many cases [~~15~~19]. Accordingly, various new molecules **11**-**15** and **18**-**27** were synthesized and characterized with the help of spectral methods. Structure of the representative molecule **18** was confirmed with X-ray crystallography. These molecules were screened against *Mycobacterium tuberculosis* H37Ra (ATCC 25177) wherein it was observed that the compounds **11**-**15**, **18**, **22** and **25** exhibited significant antitubercular activity. Based on the docking study results for compound **15**, compounds **16** and **17** were prepared and were found to be more active than the compound **15** and the results are reported herein.

2. Results and discussion

2.1. Chemistry

The synthetic route for various compounds in the present study is shown in **Scheme 1**. The intermediate 4-oxo-2-thioxo-6-(3,4,5-trimethoxyphenyl)-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (**1**) was prepared by reaction of 3,4,5-trimethoxybenzaldehyde, ethyl cyanoacetate and thiourea by the known method [~~13~~17]. The thienopyrimidinones **3a-e** were prepared by the methods described in our earlier work [~~5-8~~9-12] involving Gewald reaction of required aldehyde/ketone with ethyl cyanoacetate and sulphur in DMF in the presence of triethylamine to get the corresponding substituted ethyl 2-aminothiophene-3-carboxylate followed by reaction with formamide in the presence of ammonium acetate. The bromides **4-10** were obtained from thienopyrimidinones **3a-e** by reacting them with corresponding dibromoalkanes in the presence of base. The reactions of 4-oxo-2-thioxo-6-(3,4,5-trimethoxyphenyl)-1,2,3,4-

tetrahydropyrimidine-5-carbonitrile (**1**) with bromides **4-8** in DMF in the presence of potassium carbonate at room temperature afforded the hybrid molecules **11-15**. The compound **15** was reacted with various (un)substituted alkyl halides, propargyl bromide, (un)substituted benzyl bromides or *p*-toluenesulfonyl chloride in DMF in the presence of potassium carbonate at room temperature to obtain novel molecules **18-27**.



Scheme 1. Reagents and conditions: **a**: K_2CO_3, DMF, 110 ^oC, 3 h; **b**: Ethyl cyanoacetate, sulphur, triethyl amine, DMF, 55 ^oC, 12 h; **c**: Ammonium acetate, formamide, 145 ^oC, 12 h; **d**: Required halide, K_2CO_3, DMF, RT, 12 h.

The structure of representative molecule **18** was confirmed by X-ray crystallography [~~16~~20] and the ORTEP diagram is shown in **Figure 1**.

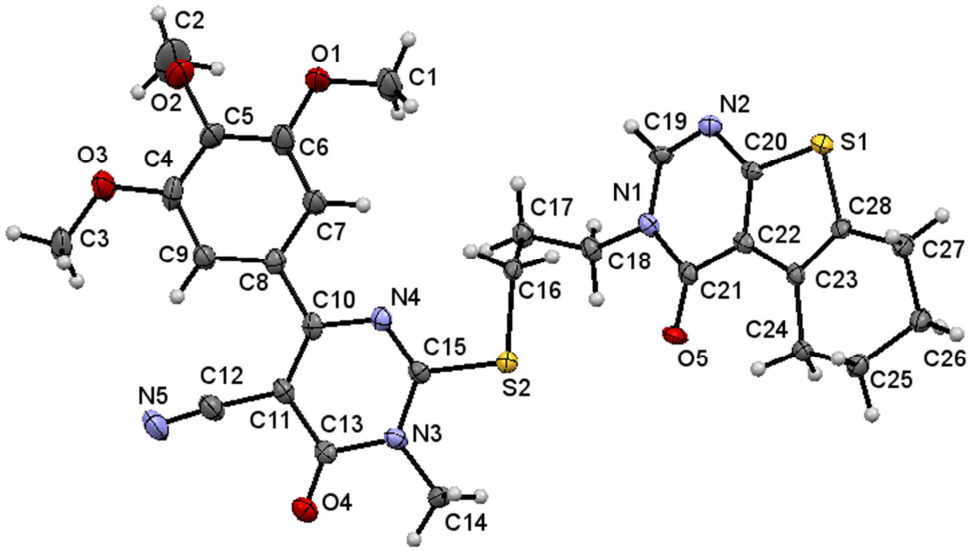


Figure 1. The Oak Ridge Thermal Ellipsoid Plot (ORTEP) of compound **18** showing the atom numbering scheme. The displacement ellipsoids are drawn at the 30% probability level and H-atoms are shown as small spheres with arbitrary radii (CCDC No. 1504297).

When the thiouracil **1** was reacted with more than one equivalents of the bromide **8**, the N,S-dialkylated compound **27** was obtained in addition to the S-alkylated compound **15**. The N,S-dialkylated compound **27** was also obtained by reacting the S-alkylated compound **15** with the bromide **8**.

Based on results of antitubercular activity screening and docking study of the above molecules, compounds **16** and **17** were synthesized by reactions similar to those used for the synthesis of compound **15**.

The 6-(4-hydroxyphenyl)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (**28**) and 4-oxo-2-thioxo-6-(p-tolyl)-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (**29**) were prepared by procedure [17] that was used for 4-oxo-2-thioxo-6-(3,4,5-trimethoxyphenyl)-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (**1**) wherein 3,4,5-trimethoxybenzaldehyde was replaced with 4-hydroxybenzaldehyde and 4-methylbenzaldehyde.

2.2. Evaluation of biological activity

The new molecules synthesized in the present work were screened for antitubercular activity (**Table 1**) against *Mycobacterium tuberculosis* H37Ra (ATCC 25177) by *in vitro* [21] and *ex vivo* methods [~~17–19~~22, 23].

Table 1. Antimycobacterial activity data for various hybrids (**11** to **27**) of thiouracils and thienopyrimidinones and thiouracils **1**, **28** and **29** against H37Ra

Entry no	Compound no	Intracellular *(Ex Vivo)*	Extracellular *(In Vitro)*	In vitro

		M. tuberculosis H37 Ra (Dormant Stage)	*M. tuberculosis* H37 Ra (Active Stage)	*M. tuberculosis* H37 Ra (Dormant Stage)	*M. tuberculosis* H37 Ra (Active Stage)	Cytotoxicity against THP-1 monocytes
		MIC (µg/mL)		MIC (µg/mL)		GI_{50} (µg/mL)
1	**11**	**11.9**±1.80	>30	**19.1**±0.16	>30	>100
2	**12**	**9.8**±0.81	>30	**12.3**±0.27	>30	>100
3	**13**	**7.1**±0.91	>30	**8.7**±1.11	>30	54.3±4.78
4	**14**	**6.91**±0.12	>30	**7.6**±0.38	>30	68.2±0.39
5	**15**	**26.1**±1.45	**28.8**±3.0	**23.4**±1.42	**25.4**±0.65	97.6±1.84
6	**16**	**18.8**±0.17	**17.1**±0.61	**19.1**±0.56	**15.6**±0.59	96.4±1.55
7	**17**	**13.6**±0.23	**11.1**±2.6	**17.1**±0.85	**11.1**±0.15	88.1±3.61
8	**18**	**21.5**±1.23	**26.9**±1.66	**26.1**±1.52	**27.9**±1.82	89.7±0.93
9	**19**	>30	>30	>30	>30	>100
10	**20**	>30	>30	>30	>30	>100
11	**21**	>30	>30	>30	>30	>100
12	**22**	**8.8**±0.20	>30	**11.7**±	>30	>100
13	**23**	>30	>30	>30	>30	>100
14	**24**	>30	>30	>30	>30	>100
15	**25**	**26.5**±1.62	>30	**28.3**±	>30	>100
16	**26**	>30	>30	>30	>30	>100
17	**27**	>30	>30	>30	>30	81.4±1.11
18	**1**	**11.3**±0.49	>30	**17.3**±0.44	>30	>100
19	**28**	>30	>30	>30	>30	93.0±1.35
20	**29**	>30	>30	>30	>30	88.9±1.92
21	**Rifampicin**	**0.75**±0.014	**0.51**±0.012	**0.48**±0.016	**0.41**±0.02	**0.1374**±0.12

Ex vivo: Intracellular antitubercular activities of each agent in differentiated THP-1 cells.

