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Synthesis, anti-thymidine phosphorylase activity and molecular docking of 5-thioxo-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-7-ones

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ABSTRACT

In our lead finding program, a series of 5-thioxo-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-7-ones and their 5-thio-alkyl derivatives were designed and synthesized which contained different substituents at *ortho*-position of 2-phenyl ring attached to the fused ring structure. The preliminary pharmacological evaluation demonstrated that the synthesized compounds exhibited a varying degree of inhibitory activity towards thymidine phosphorylase (TP), comparable to reference compound, 7-Deazaxanthine (**7-DX**, **2**) (IC_{50} value = 42.63 μM). The study also inferred that the *ortho*-substituted group at the phenyl ring and 5-thio-alkyl moiety imparted steric hindrance effects in the binding site of the enzyme, leading to a reduced inhibitory response. In addition, compound **3a** was identified as a mixed-type inhibitor of TP. Moreover, computational docking study was performed to illustrate the important structural information on the plausible ligand-enzyme binding interactions.

1. Introduction

During the last few decades, target-based drug design for anticancer therapy has gained increasing attention [1]. This approach has produced rationally designed inhibitors of dihydrofolate reductase [2], thymidylate synthase [3], purine nucleoside phosphorylase [4], glycinamide ribonucleotide formyltransferase [5] and matrix metalloproteases [6] that can either inhibit DNA replication or block critical biochemical pathways, resulting in prevention of growth of cancer cells. Continuous efforts in this field identified thymidine phosphorylase (TP, EC 2.4.2.4) as a potential therapeutic target for cancer therapy [7]. Several studies demonstrated that thymidine phosphorylase (TP), also known as platelet derived endothelial cell growth factor (PD-ECGF) and gliostatin, is overexpressed in many solid tumors and plays a crucial role in cancer angiogenesis [8]. TP exerts angiogenesis *via* the release of its metabolic product, 2-deoxy-D-ribose that stimulates the secretion and/or expression of many angiogenic factors, including MMP-9, MMP-2, VEGF, IL-8 and others [9]. Subsequently, it stimulates a cascade of events, including the migration of endothelial cells and rapid formation of long-lasting functional neo-vessels, leading to increased metastasis and tumour growth [8,10]. Therefore, inhibition of thymidine phosphorylase may be viewed as a plausible strategy to overcome its pathological effects. Pioneering research in this field has discovered several potent TP inhibitors, most of these inhibitors are derivatives of pyrimidin-2,4-dione with only a few that are fused bicyclic heterocycles [11]. In this context, **TPI** and **7-DX** have emerged as leading TP inhibitor candidates (Fig. 1) [12,13].

Recently, based on structural similarities with the reference compounds, a series of 1,3,5-triazin-2,4-dione and their fused analogues was designed, synthesized and their *in vitro* TP inhibitory potential was evaluated. Among them, 2-phenyl-5-thioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one showed inhibitory activity against TP [14]. The lead structure was modified by introducing different substituents at *meta*- and/or *para*- positions of 2-phenyl ring attached to the fused ring scaffold with the aim to improve the TP inhibition potency. The generated compounds exhibited varying degrees of inhibitory activity towards TP, comparable or better than parent compound [15].

The success of these projects has led us to investigate the hypothesis that the structural modification of 2-phenyl-5-thioxo-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-7-one by inserting various electron withdrawing substituents on the *ortho*-position of the phenyl ring would exhibit TP inhibitory action. Moreover, it was assumed that the corresponding 5-thio-alkyl derivatives of these compounds would demonstrate inhibitory properties towards TP. To address these hypotheses, herein, a library of 1,2,4-triazolo[1,5-*a*][1,3,5]triazines (Fig. 2) was synthesized *via* a practical synthetic

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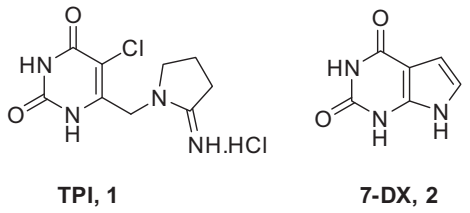


Fig. 1. Structures of known TP inhibitors.

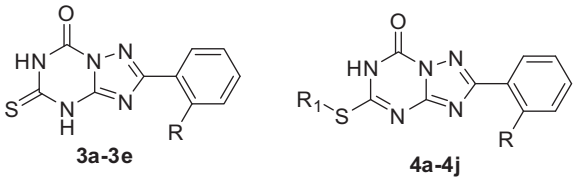


Fig. 2. Structures of target compounds to be synthesized.

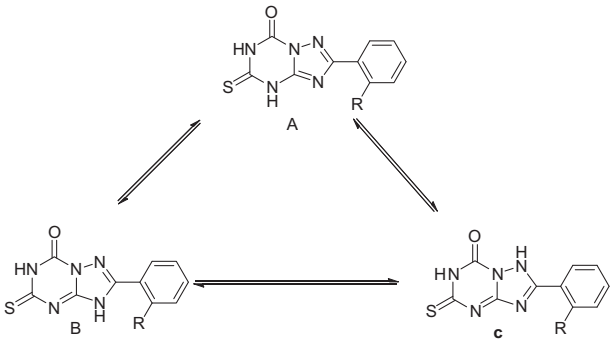
approach and the target compounds were evaluated for TP inhibition using an *in vitro* enzyme assay to explore potential lead TP inhibitors. In addition, a computational docking study was performed to illustrate the binding site and the plausible ligand-enzyme binding interactions of the compounds.

