European Journal of Medicinal Chemistry 67 (2013) 325–334

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

A structure–activity relationship study of 1,2,4-triazolo[1,5-*a*][1,3,5]triazin-5,7-dione and its 5-thioxo analogues on anti-thymidine phosphorylase and associated anti-angiogenic activities

Hriday Bera [a,b,*], Bee Jen Tan [b], Lingyi Sun [b], Anton V. Dolzhenko [b,c], Wai-Keung Chui [b,**], Gigi Nagar Chee Chiu [b]

[a] Gokaraju Rangaraju College of Pharmacy, Bachupally, Hyderabad 500090, Andhra Pradesh, India
[b] Department of Pharmacy, Faculty of Science, National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore
[c] School of Pharmacy, Monash University Sunway Campus, Jalan Lagoon Selatan, Bandar Sunway, Selangor, WA 46150, Malaysia

ARTICLE INFO

Article history:
Received 9 May 2013
Received in revised form
14 June 2013
Accepted 19 June 2013
Available online 3 July 2013

Keywords:
1,2,4-Triazolo[1,5-*a*][1,3,5]triazine
Thymidine phosphorylase inhibitors
SAR
Mixed-type inhibition
Breast cancer
Anti-angiogenesis

ABSTRACT

Thirty-three 1,2,4-triazolo[1,5-*a*][1,3,5]triazin-5,7-dione and its 5-thioxo analogues were designed and synthesized which contained different substituents at *meta*- and/or *para*-positions of 2-phenyl or 2-benzyl ring attached to the fused ring structure. The preliminary pharmacological evaluation demonstrated that the 5-thioxo analogues of 1,2,4-triazolo[1,5-*a*][1,3,5]triazine exhibited a varying degree of inhibitory activity towards thymidine phosphorylase, comparable or better than reference compound, 7-Deazaxanthine (7-DX, **2**) (IC_{50} value = 42.63 μM). Moreover, compounds **5q** and **6i** displayed a mixed-type of inhibitory mechanism in the presence of variable concentrations of thymidine (dThd). In addition, selected compounds were found to have a noticeable inhibitory effect on the expression of angiogenesis markers, including VEGF and MMP-9 in MDA-MB-231 breast cancer cells.

1. Introduction

Angiogenesis is the formation of new capillaries from pre-existing blood vessels. Pioneering research has identified angiogenesis as a key modulator in the process of cancer development [1–3]. The lack of oxygen in the centre of tumour stimulates the secretion of vascular endothelial growth factor (VEGF) which binds to the receptors present on endothelial cells of existing blood vessels [4]. Subsequently, it stimulates a cascade of events, including the secretion of metalloproteases and other matrix-degrading enzymes (MMPs), proliferation and migration of endothelial cells to the tumour tissue, allowing for rapid formation of long-lasting functional neo-vessels [5]. As it is a complex process, there is a continued need for the development of novel angiogenesis inhibitors that would target multiple angiogenic molecules or pathways to combat the spectrum of heterogeneous tumours occurring clinically [6]. Among several pro-angiogenic factors, thymidine phosphorylase (TP) has been identified as an important angiogenic protein. Several studies have demonstrated that TP has the ability to induce tumour vascularization *via* multiple pathways, resulting in the promotion of tumour growth and metastasis [7,8].

Thymidine phosphorylase (TP, EC 2.4.2.4), which is synonymous with platelet derived endothelial growth factor (PD-ECGF), is overexpressed in the hypoxic regions of solid tumours. Moreover, TP is involved in the catalysis of pyrimidine nucleotide salvage pathway. It is reported that 2-deoxy-α-D-ribose, a product generated during the catalytic reaction, stimulates the secretion and/or expression of angiogenic factors, including VEGF and MMPs. These events eventually lead to angiogenesis and cancer metastasis [8,9]. Therefore, TP may be viewed as an important target for the development of new anti-angiogenic therapy [10]. As a consequence, extensive efforts have been devoted to generate numerous TP inhibitors belonging to diverse chemical classes [11]. Among them, 5-chloro-6-[1-(2-iminopyrrolidinyl)methyl] uracil hydrochloride (TPI, **1**) (Fig. 1), the most potent inhibitor of human TP so far, inhibited the migration and basement membrane invasion of TP-overexpressing KB cells. Moreover, TPI was found to attenuate TP-induced angiogenesis in the mouse dorsal air sac assay

* Corresponding author. Gokaraju Rangaraju College of Pharmacy, Bachupally, Hyderabad 500090, Andhra Pradesh, India. Tel.: +91 8977726256.
** Corresponding author. Tel.: +65 65162933.
E-mail addresses: hriday.bera@gmail.com (H. Bera), phacwk@nus.edu.sg (W.-K. Chui).

0223-5234/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2013.06.051

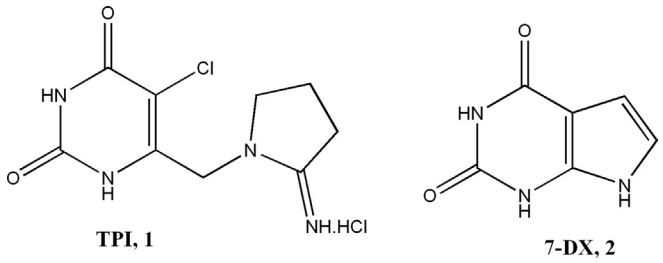


Fig. 1. Structures of known TP inhibitors.

model [12]. It is also reported that 6-(2-aminoethyl)amino-5-chlorouracil (AEAC), in combination with the VEGF-Trap, exhibited anti-angiogenic and anti-tumour activities [13]. The first purine derivative, 7-deazaxanthine (7-DX, **2**) (Fig. 1) was able to efficiently suppress the neovascularization in the CAM assay [14]. In addition, a series of phosphonate derivatives [15–17], *N*-phenyl homophthalimide analogues [18], purine riboside derivative [19,20] and prodrugs of known TP inhibitors [21,22] were designed, synthesized and tested. These structurally diverse compounds displayed TP inhibitory activity by different mechanisms and showed anti-angiogenetic potential in various *in vivo* and *in vitro* model systems.

1,2,4-Triazolo[1,5-*a*][1,3,5]triazine, a fused heterobicyclic system, constitutes an important contribution in medicinal chemistry. A wide variety of attractive pharmacological effects was attributed to the derivatives of 1,2,4-triazolo[1,5-*a*][1,3,5]triazine [23]. However, to date, no studies have been conducted to examine the inhibitory potency of this scaffold against TP. It is worthy to note that 1,2,4-triazolo[1,5-*a*][1,3,5]triazin-5,7-dione may be considered as a bioisoster of xanthine that has its carbon atom at C5 replaced by nitrogen atom, henceforth it is known as 5-azaxanthine. It is hypothesized that 5-azaxanthine and its thioxo analogues (Fig. 2) would exhibit anti-TP and associated anti-angiogenic activities due to their close structural resemblance to 7-deazaxanthine (7-DX, **2**), a leading TP inhibitor candidate. To test this hypothesis, a series of 1,2,4-triazolo[1,5-*a*][1,3,5]triazin-5,7-dione and its 5-thioxo analogues (Fig. 2) were synthesized *via* a practical synthetic method and their *in vitro* TP inhibitory potential was determined. To elucidate the mechanism of enzyme inhibition, a brief kinetic study was attempted. In addition, selected compounds endowed with promising TP inhibitory activity, were tested to explore their anti-angiogenic property by performing experiments that demonstrate an inhibition of MMP-9 and VEGF expression in MDA-MB-231 cells.

2. Results and discussion

2.1. Chemistry

The synthesis of the title compounds (**3**, **4** and **5**–**5s**, **6**–**6i**) was achieved *via* annulation of 1,3,5-triazine ring onto 3(5)-amino-1,2,4-triazoles as illustrated in Scheme 1. The adopted synthetic method is based on the chemistry developed by Bokaldere and co-workers [24]. It allowed the construction of 1,2,4-triazolo[1,5-*a*][1,3,5]triazine derivatives with varying substitutions at *meta*- and/or *para*-positions of 2-phenyl or 2-benzyl ring attached to the fused ring system.