It was found that the compounds **15, 16, 17** and **18** exhibited very good antitubercular activity against dormant as well as active stage of *M. tuberculosis* H37Ra (MIC 11-29 µg/ml, 19-51 µM) while compounds **1, 11, 12, 13, 14, 22** and **25** exhibited very good antitubercular activity against dormant stage of *M. tuberculosis* H37Ra. The cytotoxicity studies [~~19-21~~24-25] against THP-1 monocytes (GI_{50} values >50 µg/mL) showed that these compounds were non-toxic. The compounds synthesized in the present study were further studied for cytotoxicity against three human cancer cell lines and the results are shown in **Table 2**.

Table 2. Cytotoxicity profile of various hybrids (**11** to **27**) of thiouracils and thienopyrimidinones and thiouracils **1**, **28** and **29** against human cancer cell lines

Entry no	**Comp no**	**A549 (Human lung)**	**PANC 1 (Human pancreas)**	**HeLa (Human**

				cervix)
		GI_{50} (µg/mL)	**GI_{50} (µg/mL)**	**GI_{50} (µg/mL)**
1	**11**	>100	>100	52.5±4.91
2	**12**	>100	72.8±3.81	75.8±4.32
3	**13**	90.2±2.73	28.1±7.10	67.0±2.76
4	**14**	90.5±3.54	>100	76.1±3.65
5	**15**	91.4±3.54	52.8±7.04	55.7±6.77
6	**16**	96.8±1.40	36.5±2.60	76.3±6.84
7	**17**	79.7±7.32	72.6±6.48	75.4±3.40
8	**18**	95.7±4.18	24.8±2.53	70.7±0.84
9	**19**	>100	>100	73.0±3.55
10	**20**	>100	>100	>100
11	**21**	>100	>100	>100
12	**22**	>100	>100	>100
13	**23**	>100	>100	66.3±1.83
14	**24**	>100	51.9±4.80	72.1±2.12
15	**25**	>100	>100	>100
16	**26**	>100	>100	64.2±3.91
17	**27**	83.1±4.42	>100	89.6±2.86
18	**1**	>100	>100	63.7±4.71
19	**28**	87.4±2.12	89.1±6.93	90.8±1.43
20	**29**	86.7±4.33	>100	67.6±3.12
21	**Paclitaxel**	0.0035±0.0014	0.1279±0.022	0.0048±0.0012

It was observed that the GI_{50} values for all compounds studied were >50 µg/mL indicating that the compounds ~~are~~were non-toxic.

The results of selectivity of hybrid molecules **11** to **27** towards human cell lines against H37Ra in terms of the selectivity index are shown in **Table 3**. The selectivity index reflects the concentration of the compound at which it is active against *mycobacteria* but is not toxic towards host cells. According to the study of Hartkoorn [26] on the drug susceptibility of TB, antimycobacterial activity was considered to be specific when the selectivity index was >10. Some of the compounds e.g. **14** and **22** showed SI >10 against dormant *M. tuberculosis* H37Ra, which were found to be good inhibitors of dormant *M. tuberculosis* H37Ra. Although the selectivity index values for other compounds studied in the present work were <10, it is important to consider the significance of this study with respect to the antitubercular activity exhibited by the compounds studied, flexibility of synthetic strategies used in the present work and occurrence of multidrug resistant tuberculosis (MDR-TB) as well as emergence of extensively drug-resistant tuberculosis (XDR-TB). As the synthetic strategies used in the present work have potential to prepare a large number of compounds for further refinement of structures, the present preliminary results will be very useful in the development of a new class of

antimycobacterial agents with selectivity index in the desired range by suitable structural modifications.

Table 3. Selectivity index ratio of various hybrids (**11** to **27**) of thiouracils and thienopyrimidinones and thiouracils **1**, **28** and **29** against three human cancer cell lines

Entry no	Comp no	SI against dormant *M. tuberculosis* H37Ra						SI against active *M. tuberculosis* H37Ra					
		A549		**PANC-1**		**HeLa**		**A549**		**PANC-1**		**HeLa**	
		A	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
1	**11**	>8	>5	>8	>5	4	3	>3	>3	>3	>3	2	2
2	**12**	>10	>8	>7	>6	8	6	>3	>3	>2	>2	3	3
3	**13**	13	10	4	3	9	8	3	3	1	1	2	2
4	**14**	13	12	14	13	11	10	3	3	3	3	3	3
5	**15**	3	4	2	2	2	2	3	4	2	2	2	2
6	**16**	5	5	2	2	4	4	6	6	2	2	4	5
7	**17**	6	5	5	4	6	4	7	7	6	7	7	7
8	**18**	4	4	1	1	3	3	4	3	1	1	3	3
9	**19**	>3	>3	>3	>3	2	2	>3	>3	>3	>3	2	2
10	**20**	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3
11	**21**	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3
12	**22**	>11	>8	>11	>8	>11	>8	>3	>3	>3	>3	>3	>3
13	**23**	>3	>3	>3	>3	2	2	>3	>3	>3	>3	2	2
14	**24**	>3	>3	2	2	2	2	>3	>3	2	2	2	2
15	**25**	>4	>4	>4	>4	>4	>4	>3	>3	>3	>3	>3	>3
16	**26**	>3	>3	>3	>3	2	2	>3	>3	>3	>3	2	2
17	**27**	3	3	>3	>3	3	3	3	3	>3	>3	3	3
18	**1**	>9	>6	>9	>6	6	4	>3	>3	>3	>3	2	2
19	**28**	3	3	3	3	3	3	3	3	3	3	3	3
20	**29**	3	3	>3	>3	2	2	3	3	>3	>3	2	2
21	**Rif**	133	208	133	208	133	208	196	244	196	244	196	244

A: *Ex vivo*; B: *In vitro*; Rif: Rifampicin

The antitubercular screening results indicated following points regarding the structure-activity relationship of the compounds studied in the present work.

1. The hybrid molecules **11** to **17** with free NH in thiouracil moiety exhibited antitubercular activity against *M. tuberculosis* H37Ra.

2. The compounds **11** to **14** with three-carbon linker between thienopyrimidinone and thiouracil moieties and having alkyl substituent on thiophene ring of thienopyrimidinone exhibited antitubercular activity against dormant stage of *M. tuberculosis* H37Ra while corresponding compound **15** with three-carbon linker between thienopyrimidinone and thiouracil moieties and having cycloalkyl substituent on thiophene ring of thienopyrimidinone exhibit~~s~~ed antitubercular activity against dormant as well as active stage of *M. tuberculosis* H37Ra.

3. The compounds **16** and **17** with two-carbon linker between thienopyrimidinone and thiouracil moieties and having alkyl substituent on thiophene ring of thienopyrimidinone also exhibited antitubercular activity against dormant as well as active stage of *M. tuberculosis* H37Ra.