2. Results and discussion

2.1. Chemistry

The synthesis of the title compounds (**3a**–**3e** and **4a**–**4j**) was achieved *via* annulation of 1,3,5-triazine ring onto 3(5)-amino-1,2,4-triazoles as described in Scheme 1 [14,15]. The adopted synthetic method was previously disclosed by Bokaldere and co-workers [16].

The reaction of 5-amino-1,2,4-triazoles (**8**) with ethoxycarbonyl isothiocyanate in DMF gave the thiourea (**9**) derivatives which underwent base catalyzed intramolecular heterocyclization, leading to the generation of target compounds (**3a**–**3e**). Compounds **4a**–**4j**, corresponding 5-thio-alkyl derivatives of **3a**–**3e**, were subsequently produced by treating **3a**–**3e** with alkyl halide in aqueous alkali. This approach afforded products of acceptable yields (32–86%) and high purity. Moreover, an environmental benign and ease workup protocol was utilized to produce 5-amino-1,2,4-triazoles (**8**) [17,18]. The two-step reaction involved the synthesis of amidoguanidines (**7**) from commercially available substituted acid chlorides (**5**) followed by microwave-assisted



Scheme 2. The prototropic interconversion between tautomeric forms of compounds **3a**–**3e.**

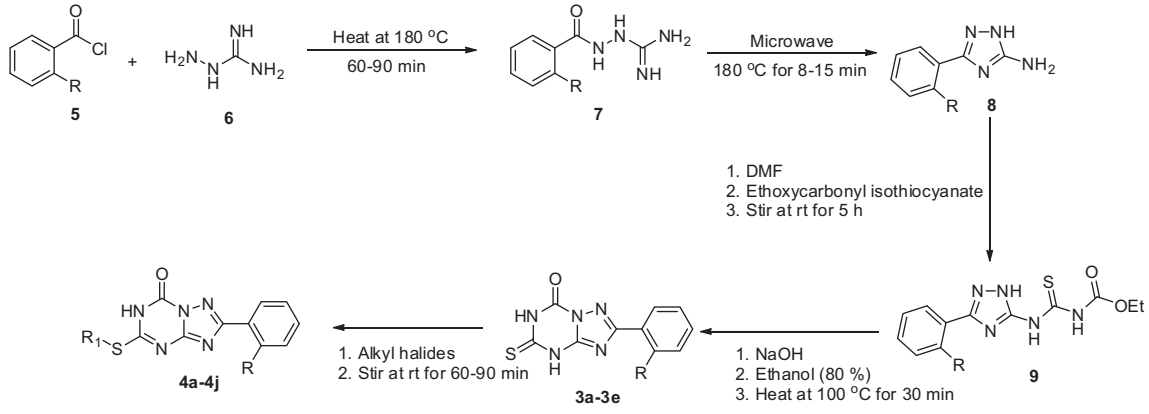
cyclocondensation in water (Scheme 1). A substantial improvement in yield of key intermediates (**8**) was observed for this approach. Moreover, the overall reaction time was reduced to 8–15 min as compared to several hours by the conventional heating process.

All the synthesized compounds (**3a**–**3e** and **4a**–**4j**) were characterized by melting points and different spectroscopic techniques (^{1}H NMR, ^{13}C NMR and MS). Interestingly, the ^{13}C NMR spectroscopy was used as an important tool to confirm and distinguish the structures of the target compounds. The ^{13}C peak of the thiocarbonyl (C═S) carbon of **3a** was found to appear at around 175.8 ppm. The appearance of the peak at about 13.9 ppm assigned to SMe in the ^{13}C NMR spectrum confirmed the formation of product **4a.** The purity of the compounds was examined by reverse phase HPLC method and elemental analysis. The purity of all compounds was satisfactory (above 95%).

Due to annular tautomerism, target compounds **3a**–**3e** can exist in dynamic equilibrium of (A) 4-, (B) 3- and (C) 1*H*-forms (Scheme 2). The prototropic interconversion between these tautomeric forms resulted in appearance of a broad 4-N(H) signal in ^{1}H NMR spectra of compounds in DMSO. The broad signal was found to disappear on heating, due to rapid exchange of the NH proton.

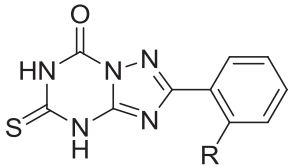
2.2. Anti-thymidine phosphorylase activity

Fifteen compounds (**3a**–**3e** and **4a**–**4j**) were evaluated for TP inhibitory activity by a spectrophotometric assay that used recombinant human thymidine phosphorylase, expressed in *E. coli* (T2807 – Sigma Aldrich) as the enzyme and thymidine as the substrate. The original method developed by Krenitsky and Bushby [19] was modified and adopted. The results of the enzyme

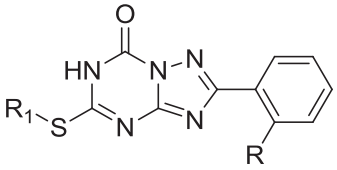


Scheme 1. Synthesis of target compounds (**3a**–**3e** and **4a**–**4j**).