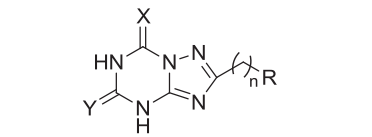


Fig. 2. Structures of target compounds.

The reaction of 5-amino-1,2,4-triazoles (**9**) with ethyl isocyanoformate or ethoxycarbonyl isothiocyanate in DMF afforded the urea (**11**) and thiourea (**12**) derivatives which underwent intramolecular heterocyclization in the presence of base, resulting in the formation of target compounds (**3**, **4** and **5**–**5s**, **6**–**6i** respectively) within 20 min. The obtained compounds were recrystallized with suitable solvents and this process gave high purity products (**3**, **4**, **5** and **6**) in 55–90% yield. Moreover, an efficient and expeditious synthetic approach was utilized to produce 5-amino-1,2,4-triazoles (**9**) [25,26]. The two-step reaction involved the synthesis of amidoguanidines (**8**) from readily available substituted acid chlorides (**10**) or hydrazides (**7**), followed by microwave-assisted cyclocondensation in water (Scheme 1). A significant enhancement in yield of 1,2,4-triazoles (**9**) was observed for the protocol, and the overall reaction time was shortened to 5–8 min compared to several hours of the conventional heating procedure.

All the synthesized compounds (**3**, **4** and **5**–**5s**, **6**–**6i**) were characterized by melting points, and different spectroscopic techniques (^{1}H NMR, ^{13}C NMR and MS). The purity of the compounds was assessed by reverse phase HPLC method and elemental analysis. The structures of the target compounds were readily distinguished and confirmed by the use of ^{13}C NMR spectroscopy. The ^{13}C peak of the thiocarbonyl (C=S) carbon of compounds **5** and **6** appeared at around 175.8 ppm; while the two signals of the carbonyl (C=O) groups of compounds **3** and **4** appeared at about 152.5 and 162.2 ppm and 152.1 and 164.8 ppm respectively. The purity of all compounds was satisfactory (above 95%).

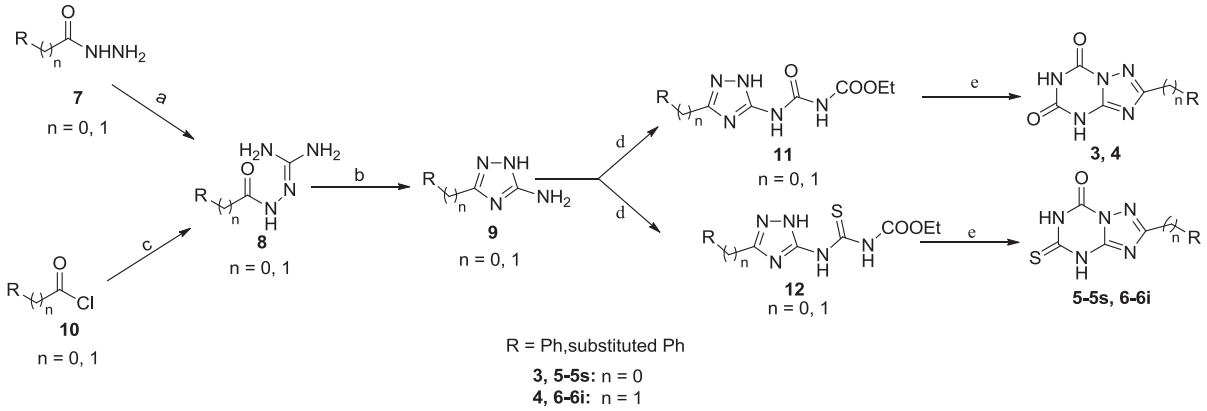
Due to annular tautomerism, target compounds **3**, **4**, **5**–**5s** and **6**–**6i** existed in dynamic equilibrium of A) 4-, B) 3- and C) 1*H*-forms. The prototropic interconversion between these tautomeric forms resulted in broadening of the 4-N(H) signals in ^{1}H NMR spectra of compounds in DMSO. On heating, the broad signal disappeared due to rapid exchange of the NH proton.

2.2. Biological activities

2.2.1. Evaluation of anti-thymidine phosphorylase (anti-TP) activity

Thirty three compounds (**3**–**6**) were evaluated for TP inhibitory activity by a spectrophotometric assay that used recombinant human thymidine phosphorylase, expressed in *Escherichia coli* (T2807-Sigma–Aldrich) as the enzyme and thymidine as the substrate. The original method developed by Krenitsky (Krenitsky et al., 1979) [27] was modified and adopted. The results of the enzyme inhibitory potency were expressed in terms of IC_{50} values and were compared with the positive control, 7-DX (IC_{50} = 42.63 μM) (Table 1). The inhibitory effect of compounds **3**, **4**, **5** and **6** suggested the following structure–activity relationships: (1) Introduction of the homophthalimide moiety led to the complete loss of inhibitory activity. This result was not consistent with the previous report, indicating that most TP inhibitors possessed a homophthalimide moiety, assumed to be the most essential for the binding interactions [11]. (2) Isosteric replacement of thiocarbonyl moiety at C5 significantly enhanced the inhibition profile. (3) Insertion of a methylene bridge into the structure of compound **5** (IC_{50} = 39.56 μM) did not demonstrate substantial improvement in binding interactions, as evident in compound **6** (IC_{50} = 43.32 μM).

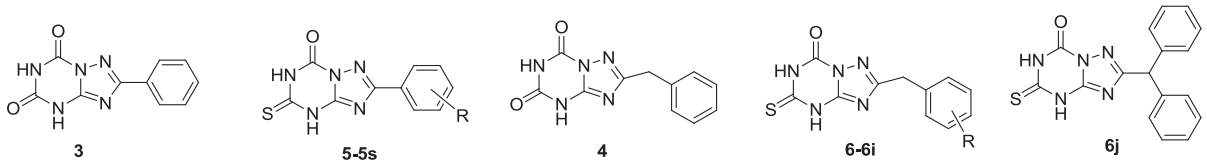
In an attempt to improve the inhibition profile of compounds **5** and **6**, Craig plot directed structural optimization approach [28] was employed. Various substituents scattered around all four Craig plot quadrants, i.e., R = F, Cl, Br, CF_3 (+π, +σ), R = OCH_3, OH (−π, −σ), R = CH_3 (+π, −σ), R = CN (−π, +σ) were selected for this study. This approach generated analogues of **5** and **6** which displayed a varying



Scheme 1. Synthesis of target compounds (**3**, **4** and **5**–**5s**, **6**–**6i**). Reagents and conditions: (a) s-methylisothiourea sulphate, NaOH, r.t., 72 h; (b) water, MW irradiation, 180 °C, 5–7 min; (c) aminoguanidine hydrochloride, fusion at 180 °C, 5 M NaOH, 20 min; (d) ethyl isocyano formate/ethoxycarbonyl isothiocyanate, DMF, r.t., 5 h; (e) NaOH, 80% ethanol (aq.), 100 °C, 20 min.

degree of TP inhibitory activity with IC_{50} values ranged from 3.0 to 64.0 μM (Table 1). With few exceptions (**5e**, **5h** and **6h**), compounds with substituents, no matter electron-withdrawing or electron-donating, showed comparable or better activity than the parent compounds (**5** and **6**) and reference inhibitor (7-DX). Interestingly, di-substituted analogues conferred significantly higher binding affinity than the mono-substituted compounds. Compound **5q** (IC_{50} = 10.84 μM) carrying chloro group in both *meta* and *para*

Table 1
Thymidine phosphorylase inhibitory activity of the synthesized compounds.