4. Methyl group ~~is~~was tolerated on thiouracil moiety of compound **15** but alkylation with longer side chains result~~s~~ed in loss of activity (compound **15** v/s compound **18** v/s compounds **19**, **20** and **21**).

5. The reaction of **15** with propargyl bromide, benzyl bromide, p-toluenesulfonyl chloride *etc* result~~s~~ed in loss of activity indicating that free NH ~~is~~was preferred (compound **15** v/s compounds **23**, **24** and **26**). The compound **27** ~~is~~was also inactive supporting the above ~~observation~~indication.

6. The compounds **20** and **24** ~~are~~were inactive while the corresponding fluorinated compounds **22** and **25** ~~are~~were active against dormant stage of *M. tuberculosis* H37Ra indicating that introduction of fluorine atoms help~~s~~ed to get better antitubercular activity.

The structure-activity relationship studies clearly indicated that it would be possible to get molecules with better antitubercular activity/selectivity index by suitable structural modifications.

2.3. Docking Studies

In silico based approaches have provided a new perspective in the development of highly efficient chemical leads and have huge potential to impart as starting points in the development of new chemical entities against TB. So, docking studies were performed against mycobacterial pantothenate synthetase due to availability of limited resources ~~availability~~ for carrying out enzyme-based experimental studies to find out the best possible mode of action of the synthesized hybrids of thienopyrimidinones and thiouracil derivatives

A distinctive characteristic of *M. Tuberculosis* is that its cell wall is enriched with the lipids which are essential for its intracellular endurance, pathogenicity and also it is believed that it makes entry of antimicrobial agents in to the cells difficult [~~22~~27]. In *M. Tuberculosis* genome huge numbers of genes encoding several enzymes are involved in the metabolism of fatty acids [~~23~~28], so inhibition of this pathway can be an important target in antitubercular drug discovery. The enzyme pantothenate synthetase (PS or PanC) is encoded by the gene panC, which is important for the synthesis of pantothenate in bacteria [~~22~~27]. Pantothenate is required for biosynthesis of coenzyme A (CoA) and acyl carrier protein (ACP) which are the important

elements for fatty acid synthesis [~~23~~28]. In the *in vitro* studies it was found that the gene encoding panC is essential for the optimum growth of bacteria and when it was genetically broken down in *M. tuberculosis*, it made the strain auxotrophic and required supplementation of pantothenate for its growth [~~24-26~~29-31]. Also, its pathogenicity is weakened in this strain [~~27~~32]. In mammals, panC is not present [~~28,29~~33,34] so targeting this enzyme will have huge potential for developing drugs which will not have any side effects in the hosts. Thus, it makes pantothenate synthetase an important target for drug discovery against tuberculosis (TB). To continue our ongoing endeavor for discovering new potent antitubercular agents [~~6~~10], this study provides~~d~~ valuable guidance for rationally designing more potent inhibitors for treatment of TB.

The antitubercular activity exhibited by compounds **11-15** prompted us to carry out docking studies [35-40] of compound **15** (**Figure 2**). From the docking studies (details given in supporting information), it ~~is~~was clear that compound **15** ~~is~~was showing hydrogen bonding interactions with Gln72 with the distance of 2.10A° and 2.18A° and with Gly158 with the distance of 2.16A°. Also, it ~~is~~was observed that thiophene ring showse~~d~~ weak π- π stacking interaction with amino acid Arg198 which ~~leads~~led to weak binding of compound **15** in the active site of PanC with the docking score of -5.863.

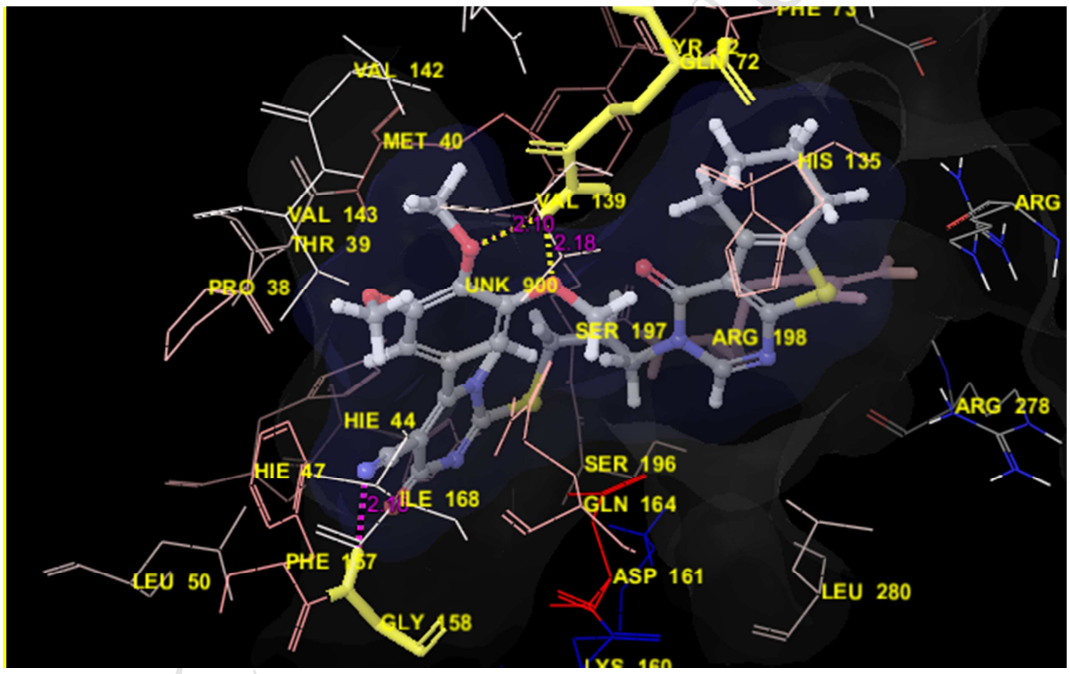


Figure 2. 3D view of binding of compound **15** with the active site of mycobacterial PanC

Encouraged by the antitubercular activity of compounds **11-15** and docking study of compound **15** indicating its binding in the active site of pantothenate synthetase, compounds **16** and **17** were designed and subjected to docking studies (details given in supplementary information). The docking scores of compounds **16** and **17** were -7.949 and -8.666 respectively. Molecular binding interactions (3D and 2D views) of compound **17** with the active site of mycobacterial pantothenate synthetase are shown in **Figures 3** and **4**.