Table 1
Thymidine phosphorylase inhibitory activity of the synthesized compounds (**3**–**4**).



3a-3e



4a-4j

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Entry	Cpd.	R	R_1	TP inhibition activity[a] IC_{50} (μM)
1	**3a**	H	–	39.56 ± 1.76
2	**3b**	F	–	45.45 ± 2.45
3	**3c**	Cl	–	75.30 ± 4.51
4	**3d**	Br	–	80.41 ± 3.12
5	**3e**	NO_2	–	88.55 ± 2.38
6	**4a**	H	CH_3	95.05 ± 4.42
7	**4b**	F	CH_3	107.11 ± 5.23
8	**4c**	Br	CH_3	145.32 ± 4.37
9	**4d**	NO_2	CH_3	160.08 ± 4.45
10	**4e**	H	C_2H_5	102.25 ± 2.49
11	**4f**	F	C_2H_5	110.12 ± 3.57
12	**4g**	Br	C_2H_5	155.82 ± 4.43
13	**4h**	NO_2	C_2H_5	164.8 ± 2.98
14	**4i**	H	C_3H_7	104.6 ± 3.13
15	**4j**	H	CH_2Ph	111.2 ± 4.22
16	**7-DX**	–	–	42.63 ± 5.23

[a] Experiments carried out in triplicate.

inhibitory potency were expressed in terms of IC_{50} values and were compared with that of **7-DX** (IC_{50} = 42.63 μM) (Table 1).

Compounds **3a**–**3e** and **4a**–**4j** demonstrated varying degrees of TP inhibition with IC_{50} values ranging between 40 and 165 μM and compound **3a** was observed to have best anti-TP activity (IC_{50} = 39.56 μM) among the two groups of compounds evaluated in this study. The structural modification of compound **3a** with the introduction of electron withdrawing substituents at *ortho*-position of phenyl ring resulted in a decrease in the binding interactions. The *ortho*-substituted groups were assumed to impart a steric hindrance effect in the binding pocket of enzyme, leading to a decreased inhibitory effect. Moreover, the inhibition activity of the compounds bearing substituents at *ortho*-position was in the descending order of H > F > Cl > Br > NO_2. This observation clearly implied that the activity was dependent on the relative size of substituents. The inhibitory potency of compounds was found to decrease gradually with increasing size of the moiety.

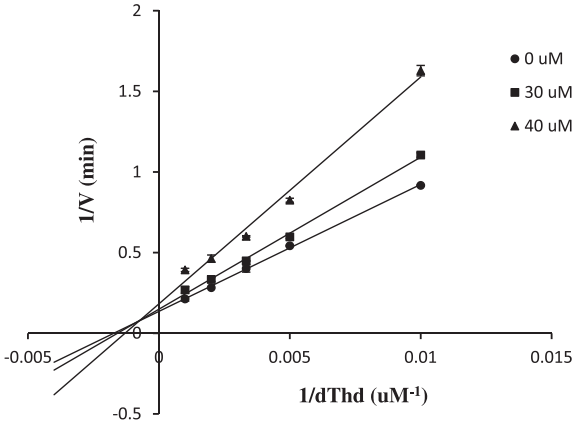

Fig. 3. Lineweaver–Burk plots of TP inhibition by **3a** with respect to thymidine (dThd) as a variable substrate. Results are presented as means ± SD; SD denoted by error bars (Experiments carried out in triplicate).

Further structural modification of **3a**–**3e** by replacing 5-thioxo moiety with thiomethyl group, resulting in subsequent elimination of active hydrogen from N4, led to dramatic decrease in inhibition property, as observed in compounds **4a**–**4d** (IC_{50} = 95–160 μM). In addition, as the alkyl chain length and bulk increased, a substantial reduction in TP inhibitory activity was observed. Therefore, it can be inferred that the 5-thioxo moiety (C=S) was optimal in the structure of the TP inhibitors. In other word, TP inhibitors having triazolo[1,5-*a*][1,3,5]triazine scaffold, must carry a C(=O)—NH—C(=S) fragment in the structure.

A brief kinetic study of compound **3a** was performed to elucidate the mechanism of inhibition according to previously disclosed method [15]. Lineweaver–Burk plots were constructed using the data obtained from the study (Fig. 3). The results demonstrated that compound **3a** was a mixed-type inhibitor of TP in the presence of thymidine as a variable substrate since the straight lines corresponding to different concentrations of the inhibitor (0, 30 and 40 μM) intersected in the left panel of the reciprocal plot. A similar type of inhibition mechanism was observed with a structurally resemble TP inhibitor [15].