Entry	Cpd	R	TP inhibition activity[a] IC_{50} (μM)[b]
1	**3**	–	>100
2	**4**	–	>100
3	**5**	H	39.56 ± 1.77
4	**6**	H	43.32 ± 5.03
5	**5a**	3-NO_2	22.63 ± 5.18
6	**5b**	3-CF_3	22.14 ± 3.32
7	**5c**	3-Cl	30.29 ± 5.44
8	**5d**	3-Br	31.78 ± 3.55
9	**5e**	3-F	48.24 ± 9.50
10	**5f**	3-Me	34.42 ± 3.21
11	**5g**	3-MeO	23.18 ± 5.82
12	**5h**	3-CN	48.54 ± 2.73
13	**5i**	4-NO_2	36.56 ± 3.75
14	**5j**	4-CF_3	22.68 ± 3.73
15	**5k**	4-Cl	25.98 ± 4.34
16	**5l**	4-Br	22.06 ± 4.41
17	**5m**	4-F	32.21 ± 4.78
18	**5n**	4-Me	31.58 ± 3.10
19	**5o**	4-MeO	21.91 ± 2.26
20	**5p**	4-CN	33.06 ± 1.71
21	**5q**	(3,4)-Cl_2	10.84 ± 2.82
22	**5r**	(3,4)-F_2	21.90 ± 7.33
23	**5s**	3-Me, 4-Br–	13.09 ± 1.84
24	**6a**	4-CF_3	9.92 ± 2.96
25	**6b**	4-Cl	8.54 ± 1.91
26	**6c**	4-Br	13.71 ± 4.83
27	**6d**	4-F	22.43 ± 2.69
28	**6e**	4-Me	19.80 ± 6.05
29	**6f**	4-MeO	33.95 ± 7.56
30	**6g**	4-OH	33.78 ± 1.65
31	**6h**	4-CN	64.33 ± 5.87
32	**6i**	(3,4)-Cl_2	2.95 ± 0.74
33	**6j**	–	36.37 ± 4.25
34	7-DX	–	42.63 ± 5.24

[a] Values are means of three experiments.
[b] Values are presented as means ± SD.

positions, was about 3.6 and 3.9-fold more potent than compound **5** and positive control, 7-DX respectively. Analogously, di-chloro substituted analogue of **6**, *viz*., compound **6i** ($IC_{50} = 2.95$ μM) was observed to have best anti-TP activity among the four groups of compounds evaluated in this study and it exhibited 14.7, 13.4 and 14.5-fold enhancement of inhibitory activity as compared to compounds **6**, **5** and 7-DX respectively.

In series **5**, compounds which contained electron-donating and -π hydrophobic substituents, implied an improvement in activity compared to compounds bearing electron-donating and +π hydrophobic groups. In other words, compounds **5g** and **5o** with a methoxy moiety presented better activity than compounds **5f** and **5n** which possessed a methyl substituent in the phenyl ring. In contrast, in series **6**, methoxy substitution in the phenyl ring was less favourable than methyl group. The methoxy moiety may impart steric effect with the introduction of a methylene bridge, resulting in a reduction of binding potential as found in compound **6f**. In addition, among the compounds having (+π, +σ) substituents, compounds **5b**, **5j** and **6a** with a trifluoromethyl moiety in the phenyl ring, displayed better binding affinity. The higher lipophilicity of trifluoromethyl was believed to be compatible with improved hydrophobic interactions into the binding site, leading to an enhancement of inhibition profile of compounds **5b**, **5j** and **6a**. Encouraged by these results, we were impelled to modify the structure of compound **6** by introducing hydrophobic diphenyl motif that generated compound **6j.** However, such effort did not demonstrate any substantial improvement in activity. The unexpected lower inhibitory activity of compound **6j** may be due to the steric hindrance effects of diphenyl ring.

2.2.2. Enzyme inhibition kinetic study of selected compound

Based on the results of TP inhibition analysis, we selected compounds **5q** ($IC_{50} = 10.84$ μM) and **6i** ($IC_{50} = 2.95$ μM) from **5** and **6** series respectively and performed enzyme inhibition kinetic study. The data obtained were analysed and Lineweaver–Burk plots (Fig. 3) were constructed. The results demonstrated that compounds **5q** and **6i** were mixed-type inhibitors with respect to thymidine as a variable substrate, since the reciprocal plots of these compounds showed straight lines that intersected each other in the left panel. This was further confirmed by the fact that increasing inhibitor concentration resulted in an increase of K_m values and a decrease of V_{max} values. For the mixed-type inhibition, we adopted the model of Scheme 2. Thus, the inhibitors may bind to the free enzyme (E) and to the enzyme–substrate complex (ES) by weak bonds at, near or inside the active site, which inactivated the enzyme but did not affect the binding of substrate. In addition, we calculated the values of K_i and αK_i by re-plotting the slopes and intercepts of the double reciprocal plot against the inhibitor concentrations (Table 2). The values of inhibition constants (K_i and αK_i) indicated that the inhibitors had stronger affinity towards the free enzyme than enzyme–substrate complex ($\alpha > 1$). Moreover, the enzyme–inhibitor complex exhibited lower affinity for substrate compared to free enzyme ($\alpha > 1$) (Scheme 2). Due to structural modification of the core ring structure, compounds **5q** and **6i** showed dissimilar interaction mode compared to 7-DX which behaved as a competitive or mixed-type inhibitor in the presence of variable concentrations of thymine [29].

2.2.3. Evaluation of anti-angiogenic activity: inhibition of VEGF expression

It is reported that TP and its metabolite overexpress the vascular endothelial growth factor (VEGF) and play a crucial role in tumour angiogenesis by inducing endothelial cell proliferation, migration and survival [30]. An attempt was made to assess the ability of the selected compounds to inhibit VEGF expression in MDA-MB-231 cells. The di-substituted compounds (**5q**, **5s** and **6i**) endowed with attractive TP inhibition (IC_{50} values >15 μM) were chosen for this assay. The expression level of VEGF protein in the conditioned cell medium was measured using an enzyme-linked immunosorbent assay (ELISA) [31]. As depicted in Fig. 4, all compounds, at doses ranging between 25 μM and 200 μM, markedly prevented VEGF expression compared to vehicle control ($p < 0.05$). A large number of studies reported that the expression of VEGF and TP in solid tumours is significantly correlated as both pro-angiogenic factors share the same transcription site [32,33].

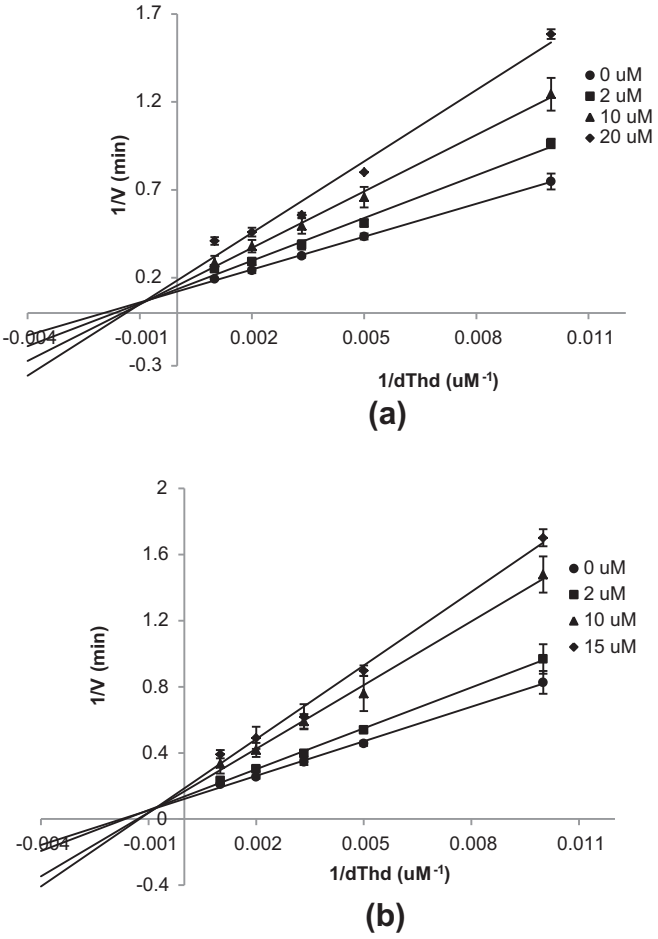
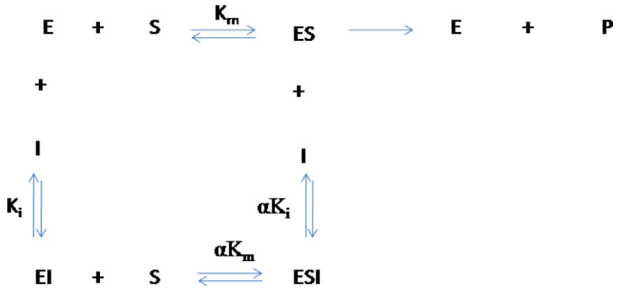


Fig. 3. Lineweaver–Burk plots of TP inhibition by **5q** (**a**) and **6i** (**b**), in the presence of variable concentrations of dThd demonstrating mixed-type enzyme inhibition. Results are presented as means ± SD; SD denoted by error bars (Experiments carried out in triplicate).