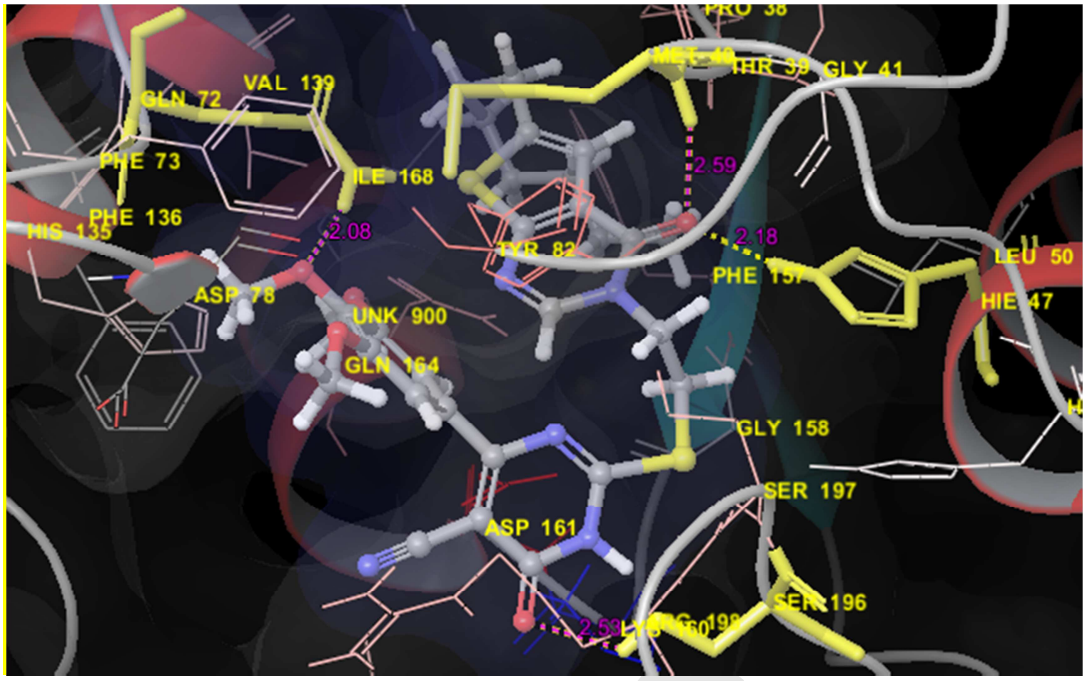


Figure 3. 3D view of the binding of compound **17** with the active site of mycobacterial pantothenate synthetase

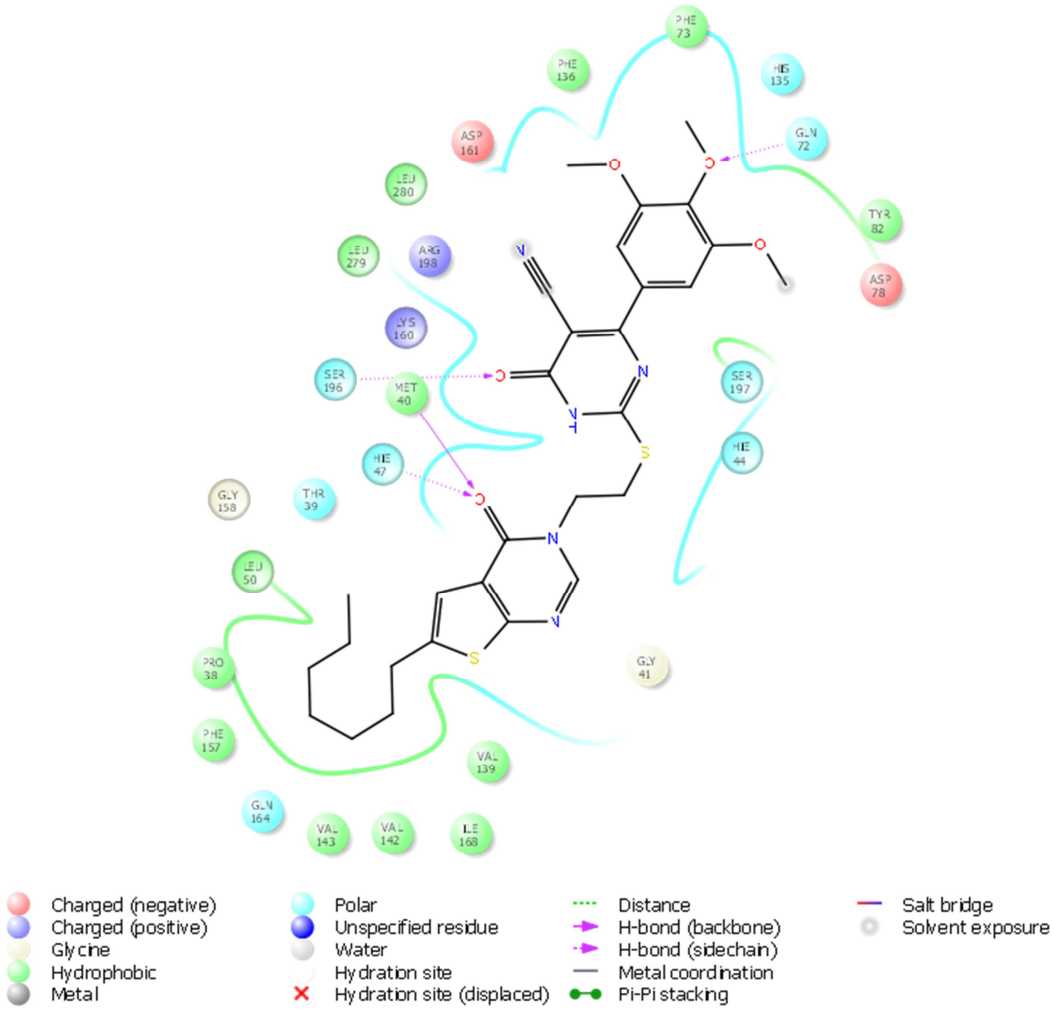


Figure 4. 2D view of the binding of compound **17** with the active site of mycobacterial pantothenate synthetase

From the lowest energy docking pose of compound **17**, it was noticed that strong hydrogen bonding interactions were observed between methoxy groups present on the phenyl ring and amino acid residue Gln72 with the distance of 2.08A°. Oxo group attached to the pyrimidine ring in compound **17** showsed the hydrogen bonding with Ser196 with the distance of 2.53A°. Oxo group attached to thienopyrimidinone showsed the hydrogen bonding interactions with the amino acid residue Met40 with the distance of 2.59A°. Further, the heptyl group attached to thienopyrimidinone ring of compound **17** ~~fits~~fitted into hydrophobic pocket formed by amino acid residues Phe157, Val142, Val143 and Ile168 present within the active site of mycobacterial pantothenate synthetase as seen in **Figure 4**. The strong hydrogen bonding and hydrophobic interaction ~~leads~~led to orientation of ligands within the active site so that they formed strong steric and electrostatic interactions with the amino acid residues present in the active site of mycobacterial pantothenate synthetase.

The ~~compound~~ compound **17** showsed firm binding with the active site of mycobacterial pantothenate synthetase with the binding energy of -54.413 kcal/mol. Many favorable van der

Waals interactions (details given in supporting information) were observed with amino acid residues present in the active site. Also, several strong electrostatic interactions (details given in supporting information) were seen with amino acid residues which stabilized the compound **17** in to the active site of mycobacterial pantothenate synthetase.

Validation of docking procedure: Co-crystallized ligand FG6 present in the active site of mycobacterial pantothenate synthetase was extracted and was docked again into the active site. Root mean square deviation (RMSD) value was found to be below 1.5Å which validatesed our docking studies.

Accordingly, compounds **16** and **17** were synthesized and screened for antitubercular activity (Table 1, entries 6 and 7) wherein it was found that these compounds were more active than the corresponding compounds **13** and **14** as well as compound **15**. Thus, from the docking studies and antitubercular activity results, it ~~is~~was clear that hybrids of thienopyrimidinones and thiouracils ~~have~~had significant binding with the active site of mycobacterial pantothenate synthetase. So, the mechanism of action for antitubercular activity of these compounds might be through the inhibition of mycobacterial pantothenate synthetase.