2.3. Molecular docking

To investigate the plausible binding orientations and molecular interactions of the synthesized compounds, a molecular docking study was performed using geometry optimized structure of the compounds. The compounds were allowed to dock at the active site of TP (PDB code: 2WK6) [20] using SYBYL-X 1.3. To analyze the docking results, the lowest energy conformation of the compounds was adopted. Initially, the docking protocol was validated by comparing the binding interactions of **TPI** in the active site of TP with the previously reported results. The predicted binding mode and interaction pattern of **TPI** generated by our software were found to be in accordance with the reported crystallographic study [21] with a RMS deviation of 0.5 Å (Fig. 5a and 5b in supplementary material).

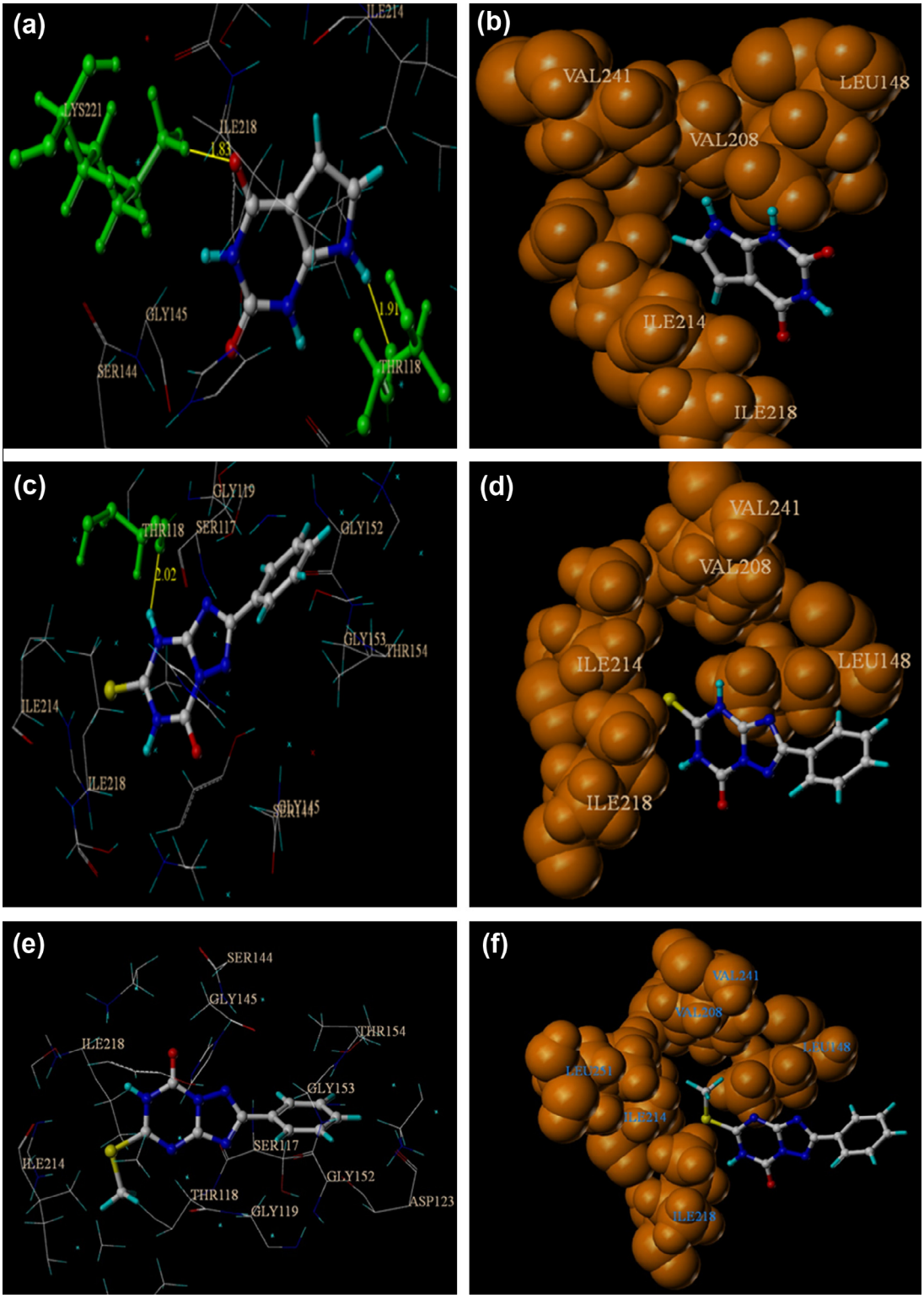

Fig. 4. Graphical representation of interactions between TP and its inhibitors analyzed by computational docking: (a) hydrogen bonding of **7-DX** (colored ball and stick) with Lys221, Thr118 (green colored ball and stick); (b) hydrophobic interaction of **7-DX** (colored ball and stick) with amino acid (orange colored sphere); (c) hydrogen bonding of **3a** (colored ball and stick) with Thr118 (green colored ball and stick); (d) hydrophobic interaction of **IVb** (colored ball and stick) with amino acid (orange colored sphere); (e) hydrogen bonding of **4a** (colored ball and stick) with Lys221 and Thr118 (green colored ball and stick); and (f) hydrophobic interaction of **4a** (colored ball and stick) with amino acid (orange colored sphere). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The analysis of docking interactions between TP and reference compound, **7-DX** revealed that the carbonyl group at C4 and NH-7 of **7-DX** created strong hydrogen bonding contacts with Lys221 and Tyr118 respectively, located in the active site of enzyme (Fig. 4a) [21]. The NH group neighboring to the thiocarbonyl moiety in compound **3a** displayed hydrogen bonding interaction with Tyr118, while the thiocarbonyl moiety positioned itself into a hydrophobic pocket formed by some amino acid residues like