Scheme 2. Enzyme kinetic model for mixed type inhibition.

Table 2
A summary of the kinetic parameters of **5q** and **6i**.

Cpd	Substrate	K_i value (μM)[a,b]	α	Type of inhibition
5q	dThd	20.09 ± 1.25	2.12	Mixed type
6i	dThd	19.57 ± 5.62	5.41	Mixed type

[a] Values are means of three experiments.
[b] Values are presented as means ± SD.

2.2.4. Evaluation of anti-angiogenic activity: inhibition of MMP-9 expression

The MMPs have been reported to play a pivotal role in the degradation and remodelling of the basement membrane. Consequently, they promote tumour cell invasion, migration and metastasis. The expression of MMPs in tumour cells has been found to be up-regulated by TP [34]. To explore the inhibitory effects of the selected compounds (**5q**, **5s** and **6i**) on PMA-induced MMP-9 expression, gelatine zymography [35] was conducted in MDA-MB-231 cells. The cells were treated with increasing doses of compounds (50–200 μM) in the presence of PMA (80 nM). Interestingly, a decrease in band intensity compared to vehicle control was detected for all compounds at 200 μM. These results clearly implied that the selected compounds inhibited the proteolytic activity of MMP-9 (band corresponded to the MW of 92 kDa) at the 200 μM concentration (Fig. 5a).

Quantification of the band intensities in the zymograms using Image Gauge 4.0 software revealed that compounds **5q** and **5s** at 100 and 200 μM had significantly ($p < 0.05$) inhibited the MMP-9 activity (Fig. 5b). However, compound **6i**, being the most active TP inhibitor ($IC_{50} = 2.95$ μM), did not display considerable inhibition of MMP-9 secretion at the 50 and 100 μM concentrations. It was assumed that compound **6i** bearing methylene bridge may interact in a slightly different orientation as compared to others (**5q** and **5s**), resulting in insufficient abrogation of MMP-9 expression. The results also suggested that a PMA-induced elevation of MMP-9 expression was attenuated dose-dependently by all the tested compounds (Fig. 5b).

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 bearing 5-thioxo-5,6-dihydro[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-7(4*H*)-one scaffold. Several compounds, in particular those that bear di-chloro group on the phenyl ring, *viz*., compounds **5q** and **6i** exhibited most promising activity as mixed type inhibitors of TP. Moreover, selected TP inhibitors demonstrated anti-angiogenic potential as they attenuated the expression of angiogenesis markers, including MMP-9 and VEGF in MDA-MB-231 cells. Thus, these compounds constitute a new direction for design and synthesis of novel TP inhibitors with promising anti-angiogenic property.

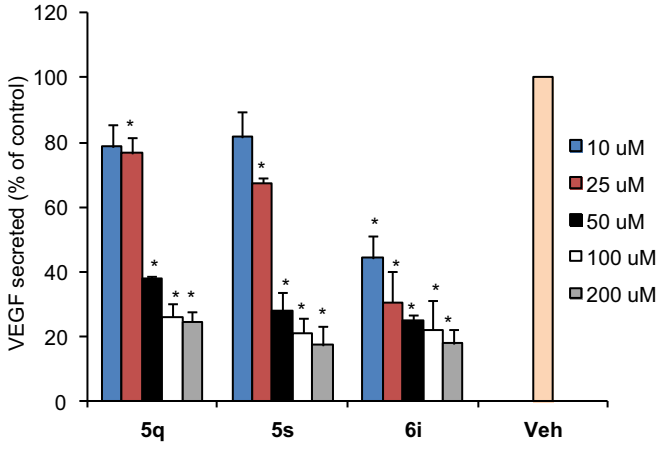


Fig. 4. Effect of selected compounds (**5q**, **5s** and **6i**) on VEGF expression in MDA-MB-231 cells after 24 h treatment. Results are presented as means ± SD; SD denoted by error bars (Experiments carried out in triplicate). *$p < 0.05$.

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 aluminium plate.

*4.1.2. General procedure for the synthesis of 1,2,4-triazolo[1,5-a][1,3,5]triazin-5,7-dione and its 5-thioxo analogues (**3**, **4** and **5**–**5s**, **6**–**6j**)*

Initially, amidoguanidines (**8**) were prepared through the reaction of S-methylisothiourea sulphate (2.8 g, 10 mmol) and corresponding hydrazides (**7**) (3.0 g, 20 mmol) in alkali medium as described previously [25]. Alternatively, the intermediates (**8**) were synthesized *via* the reaction of substituted benzoyl chloride or phenyl acetyl chloride (**10**) (10 mmol) and aminoguanidine hydrochloride (20 mmol). The resulting amidoguanidines (**8**) were then treated with microwave irradiation power (100 W) for 5–8 min in water to obtain 5-amino-1,2,4-triazoles (**9**) [26]. In the next step, the reaction between 5-amino-1,2,4-triazoles (**9**) (3 mmol) and ethyl isocyano formate/ethoxycarbonyl isothiocyanate (3.3 mmol) in anhydrous DMF (4 ml) at room temperature yielded corresponding urea (**11**) or thiourea (**12**) derivatives. Subsequently, to a stirred solution of sodium hydroxide (9 mmol) in ethanol (80%, 20 ml), urea (**11**) or thiourea (**12**) derivatives were added and the reaction mixture was heated on a water bath for 20 min with continuous stirring. After cooling, the solvent was evaporated under vacuum and the residue was suspended in water (25 ml). The resulting suspension was acidified up to pH 1–3 using 2.5 M HCl. The precipitated product (**3**, **4** and **5**–**5s**, **6**–**6j**) was filtered off, recrystallized with suitable solvents and dried under vacuum.

*4.1.3. 2-Phenyl-[1,2,4]triazolo[1,5-a][1,3,5]triazin-5,7(4H,6H)-dione (**3**)*

The title compound was isolated as white powder (54%); mp 318–319 °C (EtOH–water), ESI-MS *m/z* 228.2 $(M - 1)^+$; purity >95%; t_R 2.31 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.52–7.54 (m, 3H, H-3′, H-4′ and H-5′), 8.03–8.05 (m, 2H, H-2′ and H-6′), 11.94 (s, 1H, NH), 12.90 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 127.0 (C-2′ and C-6′), 129.4 (C-3′ and C-5′), 130.0 (C-4′), 131.0 (C-1′), 143.9 (C-2), 149.3 (C-9), 152.5 (C-5), 162.2 (C-7); Anal. calcd. for $C_{10}H_7N_5O_2$: C, 52.40; H, 3.08; N, 30.56. Found: C, 51.88; H, 3.01; N, 29.89.