3. Conclusions

Synthesis of hybrid molecules containing thiouracil and thienopyrimidinone moieties was achieved. The new chemical entities thus synthesized were tested against *Mycobacterium tuberculosis* H37Ra and it was observed that the compounds **11-14** exhibited antitubercular activity against dormant stage while compound **15** exhibited antitubercular activity against dormant as well as active stage. Structural modifications of compound **15** were carried out to study the structure-activity relationship and it was observed that compound **18** exhibited antitubercular activity comparable to compound **15** while compounds **22** and **25** exhibited antitubercular activity against dormant stage. Cytotoxicity studies revealed that these molecules were non-toxic. The docking study of compound **15** showed that there ~~is~~was binding with the active site of mycobacterial pantothenate synthetase with the docking score of -5.863. Further, the compounds **16** and **17** were designed based on docking studies and their docking scores were found to be -7.949 and -8.666 respectively indicating the possibility of these compounds to be more active. The synthesis and antitubercular activity screening of the compounds **16** and **17** was carried out and it was found that these compounds were having potent antitubercular activity supporting the binding of these compounds with mycobacterial pantothenate synthetase. Hence, the compounds **15-18** can be used as starting points for further optimization. The selectivity index for the compounds studied needs to be improved but t~~T~~he synthetic strategies used in the present work have potential to prepare a large number of compounds for further refinement of structures and the present results will be very useful in the development of new class of antimycobacterial agents.

4. Experimental section

4.1. Chemistry

All reagents and solvents were used as received from the manufacturers. Melting points are recorded in capillary tubes and are uncorrected and the temperatures are in centigrade scale. ^{1}H (200, 400 and 500 MHz) and ^{13}C (50, 100 and 125 MHz) NMR spectra were recorded on AC

200 MHz, AV 400 MHz or AV-500 MHz NMR spectrometers using $CDCl_3$ or DMSO-d_6 as solvent. The chemical shifts (δ) and coupling constants (Hz) are reported in the standard fashion with reference to chloroform, δ 7.27 (for ^1H) or the central line (77.0 δ) of $CDCl_3$ (for ^{13}C). In the ^{13}C NMR spectra, the natures of the carbons (C, CH, CH_2, or CH_3) were determined by recording the DEPT-135 spectra. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. FTIR spectra were recorded using KBr plate. The mass spectra were recorded on Orbitrap MS (LC-HRMS). The reaction progress was monitored by the TLC analysis using thin layer plates precoated with silica gel 60 F_{254} and visualized by UV light or iodine or by charring after treatment with *p*-anisaldehyde.

Synthesis of molecules

The thiouracils **1**, **28** and **29** were prepared by the reported procedure [~~13~~17].

4-Oxo-2-thioxo-6-(3,4,5-trimethoxyphenyl)-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (1)
Yield: 82%, White solid, Melting point: 283 °C. ^1H NMR (200 MHz, $CDCl_3$ + DMSO-d_6): δ 3.47 (s, 3H), 3.51 (s, 6H), 6.60 (s, 2H). ^{13}C NMR (50 MHz, $CDCl_3$ + DMSO-d_6): δ 55.2 (2C), 59.6, 62.4, 105.4 (2C), 113.6, 122.3, 140.2, 151.7 (2C), 157.6, 158.9, 175.3. IR ($CHCl_3$): 1214, 1689, 2405, 3417 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{14}H_{13}N_3O_4S$+H]: 320.0700, found: 320.0696; [$C_{14}H_{13}N_3O_4S$ +Na]: 342.0519, found: 342.0516.

6-(4-Hydroxyphenyl)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (28)
Yield: 75%, White solid, Melting point: >295 °C. ^1H NMR (200 MHz, $CDCl_3$ + DMSO-d_6): δ 6.00 (d, *J* = 8 Hz, 2H), 6.94 (d, *J* = 8 Hz, 2H), 9.06 (bs, 1H). ^{13}C NMR (50 MHz, $CDCl_3$ + DMSO-d_6): δ 82.8, 113.0 (2C), 117.4, 126.1, 128.6 (2C), 158.0, 161.6, 165.3, 180.3. IR ($CHCl_3$): 1225, 1625, 2209 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{11}H_7N_3O_2S$+H]: 246.0332 found: 246.0330.

4-Oxo-2-thioxo-6-(p-tolyl)-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (29)
Yield: 79%, White solid, Melting point: >295 °C. ^1H NMR (200 MHz, DMSO - d_6): δ 2.40 (s, 3H), 7.39 (d, *J* = 8 Hz, 2H), 7.60 (d, *J* = 8 Hz, 2H), 13.05 (bs, 1H). ^{13}C NMR (50 MHz, DMSO-d_6): δ 21.4, 90.6, 115.1, 126.6, 129.0 (2C), 129.2 (2C), 142.8, 158.8, 161.2, 176.4. IR ($CHCl_3$): 1217, 1675, 2232, 3317 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{12}H_9N_3OS$+H]: 244.0538, found: 244.0539; [$C_{12}H_9N_3OS$ + Na]: 266.0357, found: 266.0359.

Synthesis of 6-oxo-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (15)
Potassium carbonate (6.48 g, 0.047 mol, 1.5 eq) was taken in a 500 mL two-necked RB flask and heated under vacuum to remove the traces of moisture and flushed with nitrogen. 4-Oxo-2-thioxo-6-(3,4,5-trimethoxyphenyl)-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (**1**) (10 g, 0.031 mol, 1.0 eq) was added under nitrogen followed by dry DMF (125 mL) and stirred for 10 min. 3-(3-Bromopropyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (10.20 g, 0.031 mol, 1.0 eq) was added and the reaction mixture was stirred at RT for 12 h. It was then diluted with water (400 mL) and extracted with ethyl acetate (3 x 200 mL). The combined organic layer

was dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure. The residue was purified by column chromatography to afford 6-oxo-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (**15**) as off-white solid, 12.0 g (68%). Melting point: 230 °C.

^{1}H NMR (200 MHz, $CDCl_3$): δ 1.73-1.91 (m, 4H), 2.33 (t, *J* = 7 Hz, 2H), 2.75 (t, *J* = 6 Hz, 2H), 2.94 (t, *J* = 6 Hz, 2H), 3.40 (t, *J* = 7 Hz, 2H), 3.89 (s, 6H), 3.94 (s, 3H), 4.16 (t, *J* = 7 Hz, 2H), 7.33 (s, 2H), 7.99 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 21.4, 22.0, 24.4, 24.8, 27.2, 27.9, 44.3, 55.6 (2C), 60.1, 92.1, 105.8 (2C), 115.5, 121.8, 129.4, 130.6, 133.5, 140.4, 145.0, 152.2 (2C), 157.0, 161.3, 161.6, 164.7, 166.0. IR ($CHCl_3$): 1217, 1243, 1550, 1668, 2210, 2857, 2933, 3429 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{27}H_{27}N_5O_5S_2$+H]: 566.1517, found: 566.1526.

The following compounds were prepared by using procedure described for compound **15**:

6-Oxo-2-((3-(4-oxo-6-propylthieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (11)

Yield: 73%, White solid, Melting point: 185 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.91 (t, *J* = 7 Hz, 3H), 1.40-1.70 (m, 2H), 2.19 (s, 2H), 2.70 (t, *J* = 6 Hz, 2H), 3.23 (s, 2H), 3.76 (s, 6H), 3.85 (s, 3H), 4.11 (s, 2H), 7.04 (s, 1H), 7.15 (s, 2H), 8.01 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 12.7, 23.4, 26.9, 28.1, 31.6, 44.6, 55.4 (2C), 59.9, 90.5, 105.4 (2C), 116.9, 117.3, 123.6, 130.2, 139.6, 143.4, 145.3, 150.2, 152.0 (2C), 156.4, 161.6, 166.1, 167.6. IR ($CHCl_3$): 1215, 1683, 2210, 3450 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{26}H_{27}N_5O_5S_2$+H]: 554.1526, found: 554.1526; [$C_{26}H_{27}N_5O_5S_2$ +Na]: 576.1344, found: 576.1346.