Ile218, Ile214, Val 208, Val241, Val208 and Leu148 (Fig. 4c and 4d). As a result, compound **3a** showed inhibitory activity with an IC_{50} value in micromolar range being comparable to**7-DX** (Table 1). Moreover, compound **3a** showed a different binding orientation as compared to **7DX** and consequently it demonstrated a mixed mode of inhibition with respect to thymidine. It is noteworthy that reference inhibitor, **7-DX**, behaved as a competitive or mixed-type inhibitor in the presence of variable concentrations of thymine [22].

It is also evident from the interaction pattern that the compounds **4a** and other 5-thio-alkyl derivatives exhibited poor penetration to the active site probably due to the increased steric bulkiness of their thiomethyl group resulted in diminished inhibitory effect (Fig. 4e and 4f).

3. Conclusion

We have synthesized a small library of 1,2,4-triazolo[1,5-*a*][1,3,5]triazines *via* ring annelation reaction and evaluated these compounds for their thymidine phosphorylase inhibitory activity. The biological evaluation identified a number of new TP inhibitors and several compounds in particular those that bear *ortho*-substitutions on the phenyl ring, *viz.,* compounds **3a-3e.** These compounds exhibited inhibitory activity against TP with IC_{50} values being well comparable to **7-DX**. In addition, it was found that the C5 thioxo moiety is essential for interaction with the enzyme. Compound **3a** exhibited a mixed mode of inhibition towards TP with respect to thymidine as a variable substrate. The molecular docking studies suggested a justified binding mode of the active compounds in the binding site of TP. Therefore, these compounds constitute a new direction for design and synthesis of fused-ring TP inhibitors.

4. Experimental

4.1. Chemistry

4.1.1. General procedures

All reagents were purchased from Sigma–Aldrich or Alfa Aesar and were used without further purification. Melting points were determined on a Gallenkamp melting point apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX-300 spectrometer at 300 MHz and 75 MHz respectively using DMSO-d_6 as solvent and TMS as internal standard. Mass spectra were obtained on a Finnigan MAT LCQ LC-MS mass spectrometer using the electrospray ionization (ESI) mode. Reactions were monitored by TLC on silica gel (60 F_{254}) coated aluminum plate.

4.1.2. General procedure for the synthesis of 5-thioxo-(1,2,4)-triazolo[1,5-a](1,3,5)triazin-7-ones and its derivatives (**3a**–**3e**)

Initially, amidoguanidines (**7**) were prepared *via* the reaction of substituted benzoyl chloride (**5**) (10 mmol) and aminoguanidine hydrochloride (**6**) (20 mmol). The obtained intermediates (**7**) were then treated with microwave irradiation power (100 W) for 8–15 min in water to generate 5-amino-1,2,4-triazoles (**8**) [18]. Subsequently, 5-amino-1,2,4-triazoles (**8**) (3 mmol) were reacted with ethoxycarbonyl isothiocyanate (3.3 mmol) in anhydrous DMF (4 ml) at room temperature, leading to formation of thiourea derivatives (**9**).

In a well stirred solution of sodium hydroxide (9 mmol) in ethanol (80%, 20 ml), thiourea derivatives (**9**) were added and heated on a water bath for 20 min. After cooling, the solvent was evaporated under vacuum and the residue was re-suspended in water (25 ml). It was acidified up to pH 1–3 using 2.5 M HCl. The precipitated products (**3a**–**3e**) was filtered off, recrystallized with suitable solvents and dried under vacuum [14,15].

4.1.3. 2-Phenyl-5-thioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**3a**)

Yield: 78.2%; mp: 258–259 °C (Ethanol); ESI-MS *m*/*z* 244.1 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.53–7.55 (m, 3H, H-3′, H-4′ and H-5′), 8.04–8.07 (m, 2H, H-2′ and H-6′), 13.12 (s, 1H, NH), 14.31 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 127.1 (C-2′ and C-6′), 129.5 (C-3′ and C-5′), 129.7 (C-4′), 131.3 (C-1′), 141.7 (C-2), 151.9 (C-9), 162.4 (C-7), 175.8 (C-5); Anal. calcd. for $C_{10}H_7N_5OS$: C, 48.97; H, 2.88; N, 28.55. Found: C, 47.99; H, 2.71; N, 28.21.

4.1.4. 2-(2-Fluorophenyl)-5-thioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**3b**)

Yield: 50.8%; mp: 235 °C (Acetic acid); ESI-MS *m*/*z* 262.3 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.20–7.25 (m, 2H, Ar—H), 7.32–7.42 (m, 1H, Ar—H), 7.86–7.91 (m, 1H, Ar-H) 12.15 (s, 1H, NH), 14.12 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 117.3 (C-3′), 125.4 (C-1′), 180.4 (C-5′), 133.2 (C-6′), 141.7 (C-4′), 158.7 (C-2′), 159.3 (C-2), 162.11 (C-9), 175.9 (C-7), 179.9 (C-5); Anal. calcd. for $C_{10}H_6FN_5OS$: C, 45.62; H, 2.30; N, 26.60. Found: C, 45.99; H, 2.21; N, 26.28.