*4.1.4. 2-Phenyl-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5**)*

The title compound was isolated as white powder (78%); mp 258–259 °C (EtOH); ESI-MS *m/z* 244.1 $(M - 1)^+$; purity >95%; t_R

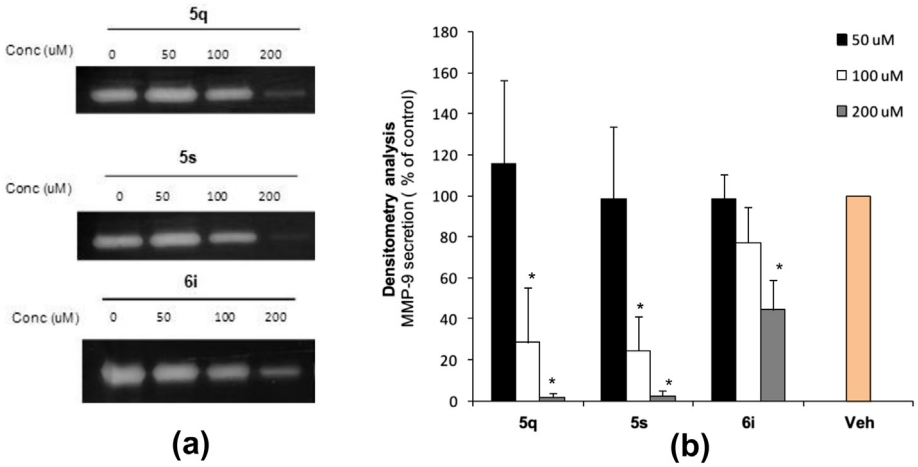


Fig. 5. Effect of selected synthesized compounds (**5q**, **5s** and **6i**) on PMA-induced MMP-9 expression in MDA-MB-231 cells: (**a**) suppressive effects of selected compounds on MMP-9 expression and their dose response; (**b**) densitometry analysis of MMP-9 expression. Results are presented as means ± SD; SD denoted by error bars (Experiments carried out in triplicate). $^{*}p < 0.05$.

2.16 min (B); ^{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, 48.28; H, 2.71; N, 28.20.

4.1.5. 2-(3-Nitrophenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5a**)

The title compound was isolated as off-white powder (80%); mp 246–248 °C (acetic acid), ESI-MS m/z 289.1 $(M-1)^+$; purity >95%; t_R 2.13 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.86 (t, 1H, H-5′, J = 8.1 Hz), 8.39 (dd, 1H, H-4′, J = 8.1, 1.3 Hz), 8.47 (d, 1H, H-6′, J = 7.5 Hz), 8.74 (s, 1H, H-2′), 13.19 (s, 1H, NH), 14.51 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 120.9 (C-2′), 125.2 (C-4′), 130.6 (C-5′), 130.9 (C-6′), 132.4 (C-1′), 141.0 (C-3′), 148.1 (C-2), 151.7 (C-9), 160.0 (C-7), 175.3 (C-5); Anal. calcd. for $C_{10}H_6N_6O_3S$: C, 41.38; H, 2.08; N, 28.95. Found: C, 41.11; H, 2.01; N, 28.26.

4.1.6. 5-Thioxo-2-(3-(trifluoromethyl)phenyl)-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5b**)

The title compound was isolated as white powder (81%); mp 268–270 °C (EtOH–water); ESI-MS m/z 312.1 $(M-1)^+$; purity >95%; t_R 2.15 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.81 (t, 1H, H-5′, J = 7.7 Hz), 7.93 (d, 1H, H-4′, J = 7.9 Hz), 8.27 (s, 1H, H-2′), 8.35 (d, 1H, H-6′, J = 7.5 Hz), 13.18 (s, 1H, NH), 14.40 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 122.6 (q, CF_3, J = 4.1 Hz), 123.8 (q, C-2′, J = 272.5 Hz), 127.2 (q, C-4′, J = 3.4 Hz), 129.7 (q, C-5′, J = 32.0 Hz), 130.2 (C-6′), 130.3 (C-3′), 130.5 (C-1′), 141.1 (C-2), 151.6 (C-9), 160.5 (C-7), 175.3 (C-5); Anal. calcd. for $C_{11}H_6F_3N_5OS$: C, 42.18; H, 1.93; N, 22.36. Found: C, 41.85; H, 1.89; N, 22.12.

4.1.7. 2-(3-Chlorophenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5c**)

The title compound was isolated as white powder (65%); mp 266–267 °C (acetic acid); ESI-MS m/z 278.0 $(M-1)^+$; purity >95%; t_R 2.18 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.55–7.63 (m, 2H, H-4′ and H-5′), 8.00–8.02 (m, 2H, H-2′ and H-6′), 13.16 (s, 1H, NH), 14.26 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 125.7 (C-6′), 126.6 (C-2′), 131.1 (C-5′), 131.6 (C-4′), 134.2 (C-3′), 141.6 (C-1′), 141.6 (C-2), 152.0 (C-9), 161.2 (C-7), 175.8 (C-5); Anal. calcd. for $C_{10}H_6ClN_5OS$: C, 42.94; H, 2.16; N, 25.04. Found: C, 41.97; H, 2.02; N, 24.88.

4.1.8. 2-(3-Bromophenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5d**)

The title compound was isolated as white crystalline powder (75%); mp 262–264 °C (acetic acid), ESI-MS m/z 324.0 $(M-1)^+$; purity >95%; t_R 2.19 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.52 (t, 1H, H-5′, J = 7.9 Hz), 7.74 (d, 1H, H-4′, J = 7.9 Hz), 8.05 (d, 1H, H-6′, J = 7.9 Hz), 8.14 (s, 1H, H-2′), 13.18 (s, 1H, NH), 14.22 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 122.6 (C-3′), 126.0 (C-6′), 129.4 (C-2′), 131.9 (C-5′), 131.9 (C-4′), 133.9 (C-1′), 141.6 (C-2), 152.0 (C-9), 161.0 (C-7), 175.8 (C-5); Anal. calcd. for $C_{10}H_6BrN_5OS$: C, 37.05; H, 1.87; N, 21.60. Found: C, 36.89; H, 1.72; N, 20.96.

4.1.9. 2-(3-Fluorophenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5e**)

The title compound was isolated as white powder (72%); mp 283–285 °C (acetic acid); ESI-MS m/z 262.3 $(M-1)^+$; purity >95%; t_R 2.15 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.39 (td, 1H, H-5′, J = 8.5, 2.3 Hz), 7.60 (dd, 1H, H-4′, J = 7.9, 13.9 Hz), 7.75 (d, 1H, H-2′, J = 9.8 Hz), 7.90 (d, 1H, H-6′, J = 7.9 Hz), 13.13 (s, 1H, NH), 14.33 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 114.1 (d, C-2′, J = 23.3 Hz), 118.7 (d, C-4′, J = 21.1 Hz), 122.7 (d, C-6′, J = 1.5 Hz), 131.3 (d, C-5′, J = 8.0 Hz), 131.5 (d, C-1′, J = 8.0 Hz), 141.1 (C-9), 151.5 (C-7), 160.7 (d, C-2, J = 2.9 Hz), 162.2 (d, C-3′, J = 244.1 Hz), 175.3 (C-5); Anal. calcd. for $C_{10}H_6FN_5OS$: C, 45.62; H, 2.30; N, 26.60. Found: C, 45.79; H, 2.22; N, 26.16.

4.1.10. 2-(3-Methylphenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5f**)

The title compound was isolated as white powder (80%); mp 259–261 °C (EtOH–water); ESI-MS m/z 258.1 $(M-1)^+$; purity >95%; t_R 2.17 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.40 (s, 3H, Me), 7.34 (d, 1H, H-4′, J = 7.5 Hz), 7.42 (t, 1H, H-5′, J = 7.5 Hz), 7.84 (d, 1H, H-6′, J = 7.9 Hz), 7.88 (s, 1H, H-2′), 13.09 (s, 1H, NH), 14.24 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 20.9 (Me), 123.7 (C-6′), 127.0 (C-2′), 128.8 (C-5′), 129.0 (C-4′), 131.3 (C-1′), 138.2 (C-3′), 141.2 (C-2), 151.3 (C-9), 161.9 (C-7), 175.2 (C-5); Anal. calcd. for $C_{11}H_9N_5OS$: C, 50.95; H, 3.50; N, 27.01. Found: C, 50.77; H, 3.42; N, 26.96.