6-Oxo-2-((3-(4-oxo-6-pentylthieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (12)

Yield: 78%, White solid, Melting point: 183 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.85 (t, *J* = 7 Hz, 3H), 1.15-1.40 (m, 4H), 1.60 (t, *J* = 7 Hz, 2H), 2.18 (s, 2H), 2.72 (t, *J* = 7 Hz, 2H), 3.22 (s, 2H), 3.77 (s, 6H), 3.86 (s, 3H), 4.11 (s, 2H), 7.05 (s, 1H), 7.14 (s, 2H), 8.00 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.9, 22.3, 28.0, 28.7, 30.5, 30.7, 31.1, 45.5, 56.1 (2C), 60.8, 90.3, 106.2 (2C), 117.9, 118.3, 118.4, 124.4, 130.3, 140.8, 145.3, 145.7, 152.7 (2C), 157.4, 162.5, 167.0, 167.1. IR ($CHCl_3$): 1257, 1651, 2215, 2932, 3415 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{28}H_{31}N_5O_5S_2$+H]: 582.1838, found: 582.1839.

2-((3-(6-Hexyl-4-oxothieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-6-oxo-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (13)

Yield: 81%, White solid, Melting point: 293 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.88 (t, *J* = 7 Hz, 3H), 1.20-1.45 (m, 6H), 1.57-1.78 (m, 2H), 2.35 (t, *J* = 7 Hz, 2H), 2.82 (t, *J* = 7 Hz, 2H), 3.42 (t, *J* = 7 Hz, 2H), 3.90 (s, 6H), 3.94 (s, 3H), 4.20 (t, *J* = 7 Hz, 2H), 7.11 (s, 1H), 7.34 (s, 2H), 8.03 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.0, 22.5, 28.3, 28.4, 28.6, 30.6, 31.0, 31.4, 45.4, 56.3 (2C), 61.0, 92.5, 106.7 (2C), 115.5, 115.6, 118.0, 124.6, 129.4, 141.8, 145.4, 145.6, 145.9, 153.0 (2C), 157.4, 162.3, 167.5. IR ($CHCl_3$): 1216, 1258, 1652, 2218, 3418 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{29}H_{33}N_5O_5S_2$+H]: 596.1995, found: 596.1996; [$C_{29}H_{33}N_5O_5S_2$+Na]: 618.1813, found: 618.1815.

2-((3-(6-Heptyl-4-oxothieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-6-oxo-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (14)

Yield: 83%, White solid, Melting point: 187 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.83 (t, J = 6 Hz, 3H), 1.16-1.33 (m, 8H), 1.59 (t, J = 7 Hz, 2H), 2.17 (t, J = 7 Hz, 2H), 2.79 (t, J = 7 Hz, 2H), 3.27 (t, J = 7 Hz, 2H), 3.76 (s, 3H), 3.83 (s, 6H), 4.10 (t, J = 7 Hz, 2H), 7.06 (s, 1H), 7.29 (s, 2H), 8.36 (s, 1H). ^{13}C NMR (200 MHz, DMSO-d_6): δ 14.1, 22.2, 27.8, 28.47, 28.50, 28.7, 29.9, 30.8, 31.4, 44.8, 56.2 (2C), 60.4, 92.2, 106.6 (2C), 116.8, 118.4, 124.0, 130.5, 140.6, 143.7, 147.7, 152.7 (2C), 156.7, 162.1, 162.2, 166.3, 166.5. IR ($CHCl_3$): 1255, 1655, 2214, 3418 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{30}H_{35}N_5O_5S_2$+H]: 610.2151, found: 610.2152; [$C_{30}H_{35}N_5O_5S_2$ +Na]: 632.1968, found: 632.1972.

2-((3-(6-Hexyl-4-oxothieno[2,3-*d*]pyrimidin-3(4*H*)-yl)ethyl)thio)-6-oxo-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (16)

Yield: 80%, White solid, Melting point: 125 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.86 (t, J = 7 Hz, 3H), 1.13-1.40 (m, 6H), 1.41-1.70 (m, 2H), 2.70 (t, J = 7 Hz, 2H), 3.64 (s, 2H), 3.87 (s, 6H), 3.93 (s, 3H), 4.39 (s, 2H), 6.89 (s, 1H), 7.16 (s, 2H), 8.15 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.0, 22.6, 29.0, 29.2, 29.5, 30.4, 30.9, 31.8, 56.1 (2C), 60.9, 106.4 (2C), 116.1, 117.8, 124.3, 129.0, 141.41, 141.42, 145.05, 145.08, 146.02, 146.03, 152.7 (2C), 157.0, 162.1, 167.6. IR ($CHCl_3$): 1223, 1672, 2213, 3403 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{28}H_{31}N_5O_5S_2$+H]: 582.1839, found: 582.1839.

2-((2-(6-Heptyl-4-oxothieno[2,3-*d*]pyrimidin-3(4*H*)-yl)ethyl)thio)-6-oxo-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (17)

Yield: 77%, White solid, Melting point: 130 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.84 (s, 3H), 1.22 (s, 8H), 1.53 (s, 2H), 2.65 (t, J = 6 Hz, 2H), 3.41 (s, 2H), 3.81 (s, 6H), 3.89 (s, 3H), 4.25 (s, 2H), 6.95 (s, 1H), 7.08 (s, 2H), 8.03 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 14.0, 22.5, 28.6, 28.9, 29.0, 30.5, 31.1, 31.7, 47.4, 56.0 (2C), 60.8, 88.5, 105.8 (2C), 117.7, 121.1, 124.1, 131.1, 139.9, 145.5, 152.7 (2C), 157.4, 162.8, 167.1, 167.2, 172.4, 174.7. IR ($CHCl_3$): 1227, 1666, 2219, 3412 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{29}H_{33}N_5O_5S_2$+H]: 596.1996, found: 596.1985.

Synthesis of 1-methyl-6-oxo-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (18)

Potassium carbonate (243 mg, 1.76 mmol, 2.0 eq) was taken in a 100 mL two-necked RB flask and heated under vacuum to remove the traces of moisture and flushed with nitrogen. 6-Oxo-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (**15**) (500 mg, 0.88 mmol, 1.0 eq) was added under nitrogen followed by dry DMF (1.5 mL) and stirred for 10 min. Iodomethane (0.08 ml, 188 mg, 1.32 mmol, 1.5 eq) was added by microlitre syringe and the reaction mixture was stirred at RT for 12 h. It was then diluted with water (10 mL) and extracted with ethyl acetate (3 x 10 mL). The combined organic layer was dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure. The residue was purified by column chromatography to afford 1-methyl-6-oxo-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (**18**) as off-white solid, 435 mg (85%). Melting point: 185 °C.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.75-1.91 (m, 4H), 2.22-2.41 (m, 2H), 2.76 (t, *J* = 6 Hz, 2H), 2.95 (t, *J* = 6 Hz, 2H), 3.42 (t, *J* = 7 Hz, 2H), 3.55 (s, 3H), 3.90 (s, 6H), 3.92 (s, 3H), 4.13 (t, *J* = 7 Hz, 2H), 7.33 (s, 2H), 7.88 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 22.1, 22.7, 25.1, 25.5, 28.3, 29.8, 30.9, 44.9, 56.3 (2C), 60.9, 92.1, 106.5 (2C), 115.7, 122.6, 129.5, 131.4, 134.7, 141.5, 145.0, 153.0 (2C), 157.8, 160.2, 161.9, 164.5, 165.3. IR ($CHCl_3$): 1218, 1669, 2219, 2358, 3411 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{28}H_{29}N_5O_5S_2$+H]: 580.1674, found: 580.1683; [$C_{28}H_{29}N_5O_5S_2$ +Na]: 602.1490, found: 602.1502.