4.1.5. 2-(2-Chlorophenyl)-5-thioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**3c**)

Yield: 60.1%; mp: 166 °C (Acetic acid); ESI-MS *m*/*z* 278.0 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.46–7.58 (m, 2H, Ar—H), 7.63 (d, 1H, Ar—H, *J* = 7.1584), 7.92 (dd, 1H, Ar-H, *J* = 1.88 and 7.15 Hz), 13.1 (s, 1H, NH), 14.2 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 131.8 (C-5′), 128.1 (C-6′), 130.8 (C-3′), 131.6 (C-4′), 131.7 (C-2′), 131.8 (C-1′), 141.1 (C-2), 150.8 (C-9), 160.7 (C-7), 175.3 (C-5); Anal. calcd. for $C_{10}H_6ClN_5OS$: C, 42.94; H, 2.16; N, 25.04. Found: C, 41.99; H, 2.01; N, 24.72.

4.1.6. 2-(2-Bromophenyl)-5-thioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**3d**)

Yield: 59.5%; mp: 144 °C (Acetic acid); ESI-MS *m*/*z* 324.0 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.38–7.65 (m, 2H, Ar—H), 7.69–7.92 (m, 2H, Ar—H) 13.15 (s, 1H, NH), 14.21(br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 120.9 (C-2′), 127.8 (C-5′), 130.3 (C-6′), 131.7 (C-4′), 131.8 (C-3′), 134.0 (C-1′), 141.1 (C-2), 150.7 (C-9), 161.6 (C-7), 175.2 (C-5); Anal. calcd. for $C_{10}H_6BrN_5OS$: C, 37.05; H, 1.87; N, 21.60. Found: C, 36.81; H, 1.75; N, 20.94.

4.1.7. 2-(2-Nitrophenyl)-5-thioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**3e**)

Yield: 40.1%, mp: 211 °C (Acetic acid), ESI-MS *m*/*z* 289.1 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.64–8.08 (m, 3H, Ar—H), 8.81–8.99 (m, 1H, Ar—H), 13.21 (s, 1H, NH), 14.21(br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 122.8 (C-3′), 124.6 (C-1′), 131.2 (C-4′), 132.4 (C-6′), 133.2 (C-5′), 141.6 (C-2′), 149.5 (C-2), 151.8 (C-9), 159.5 (C-7), 175.9 (C-5); Anal. calcd. for $C_{10}H_6N_6O_3S$: C, 41.38; H, 2.08; N, 28.95. Found: C, 41.34; H, 2.04; N, 28.22.

4.1.8. General procedure for the synthesis of thioalkyl derivatives of 3a-3e (**4a**–**4j**)

To a stirred solution of **3a**–**3e** (5 mmol) in 4 ml of 2.5 M NaOH, iodomethane/ethyl iodide, propyl iodide/benzyl bromide (7.5 mmol) was added, the stirring was continued for 2 h. A solid precipitate was observed which upon acidification (pH 1–3) with 2.5 M HCl afforded another solid. The precipitated solid (**4a**–**4j**) was filtered, washed with ice cold water and recrystallized by water–ethanol system.

*4.1.9. 2-Phenyl-5-methylthioxo- [1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**4a**)*

Yield: 58.2%; mp: 292–294 °C (Ethanol–water); ESI-MS *m/z* 258.0 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.62 (s, 3H, SMe), 7.54–7.55 (m, 3H, H-3′, H-4′ and H-5′), 13.15 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 13.9 (SMe), 127.1 (C-2′ and C-6′), 129.4 (C-3′and C-5′), 130.4 (C-4′), 131.0 (C-1′), 143.8 (C-2), 157.9 (C-9), 163.1 (C-5), 165.3 (C-7); Anal. calcd. for $C_{11}H_9N_5OS$: C, 50.95; H, 3.50; N, 27.01. Found: C, 50.26; H, 3.41; N, 26.73.

*4.1.10. 2-(2-Fluorophenyl)-5-methylthioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**4b**)*

Yield: 86.3%; mp: 231 °C (Ethanol–water); ESI-MS *m/z* 277.0 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.52 (s, 3H, CH3), 7.30–7.46 (m, 4H, Ar—H), 13.94 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 117.3 (C-3′), 118.3 (C-1′), 125.3 (C-5′), 130.9 (C-6′), 132.9 (C-4′), 143.8 (C-2′), 157.5 (C-2), 158.7 (C-9), 159.9 (C-7), 162.1 (C-5); Anal. calcd. for $C_{11}H_8FN_5OS$: C, 47.65; H, 2.91; N, 25.26. Found: C, 46.77; H, 2.82; N, 26.11.