4.1.11. 2-(3-Methoxyphenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5g**)

The title compound was isolated as white powder (90%); mp 260 °C (acetic acid), ESI-MS m/z 274.1 $(M-1)^+$; purity >95%; t_R 2.15 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 276.6. 3.84 (s, 3H,

OMe), 7.11 (dd, 1H, H-4′, J = 8.1, 2.1 Hz), 7.45 (t, 1H, H-5′, J = 7.9 Hz), 7.54 (s, 1H, H-2′), 7.64 (d, 1H, H-6′, J = 7.5 Hz), 13.10 (s, 1H, NH), 14.32 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 55.1 (OMe), 111.2 (C-2′), 116.6 (C-4′), 118.9 (C-6′), 130.1 (C-5′), 130.4 (C-1′), 141.1 (C-3′), 151.3 (C-2), 159.4 (C-9), 161.7 (C-7), 175.2 (C-5); Anal. calcd. for $C_{11}H_9N_5O_2S$: C, 47.99; H, 3.30; N, 25.44. Found: C, 47.23; H, 3.21; N, 25.16.

4.1.12. 3-(7-Oxo-5-thioxo-4,5,6,7-tetrahydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-2-yl)benzonitrile (**5h**)

The title compound was isolated as white crystalline powder (67%); mp >300 °C (acetic acid); ESI-MS m/z 287.0 $(M-1)^+$; purity >95%; t_R 4.07 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.48–8.57 (m, 4H, Ar–H), 13.10 (s, 1H, NH), 14.38 (br. s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ 126.5 (C-3′), 129.7 (CN), 129.8 (C-2′), 130.0 (C-5′), 130.1 (C-1′), 135.6 (C-6′), 141.9 (C-4′), 152.2 (C-2), 162.1 (C-9), 168.1 (C-7), 176.0 (C-5); Anal. calcd. for $C_{11}H_6N_6OS$: C, 48.88; H, 2.24; N, 31.09. Found: C, 48.12; H, 2.11; N, 30.15.

4.1.13. 2-(4-Nitrophenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5i**)

The title compound was isolated as off-white powder (65%); mp >300 °C (EtOH–water); ESI-MS m/z 289.1 $(M-1)^+$; purity >95%; t_R 2.13 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.29 (d, 2H, H-3′ and H-5′, J = 8.7 Hz), 8.39 (d, 2H, H-2′ and H-6′, J = 9.0 Hz), 13.21 (s, 1H, NH), 14.28 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 124.3 (C2′ and C-6′), 127.7 (C-3′ and C-5′), 135.0 (C-4′), 141.1 (C-1′), 148.5 (C-2), 151.7 (C-9), 160.1 (C-7), 175.3 (C-5); Anal. calcd. for $C_{10}H_6N_6O_3S$: C, 41.38; H, 2.08; N, 28.95. Found: C, 41.25; H, 2.05; N, 28.12.

4.1.14. 5-Thioxo-2-(4-(trifluoromethyl)phenyl)-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5j**)

The title compound was isolated as white powder (69%); mp 280 °C (EtOH–water); ESI-MS m/z 312.0 $(M-1)^+$; purity >95%; t_R 2.15 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.91 (d, 2H, H-3′ and H-5′, J = 8.3), 8.26 (d, 2H, H-2′ and H-6′, J = 8.3), 13.18 (s, 1H, NH), 14.36 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 123.9 (q, CF_3, J = 272.2 Hz), 125.9 (q, C-3′ and C-5′, J = 3.4 Hz), 127.3 (C-2′ and C-6′), 130.5 (q, C-4′, J = 32.0 Hz), 133.0 (C-1′), 141.1 (C-2), 151.7 (C-9), 160.6 (C-7), 175.3 (C-5); Anal. calcd. for $C_{11}H_6F_3N_5OS$: C, 42.18; H, 1.93; N, 22.36. Found: C, 41.54; H, 1.82; N, 22.21.

4.1.15. 2-(4-Chlorophenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5k**)

The title compound was isolated as white powder (66%); mp 281–282 °C (EtOH–water); ESI-MS m/z 278.0 $(M-1)^+$; purity >95%; t_R 2.18 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.61 (d, 2H, H-3′ and H-5′, J = 8.3), 8.05 (d, 2H, H-2′ and H-6′, J = 8.3), 13.14 (s, 1H, NH), 14.31 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 128.01 (C-4′), 128.26 (C2′ and C-6′), 129.08 (C-3′ and C-5′), 135.32 (C-1′), 141.08 (C-2), 151.46 (C-9), 160.89 (C-7), 175.22 (C-5); Anal. calcd. for $C_{10}H_6ClN_5OS$: C, 42.94; H, 2.16; N, 25.04. Found: C, 41.26; H, 2.11; N, 24.29.

4.1.16. 2-(4-Bromophenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5l**)

The title compound was isolated as white powder (73%); mp 292–293 °C (acetic acid); ESI-MS m/z 324.0 $(M-1)^+$; purity >95%; t_R 2.21 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.75 (d, 2H, H-3′ and H-5′, J = 8.3), 7.98 (d, 2H, H-2′ and H-6′, J = 8.3), 13.13 (s, 1H, NH), 14.35 (br. s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ 124.8 (C-4′), 128.9 (C-1′), 129.1 (C2′ and C-6′), 132.6 (C-3′ and C-5′), 141.7 (C-2), 152.0 (C-9), 161.6 (C-7), 175.8 (C-5); Anal. calcd. for $C_{10}H_6BrN_5OS$: C, 37.05; H, 1.87; N, 21.60. Found: C, 36.54; H, 1.75; N, 20.82.

4.1.17. 2-(4-Fluorophenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5m**)

The title compound was isolated as white powder (67%); mp 262 °C (EtOH–water); ESI-MS m/z 262.3 $(M-1)^+$; purity >95%; t_R 2.13 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.37 (dd, 2H, H-3′ and H-5′, J = 8.9, 8.9 Hz), 8.09 (dd, 2H, H-2′ and H-6′, J = 8.7, 5.7 Hz), 13.12 (s, 1H, NH), 14.20 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 116.0 (d, C-3′ and C-5′, J = 21.8 Hz), 125.7 (d, C-1′, J = 2.9 Hz), 128.9 (d, C-2′ and C-6′, J = 8.7 Hz), 141.1 (C-2), 151.4 (C-9), 161.0 (C-7), 163.5 (d, C-4′, J = 247.8 Hz), 175.2 (C-5); Anal. calcd. for $C_{10}H_6FN_5OS$: C, 45.62; H, 2.30; N, 26.60. Found: C, 45.24; H, 2.14; N, 26.28.

4.1.18. 2-(4-Methylphenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5n**)

The title compound was isolated as white powder (78%); mp 296–297 °C (EtOH–water); ESI-MS m/z 258.1 $(M-1)^+$; purity >95%; t_R 2.19 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.38 (s, 3H, ArMe), 7.34 (d, 2H, H-3′ and H-5′, J = 7.9), 7.94 (d, 2H, H-2′ and H-6′, J = 7.9), 13.07 (s, 1H, NH), 14.26 (br. s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ 20.95 (Me), 126.38 (C-4′), 126.46 (C2′ and C-6′), 129.45 (C-3′ and C-5′), 140.44 (C-1′), 141.11 (C-2), 151.22 (C-9), 161.86 (C-7), 175.14 (C-5); Anal. calcd. for $C_{11}H_9N_5OS$: C, 50.95; H, 3.50; N, 27.01. Found: C, 50.15; H, 3.24; N, 27.26.