The following compounds were prepared by using procedure described for compound **18** wherein the required halide was used in place of iodomethane:

6-Oxo-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-1-pentyl-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (19)

Yield: 74%, White solid, Melting point: 86 ºC. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.93 (t, *J* = 7 Hz, 3H), 1.30-1.52 (m, 4H), 1.70-1.96 (m, 6H), 2.20-2.42 (m, 2H), 2.67-2.85 (m, 2H), 2.91-3.06 (m, 2H), 3.26 (t, *J* = 7 Hz, 2H), 3.93 (s, 9H), 4.13 (t, *J* = 7 Hz, 2H), 4.46 (t, *J* = 7 Hz, 2H), 7.33 (s, 2H), 7.88 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 13.9, 22.1, 22.3, 22.8, 25.2, 25.6, 27.9, 28.1 (2C), 28.5, 45.3, 56.3 (2C), 60.9, 68.8, 87.6, 106.4 (2C), 114.9, 122.7, 129.8, 131.4, 134.4, 141.2, 145.2, 153.1 (2C), 157.7, 162.0, 167.8, 169.8, 174.0. IR ($CHCl_3$): 1217, 1247, 1468, 1536, 1669, 2219, 2358, 2932 3411 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{32}H_{37}N_5O_5S_2$+H]: 636.2297, found: 580.1683; [$C_{32}H_{37}N_5O_5S_2$ +Na]: 658.2114, found: 658.2128.

1-Octyl-6-oxo-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (20)

Yield: 79%, White solid, Melting point: 78 ºC. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.89 (t, *J* = 7 Hz, 3H), 1.21-1.52 (m, 12H), 1.75-1.95 (m, 6H), 2.25-2.38 (m, 2H), 2.79 (t, *J* = 7 Hz, 2H), 3.00 (t, *J* = 7 Hz, 2H), 3.27 (t, *J* = 7 Hz, 2H), 3.94 (s, 9H), 4.14 (t, *J* = 7 Hz, 2H), 4.47 (t, *J* = 7 Hz, 2H), 7.34 (s, 2H), 7.89 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1, 22.2, 22.6, 22.8, 25.2, 25.6, 25.8, 28.2, 28.5 (2C), 29.16, 29.23, 31.8, 45.3, 56.3 (2C), 61.0, 68.9, 87.7, 106.3 (2C), 115.0, 122.7, 129.9, 131.5, 134.6, 141.1, 145.2, 153.1 (2C), 157.8, 162.0, 167.9, 169.9, 174.0. IR ($CHCl_3$): 1197, 1230, 1450, 1530, 1677, 2209, 2911, 3409 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{35}H_{43}N_5O_5S_2$+H]: 678.2765, found: 678.2778.

6-Oxo-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-4-(3,4,5-trimethoxyphenyl)-1-undecyl-1,6-dihydropyrimidine-5-carbonitrile (21)

Yield: 78%, White solid, Melting point: 74 ºC. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.87 (t, *J* = 7 Hz, 3H), 1.15-1.55 (m, 16H), 1.70-1.94 (m, 6H), 2.19-2.40 (m, 2H), 2.68-2.85 (m, 2H), 2.89-3.06 (m, 2H), 3.26 (t, *J* = 7 Hz, 2H), 3.93 (s, 9H), 4.13 (t, *J* = 7 Hz, 2H), 4.45 (t, *J* = 7 Hz, 2H), 7.33 (s, 2H), 7.88 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 14.0, 22.1, 22.6, 22.8, 25.1, 25.6, 25.8, 28.2, 28.45, 28.53, 29.2 (2C), 29.4, 29.5 (2C), 31.8, 45.3, 56.3 (2C), 60.9, 68.8, 87.6, 106.4 (2C), 114.9, 122.6, 129.8, 131.4, 134.4, 141.2, 145.2, 153.1 (2C), 157.7, 162.0, 167.8, 169.8, 173.9. IR ($CHCl_3$):1247, 1280, 1422, 1542, 1638, 2139, 2891, 3401 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{38}H_{49}N_5O_5S_2$+H]: 720.3248, found: 720.3242; [$C_{38}H_{49}N_5O_5S_2$ +Na]:742.3067, found: 742.3060.

6-Oxo-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-1-(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (22)

Yield: 64%, White solid, Melting point: 140 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.73-1.96 (m, 4H), 2.21-2.42 (m, 2H), 1.54-1.87 (m, 4H), 2.89-3.07 (m, 2H), 3.28 (t, *J* = 7 Hz, 2H), 3.94 (s, 9H), 4.13 (t, *J* = 7 Hz, 2H), 4.82 (t, *J* = 7 Hz, 2H), 7.35 (s, 2H), 7.88 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 22.2, 22.8, 25.2, 25.6, 28.3, 28.6, 29.7, 30.5 (t), 45.3, 56.3 (2C), 61.0, 87.6, 106.5 (2C), 108.0-119.7 (m, 6C), 114.4, 122.7, 129.5, 131.5, 134.6, 141.5, 145.2, 153.2 (2C), 157.8, 162.0, 168.1, 169.1, 174.1. IR ($CHCl_3$): 760, 1250, 1301, 1432, 1492, 1608, 2149, 2931, 3411 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{35}H_{30}N_5O_5F_{13}S_2$+H]: 912.1553, found: 912.1554; [$C_{35}H_{30}N_5O_5F_{13}S_2$+Na]: 934.1371, found: 934.1373.

6-Oxo-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-1-(prop-2-yn-1-yl)-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (23)

Yield: 71%, White solid, Melting point: 171 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.79-1.91 (m, 4H), 2.28-2.37 (m, 2H), 2.55 (t, *J* = 2 Hz, 1H), 2.78 (t, *J* = 6 Hz, 2H), 2.99 (t, *J* = 6 Hz, 2H), 3.28 (t, *J* = 7 Hz, 2H), 3.93 (s, 9H), 4.14 (t, *J* = 7 Hz, 2H), 5.12 (d, *J* = 2 Hz, 2H), 7.34 (s, 2H), 7.90 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 22.2, 22.8, 25.2, 25.6, 28.3, 28.7, 45.4, 55.6, 56.3 (2C), 61.0, 76.2, 76.9, 87.6, 106.5 (2C), 114.5, 122.7, 129.5, 131.5, 134.5, 141.4, 145.2, 153.2 (2C), 157.8, 162.0, 168.2, 168.8, 174.2. IR ($CHCl_3$): 1220, 1655, 2219, 2230 2355, 3410 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{30}H_{29}N_5O_5S_2$+H]: 604.1675, found: 604.1683; [$C_{30}H_{29}N_5O_5S_2$+Na]: 626.1491, found: 626.1502.

1-Benzyl-6-oxo-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (24)

Yield: 64%, White solid, Melting point: 110 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.65-2.00 (m, 4H), 2.13-2.44 (m, 2H), 2.59-2.84 (m, 2H), 2.88-3.11 (m, 2H), 3.37 (t, *J* = 7 Hz, 2H), 3.90 (s, 6H), 3.93 (s, 3H), 4.04 (t, *J* = 6 Hz, 2H), 5.30 (s, 2H), 7.28-7.56 (m, 7H), 7.75 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 22.1, 22.7, 25.2, 25.6, 28.1, 30.0, 45.0, 48.0, 56.4 (2C), 61.0, 92.7, 106.6 (2C), 115.6, 122.6, 128.1 (2C), 128.4, 128.8 (2C), 129.4, 131.4, 133.6, 134.7, 141.7, 145.0, 153.0 (2C), 157.7, 160.5, 162.0, 164.5, 165.0. IR ($CHCl_3$): 1217, 1241, 1455, 1527, 1538, 1624, 1680, 2213, 2901, 3410 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{34}H_{33}N_5O_5S_2$+H]: 656.1981, found: 656.1972.