*4.1.11. 2-(2-Bromophenyl)-5-methylthioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**4c**)*

Yield: 59.2%; mp: 138 °C (Ethanol–water), ESI-MS *m/z* 337.2 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.63 (s, 1H, CH_3), 7.36–7.61 (m, 2H, Ar—H), 7.71–7.93 (m, 2H, Ar—H), 13.71 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 13.9 (CH_3), 121.6 (C-2′), 128.3 (C-5′), 131.6 (C-6′), 132.1 (C-4′), 132.4 (C-3′), 134.5 (C-1′), 143.7 (C-2), 157.3 (C-9), 163.1 (C-7), 165.5 (C-5); Anal. calcd. for $C_{11}H_8BrN_5OS$: C, 39.07; H, 2.38; N, 20.71. Found: C, 38.42; H, 2.29; N, 20.13.

*4.1.12. 2-(2-Nitrophenyl)-5-methylthioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**4d**)*

Yield: 67.8%, mp: 253 °C, ESI-MS *m/z* 303.8 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.50 (s, 3H, CH_3), 7.70–7.89 (m, 2H, Ar-H), 7.99 (d, 1H, Ar—H, *J* = 7.5 Hz), 8.08 (d, 1H, Ar—H, *J* = 7.53 Hz), 13.50 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 13.9 (CH_3), 123.4(C-3′), 124.4(C-1′), 130.9(C-4′), 132.1(C-6′), 133.0(C-5′), 143.7(C-2′), 149.7 (C-2), 157.8 (C-9), 160.1 (C-7), 166.0 (C-5); Anal. calcd. for $C_{11}H_8N_6O_3S$: C, 43.42; H, 2.65; N, 27.62. Found: C, 43.12; H, 2.56; N, 27.02.

*4.1.13. 2-Phenyl-5-ethylthioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**4e**)*

Yield: 31.8%; mp: 249 °C (Ethanol); ESI-MS *m/z* 272.2 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.37 (t, 3H, CH_3, *J* = 7.34 Hz), 3.22 (q, 2H, CH_2, *J* = 7.5 Hz), 7.49–7.57 (m, 3H, Ar—H), 8.07–8.17 (m, 1H, Ar—H), 13.65 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.8 (CH_3), 25.5(CH_2), 127.1 (C-2′ and C-6′), 129.4 (C-3′ and C-5′), 130.4 (C-4′), 130.0 (C-1′), 143.9 (C-2), 157.9 (C-9), 163.1 (C-7), 164.6 (C-5); Anal. calcd. for $C_{12}H_{11}N_5OS$: C, 52.73; H, 4.06; N, 25.62. Found: C, 51.37; H, 4.01; N, 25.11.

*4.1.14. 2-(2-Fluorophenyl)-5-ethylthioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**4f**)*

Yield: 53.4%; mp: 189 °C (Ethanol); ESI-MS *m/z* 291.0 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.40 (t, 3H, CH_3, *J* = 7.2 Hz), 3.25 (q, 2H, CH_2, *J* = 7.0 Hz), 7.27–7.47 (m, 2H, Ar—H), 7.96–8.23 (m, 2H, Ar—H), 7.97–8.23 (m, 1H, Ar—H), 13.73 (br. s, 1H, NH), ^{13}C NMR (75 MHz, DMSO-d_6): δ 117.4 (C-3′), 118.4 (C-1′), 125.3 (C-5′), 131.1 (C-6′), 132.9 (C-4′), 143.8 (C-2′), 162.1 (C-2), 164.8 (C-9), 175.8 (C-7), 179.9 (C-5); Anal. calcd. for $C_{12}H_{10}FN_5OS$: C, 49.48; H, 3.46; N, 24.04. Found: C, 48.32; H, 3.35; N, 23.29.

*4.1.15. 2-(2-Bromophenyl)-5-ethylthioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**4g**)*

Yield: 76.6%; mp: 239 °C (Ethanol–water); ESI-MS *m/z* 350.1 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.25 (t, 3H, CH_3, *J* = 7.4 Hz), 3.23 (q, 2H, CH_2, *J* = 7.3 Hz), 7.23–7.56 (m, 1H, Ar—H), 7.61–7.73 (m, 3H, Ar—H), 13.86 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.7 (CH_3), 25.5 (CH_2), 121.6 (C-2′), 128.3 (C-5′), 131.6 (C-6′), 132.1 (C-4′), 132.4 (C-3′), 134.5 (C-1′), 143.8 (C-2), 157.4 (C-9), 163.1 (C-7), 164.8(C-5); Anal. calcd. for $C_{12}H_{10}BrN_5OS$: C, 40.92; H, 2.86; N, 19.88. Found: C, 40.28; H, 2.81; N, 19.26.

*4.1.16. 2-(2-Nitrophenyl)-5-ethylthioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**4h**)*

Yield: 40.2%; mp: 188 °C; ESI-MS *m/z* 318.0 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.37 (t, 3H, CH_3), 3.24 (q, 2H, CH_2), 7.69–7.91 (m, 2H, Ar—H), 7.99–8.13 (m, 2H, Ar—H), 13.87 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.7 (CH_3), 25.5 (CH_2), 123.4 (C-3′), 124.4 (C-1′), 130.9 (C-4′), 132.1 (C-6′), 133.0 (C-5′), 143.7 (C-2′), 149.7 (C-2), 157.8 (C-9), 160.1 (C-7), 165.3 (C-5); Anal. calcd. for $C_{12}H_{10}N_6O_3S$: C, 45.28; H, 3.17; N, 26.40. Found: C, 44.34; H, 3.05; N, 26.88.