4.1.19. 2-(4-Methoxyphenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5o**)

The title compound was isolated as white crystalline powder (63%); mp 270–271 °C (acetic acid); ESI-MS m/z 288.1 $(M-1)^+$; purity >95%; t_R 2.15 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.84 (s, 3H, OMe), 7.08 (d, 2H, H-3′ and H-5′, J = 8.7 Hz), 7.99 (d, 2H, H-2′ and H-6′, J = 9.0 Hz), 13.07 (s, 1H, NH), 14.11 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 55.25 (MeO), 114.29 (C2′ and C-6′), 121.52 (C-4′), 128.17 (C-3′ and C-5′), 141.13 (C-1′), 151.20 (C-2), 161.12 (C-9), 161.75 (C-7), 175.12 (C-5); Anal. calcd. for $C_{11}H_9N_5O_2S$: C, 47.99; H, 3.30; N, 25.44. Found: C, 47.56; H, 3.19; N, 25.02.

4.1.20. 4-(7-Oxo-5-thioxo-4,5,6,7-tetrahydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-2-yl)benzonitrile (**5p**)

The title compound was isolated as white powder (69%); mp >300 °C (EtOH–water); ESI-MS m/z 287.0 $(M-1)^+$; purity >95%; t_R 4.06 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.02 (d, 2H, H-3′ and H-5′, J = 8.4 Hz), 8.12 (d, 2H, H-2′ and H-6′, J = 8.4 Hz), 13.11 (s, 1H, NH), 14.44 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 126.3 (C-3′ and C-5′), 128.1 (C-2′ and C-6′), 131.6 (CN), 136.0 (C-4′), 141.2 (C-1′), 151.7 (C-2), 161.2 (C-9), 167.2 (C-5), 175.4 (C-7); Anal. calcd. for $C_{11}H_6N_6OS$: C, 48.88; H, 2.24; N, 31.09. Found: C, 48.52; H, 2.08; N, 30.58.

4.1.21. 2-(3,4-Dichlorophenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5q**)

The title compound was isolated as white powder (74%); mp 271–273 °C (acetic acid); ESI-MS m/z 314.0 $(M-1)^+$; purity >95%; t_R 2.23 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.82 (d, 1H, H-6′, J = 8.3 Hz), 8.01 (dd, 1H, H-5′, J = 8.3 Hz, J = 1.9 Hz), 8.16 (d, 1H, H-2′, J = 1.9 Hz), 13.17 (s, 1H, NH), 14.35 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 127.1 (C-6′), 128.5 (C-2′), 130.3 (C-5′), 132.0 (C-4′), 132.4 (C-3′), 133.9 (C-1′), 141.6 (C-2), 152.2 (C-9), 160.4 (C-7), 175.9 (C-5); Anal. calcd. for $C_{10}H_5Cl_2N_5OS$: C, 38.23; H, 1.60; N, 22.29. Found: C, 38.02; H, 1.41; N, 21.95.

4.1.22. 2-(3,4-Difluorophenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5r**)

The title compound was isolated as white powder (68%); mp 267–269 °C (acetic acid); ESI-MS m/z 280.3 $(M-1)^+$; purity >95%; t_R 2.16 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.57–7.66 (m, 1H, H-5′), 7.89–8.00 (m, 1H, H-2′ and H-6′), 13.17 (s, 1H, NH), 14.40 (br.

s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 115.5 (d, C-3′, J = 18.9 Hz), 118.4 (d, C-5′, J = 17.4 Hz), 123.8 (dd, C-6′, J = 6.9, 3.3 Hz), 126.7 (dd, C-1′, J = 6.5, 3.6 Hz), 141.1 (C-2), 149.6 (dd, C-4′, J = 246.7, 12.7 Hz), 150.9 (dd, C-3′, J = 250.0, 12.4 Hz), 151.6 (C-9), 160.1 (C-7), 175.3 (C-5); Anal. calcd. for $C_{10}H_5F_2N_5OS$: C, 42.71; H, 1.79; N, 24.90. Found: C, 42.08; H, 1.56; N, 24.12.

4.1.23. 2-(4-Bromo-3-methylphenyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**5s**)

The title compound was isolated as white crystalline powder (77%); mp 278–280 °C (acetic acid); ESI-MS m/z 338.0 $(M-1)^+$; purity >95%; t_R 2.24 min (B); ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.44 (s, 3H, Me), 7.72–7.79 (m, 2H, H-5′ and H-6′), 8.00 (s, 1H, H-2′), 13.13 (s, 1H, NH), 14.30 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 22.9 (Me), 126.4 (C-4′), 127.2 (C-6′), 129.2 (C-2′), 129.3 (C-5′), 133.4 (C-1′), 138.7 (C-3′), 141.7 (C-2′), 152.0 (C-9), 161.6 (C-7), 175.8 (C-5); Anal. calcd. for $C_{11}H_8BrN_5OS$: C, 39.07; H, 2.38; N, 20.71. Found: C, 38.88; H, 2.26; N, 20.21.

4.1.24. 2-Benzyl-[1,2,4]triazolo[1,5-a][1,3,5]triazine-5,7(4H,6H)-dione (**4**)

The title compound was isolated as white powder (47%); mp >300 °C (EtOH–water); ESI-MS m/z 242.3 $(M-1)^+$; purity >95%; t_R 2.02 min (A); ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.00 (s, 2H, $PhCH_2$), 7.04–7.61 (m, 5H, Ar–H), 11.81 (s, 1H, NH), 12.69 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 34.8 (CH_2), 127.04 (C-4′), 128.8 (C-2′ and C-6′), 129.5 (C-3′ and C-5′), 137.5 (C-1′), 143.9 (C-2), 149.4 (C-9), 152.1 (C-7), 164.8 (C-5); Anal. calcd. for $C_{11}H_9N_5O_2$: C, 54.32; H, 3.73; N, 28.79. Found: C, 54.02; H, 3.58; N, 27.88.

4.1.25. 2-Benzyl-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**6**)

The title compound was isolated as white powder (55%); mp 246 °C (EtOH–water); ESI-MS m/z 242.3 $(M-1)^+$; purity >95%; t_R 1.68 min (A); ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.01 (s, 2H, $PhCH_2$), 7.23–7.29 (m, 5H, Ph), 12.99 (s, 1H, NH), 14.10 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 34.7 (CH_2), 127.1 (C-4′), 128.9 (C-2′ and C-6′), 129.5 (C-3′ and C-5′), 137.4 (C-1′), 141.6 (C-2), 151.5 (C-9), 165.0 (C-7), 175.8 (C-5); Anal. calcd. for $C_{11}H_9N_5OS$: C, 50.95; H, 3.50; N, 27.01. Found: C, 51.53; H, 3.38; N, 28.32.

4.1.26. 5-Thioxo-2-(4-(trifluoromethyl)benzyl)-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**6a**)

The title compound was isolated as white powder (67%); mp 280–281 °C (EtOH–water); ESI-MS m/z 326.1 $(M-1)^+$; purity >95%; t_R 4.53 min (A); ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.15 (s, 2H, $PhCH_2$), 7.54 (d, 2H, H-3′ and H-5′, J = 7.9 Hz), 7.69 (d, 2H, H-2′ and H-6′, J = 7.9 Hz), 13.03 (s, 1H, NH), 14.12 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 33.8 (CH_2), 124.3 (q, CF_3, J = 271.9 Hz), 125.1 (q, C-3′ and C-5′, J = 3.7 Hz), 127.4 (q, C-4′, J = 31.8 Hz), 129.8 (C-2′ and C-6′), 141.0 (C-1′), 141.7 (C-2), 151.1 (C-9), 163.8 (C-7), 175.2 (C-5); Anal. calcd. for $C_{12}H_8F_3N_5OS$: C, 44.04; H, 2.46; N, 21.40. Found: C, 45.23; H, 2.29; N, 21.02.

4.1.27. 2-(4-Chlorobenzyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**6b**)

The title compound was isolated as white powder (64%); mp 241–243 °C (EtOH–water); ESI-MS m/z 292.2 $(M-1)^+$; purity >95%; t_R 1.95 min (A); ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.02 (s, 2H, $PhCH_2$), 7.32 (d, 2H, H-3′ and H-5′, J = 8.6 Hz), 7.38 (d, 2H, H-2′ and H-6′, J = 8.5 Hz), 13.00 (s, 1H, NH), 14.14 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 33.9 (CH_2), 128.7 (C-2′ and C-6′), 131.3 (C-3′ and C-5′), 131.8 (C-4′), 136.4 (C-1′), 141.6 (C-2), 151.6 (C-9), 164.6 (C-7), 175.8 (C-5); Anal. calcd. for $C_{11}H_8ClN_5OS$: C, 44.98; H, 2.75; N, 23.84. Found: C, 44.22; H, 2.69; N, 23.65.