6-Oxo-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-1-((perfluorophenyl)methyl)-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (25)

Yield: 76%, White solid, Melting point: 138 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.73-2.00 (m, 4H), 2.21-2.45 (m, 2H), 2.75 (t, *J* = 5 Hz, 2H), 2.94 (t, *J* = 5 Hz, 2H), 3.31 (t, *J* = 7 Hz, 2H), 3.93 (s, 9H), 4.14 (t, *J* = 6 Hz, 2H), 5.60 (s, 2H), 7.32 (s, 2H), 7.90 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 22.1, 22.7, 25.1, 25.5, 28.3, 28.8, 45.4, 56.3 (2C), 56.8, 60.9, 87.4, 106.5 (2C), 108.4 (dt), 114.3, 122.6, 129.4, 131.4, 134.5, 136.5-146.8 (m, 5C), 141.5, 145.2, 153.1 (2C), 157.7, 162.0, 168.2, 168.8, 174.1. IR ($CHCl_3$):1233, 1321, 1442, 1502, 1628, 2159, 2922, 3401 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{34}H_{28}N_5O_5F_5S_2$+Na]: 768.1334, found: 768.1344.

6-Oxo-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-1-tosyl-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (26)

Yield: 58%, White solid, Melting point: 163 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.69-2.01 (m, 4H), 2.23-2.44 (m, 2H), 2.51 (s, 3H), 2.78 (t, *J* = 6 Hz, 2H), 3.02 (t, *J* = 6 Hz, 2H), 3.26 (t, *J* = 7 Hz, 2H), 3.93 (s, 9H), 4.10-4.28 (m, 2H), 7.32 (s, 2H), 7.47 (d, *J* = 8 Hz, 2H), 8.04 (d, *J* = 8 Hz, 2H), 8.18 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.8, 22.1, 22.8, 25.1, 25.6, 28.6, 29.0, 45.3, 56.3 (2C), 60.9, 88.7, 106.6 (2C), 113.3, 122.7, 128.7 (2C), 128.9, 130.1 (2C), 131.5, 132.5, 134.2, 141.9, 145.6, 146.8, 153.2 (2C), 157.7, 161.8, 165.1, 169.2, 175.1. IR ($CHCl_3$): 1145, 1239, 1284, 1362, 1373, 1396, 1492, 1557, 1668, 2225, 2936 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{34}H_{33}N_5O_7S_3$+H]: 720.1609, found: 720.1615; [$C_{34}H_{33}N_5O_7S_3$+Na]: 742.1427, found: 742.1434.

6-Oxo-1-(3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)-2-((3-(4-oxo-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-3(4*H*)-yl)propyl)thio)-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (27)

Yield: 60% (from compound **15** by reaction with bromide **8**), White solid, Melting point: 129 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.75-1.93 (m, 8H), 2.19-2.45 (m, 4H), 2.77 (s, 4H), 2.97 (s, 4H), 3.24 (t, *J* = 7 Hz, 2H), 3.94 (s, 9H), 4.12 (t, *J* = 7 Hz, 2H), 4.23 (t, *J* = 7 Hz, 2H), 4.56 (t, *J* = 7 Hz, 2H), 7.34 (s, 2H), 7.91 (s, 1H), 8.00 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 22.1 (2C), 22.7 (2C), 25.1 (2C), 25.5 (2C), 27.9, 28.2, 28.6, 43.6, 45.3, 56.3 (2C), 60.9, 65.1, 87.3, 106.4 (2C), 114.7, 122.6, 122.7, 129.5, 131.4, 134.3, 134.4, 141.4, 141.5, 145.2, 145.5, 153.1 (2C), 157.7, 157.8, 162.0, 162.1, 167.8, 169.4, 174.2. IR ($CHCl_3$): 1127, 1500, 1556, 1667, 2212, 2937 cm^{-1}. HRMS (ESI) m/z calculated for [$C_{40}H_{41}N_7O_6S_3$+H]: 812.2347, found: 812.2353; [$C_{40}H_{41}N_7O_6S_3$+Na]: 834.2166, found: 834.2173.

4.2 Biological Screening

Antitubercular activity

The compounds synthesized in the present work were tested for their *in vitro* ~~and *ex vivo* effects against dormant and active stages MTB using XRMA protocol [17, 18].~~ inhibitory activity against dormant (12 days incubation) and active (8 days incubation) H37Ra mycobacteria using XRMA protocol as described by Singh *et al*. [21]. The absorbance of XRMA was measured at 470 nm. *Ex vivo* activity against dormant and active stages of MTB was estimated through nitrate reductase (NR) assay reading absorbance at 540 nm as per the protocol described by Khan and Sarkar [23]. MTB (ATCC No. 25177) were grown to logarithmic phase (O. D. 1.0) in a M. pheli medium. The stock culture was maintained at -70°C and sub-cultured once in M. pheli medium before inoculation into the experimental culture. All experiments were performed in triplicates and IC_{50} and IC_{90} values were calculated from their dose–response curves ~~[19]~~.

Cytotoxicity assay

The cytotoxicity of the compounds was determined using MTT assay against three different human cancer cell lines and THP-1 monocytes in duplicate [~~19, 20, 21~~24,25]. Leukaemia THP-1, lung A549 adenocarcinoma, pancreatic PANC-1 adenocarcinoma and HeLa cervical carcinoma cell lines were obtained from the European Collection of Cell Cultures (ECCC), Salisbury, UK.

Cell lines were maintained under standard cell culture conditions at 37 ^{o}C and 5% CO_2 in a humidified environment.

Docking studies

Molecular docking studies were carried out to predict the probable mode of action of antitubercular activity of the synthesized hybrids of thienopyrimidinones and thiouracil derivatives with mycobacterium tuberculosis pantothenate synthetase (PDB id: 3IVX).

To perform docking studies Glide 7.1[~~30~~35] was used. All chemical structures were drawn using 2D-sketcher incorporated within Maestro 10.6 [~~31~~36]. LigPrep 3.8 [~~32~~37] was used to prepare 3D structures with corrected chiralities and each individual structure is refined with the optimized energy and best possible conformations. Protein obtained was initially purified by adding H-atoms wherever necessary to the amino acid residues and bond order was assigned to find out the correct ionization and tautomeric states of them. All the water molecules present within the active site were removed and missing side chains were added using Prime 4.4 [~~33~~38]. For further purification protein was refined by checking the protonation state of histidines, terminal acids and of polar hydrogens. The existing steric clashes present within the protein were relaxed using OPLS-2005 force field and was terminated once root mean square deviation reached 0.30 Å [~~34, 35~~39,40].

Grid was prepared by selecting the co-crystallized ligand FG6 and the length of enclosing cubic box was set to 20A° to cover the maximum area of active site around the co-crystallized ligand.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at

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Highlights

* New hybrids of thienopyrimidinones and thiouracils synthesized.

* Some of the compounds exhibited significant antitubercular activity against *MTB* H37Ra.

* The docking study done to predict the probable mode of action.

* Inhibition of mycobacterial pantothenate synthetase predicted.