*4.1.17. 2-Phenyl-5-propylthioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**4i**)*

Yield: 73.1%; mp: 214 °C (Ethanol–water); ESI-MS *m/z* 287.0 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.02 (t, 3H, CH_3), 1.76 (m, 2H, CH_2), 3.21 (t, 2H, SCH_2), 7.43–7.62 (m, 3H, Ar—H), 8.02–8.22 (m, 1H, Ar—H), 13.23 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 13.6 (CH_3), 22.4 (CH_2), 32.7 (SCH_2), 127.1 (C-2′ and C-6′), 129.4 (C-3′ and C-5′), 130.4(C-4′), 131.0(C-1′), 143.9 (C-2), 158.0 (C-9), 163.0 (C-7), 164.7 (C-5); Anal. calcd. for $C_{13}H_{13}N_5OS$: C, 54.34; H, 4.56; N, 24.37. Found: C, 53.98; H, 4.45; N, 24.11.

*4.1.18. 2-Phenyl-5-benzylthioxo-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-one (**4j**)*

Yield: 38.9%; mp: 231 °C (Ethanol); ESI-MS *m/z* 334.5 $(M-1)^+$; purity > 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.20 (s, 2H, CH_2), 7.21–7.30 (m,1H, Ar—H), 7.36–7.84 (m, 3H, Ar—H), 7.91–8.01 (m, 2H, Ar—H), 8.12–8.23 (m, 2H, Ar—H), 13.78 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 34.7 (CH_2), 127.0 (C-2′ and C-6′), 127.8 (C-4″), 129.0 (C-3″ and C-5″), 129.3 (C-3′ and C-5′), 129.5(C-2″ and C-6″), 130.6 (C-4′), 131.0 (C-1′), 137.6 (C-1″), 146.3 (C-2), 158.8 (C-9), 162.2 (C-7), 167.3 (C-5); Anal. calcd. for $C_{17}H_{13}N_5OS$: C, 60.88; H, 3.91; N, 20.88. Found: C, 59.15; H, 3.78; N, 20.23.

4.2. In vitro thymidine phosphorylase enzyme assay

A spectrophotometric assay method, originally developed by Krenitsky and Bushby [19], was adopted to evaluate *in vitro* TP inhibitory activity of the synthesized compounds. Briefly, thymidine (substrate) and recombinant thymidine phosphorylase, expressed in *E. coli* (T2807-Sigma Aldrich) were used for this assay. Absorbance at 290 nm was recorded on a Shimadzu UV Mini 1240 UV–Vis Spectrophotometer. The enzymatic reaction was initiated by addition of substrate (200 μl, 5 mM) into a cuvette containing 780 μl of potassium phosphate buffer (pH 7.4), 10 μl of enzyme at concentration of 1.5 U and 10 μl of test compounds dissolved in DMSO. The decrease in absorbance due to conversion of thymidine to thymine was followed after 4, 8, 12, 16 and 20 min and from the slope of the change in absorbance, the initial reaction rate was determined. The same experiments were performed using 10 μl of DMSO to calculate slope of uninhibited enzyme. The initial rates of the change in absorbance at different concentrations of

inhibitors were converted to % inhibition of enzyme and plotted against inhibitor concentrations using Graphpad Prism vs 4.0 to give the IC_{50} at 50% inhibition.

The inhibitory activity of each compound was calculated by the following formulae:

$$Activity = \frac{\text{Slope of inhibited enzyme}}{\text{Slope of uninhibited enzyme}} \times 100\%$$

$$\text{Inhibition} = 100\% - \text{Activity}$$

All the experiments were carried out in triplicate.

4.3. Enzyme inhibition kinetics study

TP enzyme inhibition was examined by monitoring the conversion of thymidine to thymine at varying thymidine (dThd) concentrations (1000, 500, 300, 200, 100 μM) in the presence of various inhibitor concentrations (0, 30 and 40 μM). The concentration of phosphate (KPi) concentration was maintained constant at 25 mM. The data obtained was analyzed by the double reciprocal plot method. All the experiments were conducted in triplicate.

4.4. Molecular docking study

The molecular docking was performed using Surflex Dock Geom X method of Sybyl X-1.3 [23]. The co-crystal structure of the protein and ligand was retrieved from Protein data Bank (PDB ID: 2WK6) [20]. The protein was prepared by deleting the identical B chain, unwanted water molecules and adding hydrogens. The protein was further energy minimized through the stage minimization tool of the Sybyl software over hundred iterations using conjugate minimization technique. The idealized active site, i.e. the protomol was generated from hydrogen-containing protein mol2 file by keeping a threshold factor of 0.5 and a bloat of 0 Å [24]. The ligands were drawn into the Sybyl package with standard bond lengths and angles and energy was minimized *via* the conjugate gradient method which applies the Gasteiger–Huckel charge, with a distance-dependent dielectric function. Subsequently, the Surflex Dock Geom X programme was run and the docking scores were ranked in molecular spread sheet.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2013.07.004.

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