4.1.28. 2-(4-Bromobenzyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**6c**)

The title compound was isolated as white powder (65%); mp 238–240 °C (EtOH–water); ESI-MS m/z 338.0 $(M-1)^+$; purity >95%; t_R 1.69 min (A); ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.00 (s, 2H, $PhCH_2$), 7.26 (d, 2H, H-3′ and H-5′, J = 8.0 Hz), 7.51 (d, 2H, H-2′ and H-6′, J = 8.1 Hz), 12.98 (s, 1H, NH), 13.88 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 33.40 (CH_2), 119.7 (C-4′), 131.1 (C-2′ and C-6′), 131.2 (C-3′ and C-5′), 136.2 (C-1′), 141.0 (C-2), 151.1 (C-9), 164.0 (C-7), 175.2 (C-5); Anal. calcd. for $C_{11}H_8BrN_5OS$: C, 39.07; H, 2.38; N, 20.71. Found: C, 40.25; H, 2.24; N, 20.12.

4.1.29. 2-(4-Fluorobenzyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**6d**)

The title compound was isolated as white powder (72%); mp 260–262 °C (EtOH–water); ESI-MS m/z 276.1 $(M-1)^+$; purity >95%; t_R 1.68 min (A); ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.01 (s, 2H, $PhCH_2$), 7.11–7.16 (m, 2H, H-3′ and H-5′), 7.32–7.35 (m, 2H, H-2′ and H-6′), 13.00 (s, 1H, NH), 14.09 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 33.8 (CH_2), 115.6 (d, C-3′ and C-5′, J = 21.3 Hz), 131.3 (d, C-2′ and C-6′, J = 8.1 Hz), 133.5 (d, C-1′, J = 3.7 Hz), 141.6 (C-2), 151.5 (C-9), 161.6 (d, C-4′, J = 242.1 Hz), 164.9 (C-7), 175.8 (C-5); Anal. calcd. for $C_{11}H_8FN_5OS$: C, 47.65; H, 2.91; N, 25.26. Found: C, 47.23; H, 2.82; N, 25.05.

4.1.30. 2-(4-Methylbenzyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**6e**)

The title compound was isolated as white powder (72%); mp 244–246 °C (EtOH–water); ESI-MS m/z 272.1 $(M-1)^+$; purity >95%; t_R 1.68 min (A); ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.26 (s, 3H, Me), 3.96 (s, 2H, $PhCH_2$), 7.10 (d, 2H, H-3′ and H-5′, J = 7.8 Hz), 7.17 (d, 2H, H-2′ and H-6′, J = 8.0 Hz), 13.00 (s, 1H, NH), 14.03 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 21.1 (Me), 34.3 (CH_2), 129.3 (C-2′ and C-6′), 129.4 (C-3′ and C-5′), 134.2 (C-4′), 136.1 (C-1′), 141.6 (C-2), 151.5 (C-9), 165.2 (C-7), 175.8 (C-5); Anal. calcd. for $C_{12}H_{11}N_5OS$: C, 52.73; H, 4.06; N, 25.62. Found: C, 51.85; H, 4.12; N, 25.26.

4.1.31. 2-(4-Methoxybenzyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**6f**)

The title compound was isolated as white crystalline powder (78%); mp 243–244 °C (EtOH–water); ESI-MS m/z 288.1 $(M-1)^+$; purity >95%; t_R 1.68 min (A); ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.72 (s, 3H, MeO), 3.93 (s, 2H, $PhCH_2$), 6.86 (d, 2H, H-3′ and H-5′, J = 8.7 Hz), 7.21 (d, 2H, H-2′ and H-6′, J = 8.7 Hz), 12.98 (s, 1H, NH), 14.09 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 33.8 (CH_2), 55.5 (MeO), 114.2 (C-2′ and C-6′), 129.2 (C-4′), 130.5 (C-3′ and C-5′), 141.6 (C-1′), 151.4 (C-2), 158.5 (C-9), 165.3 (C-7), 175.8 (C-5); Anal. calcd. for $C_{12}H_{11}N_5O_2S$: C, 49.82; H, 3.83; N, 24.21. Found: C, 50.06; H, 3.78; N, 24.38.

4.1.32. 2-(4-Hydroxybenzyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**6g**)

The title compound was isolated as white powder (65%); mp 268–270 °C (EtOH–water); ESI-MS m/z 274.0 $(M-1)^+$; purity >95%; t_R 1.75 min (A); ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.87 (s, 2H, $PhCH_2$), 6.69 (d, 2H, H-3′ and H-5′, J = 8.2 Hz), 7.08 (d, 2H, H-2′ and H-6′, J = 8.2 Hz), 9.26 (s, 1H, OH), 12.98 (s, 1H, NH), 14.05 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 33.9 (CH_2), 115.6 (C-2′ and C-6′), 127.4 (C-4′), 130.4 (C-3′ and C-5′), 141.6 (C-1′), 151.4 (C-2), 156.5 (C-9), 165.5 (C-7), 175.8 (C-5); Anal. calcd. for $C_{11}H_9N_5O_2S$: C, 47.99; H, 3.30; N, 25.44. Found: C, 47.45; H, 3.22; N, 24.78.

4.1.33. 4-((7-Oxo-5-thioxo-4,5,6,7-tetrahydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-2-yl)methyl)benzonitrile (**6h**)

The title compound was isolated as off-white powder (60%); mp >300 °C (EtOH–water); ESI-MS m/z 302.1 $(M-1)^+$; purity >95%; t_R

1.64 min (A); ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.10 (s, 2H, $PhCH_2$), 7.42 (d, 2H, H-3′ and H-5′, J = 8.2 Hz), 7.69 (d, 2H, H-2′ and H-6′, J = 8.2 Hz), 12.95 (s, 1H, NH), 13.71 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 34.0 (CH_2), 127.5 (C-4′), 129.1 (C-2′ and C-6′), 129.3 (C-3′ and C-5′), 141.1 (C-1′), 142.0 (C-2), 163.9 (C-9), 167.0 (C-7), 175.4 (C-5); Anal. calcd. for $C_{12}H_8N_6OS$: C, 50.70; H, 2.84; N, 29.56. Found: C, 50.56; H, 2.72; N, 29.12.

4.1.34. 2-(3,4-Dichlorobenzyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**6i**)

The title compound was isolated as white powder (57%); mp 265–267 °C (EtOH–water); ESI-MS m/z 326.1 $(M-1)^+$; purity >95%; t_R 1.75 min (A); ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.07 (s, 2H, $PhCH_2$), 7.30–7.33 (m, 1H, H-2′), 7.56–7.59 (m, 3H, H-5′ and H-6′), 13.04 (s, 1H, NH), 14.15 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 33.5 (CH_2), 129.8 (C-6′), 130.0 (C-2′), 130.9 (5′), 131.3 (C-4′), 138.5 (C-1′), 141.6 (C-2), 151.6 (C-9), 164.1 (C-7), 175.8 (C-5); Anal. calcd. for $C_{11}H_7Cl_2N_5OS$: C, 40.26; H, 2.15; N, 21.34. Found: C, 40.12; H, 2.12; N, 21.16.

4.1.35. 2-([1,1′-Diphenyl]-4-ylmethyl)-5-thioxo-5,6-dihydro-[1,2,4]triazolo[1,5-a][1,3,5]triazin-7(4H)-one (**6j**)

The title compound was isolated as white powder (63%); mp 210–212 °C (EtOH–water); ESI-MS m/z 334.1 $(M-1)^+$; purity >95%; t_R 1.69 min (A); ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.62 (s, 2H, $PhCH_2$), 7.21–7.41 (m, 9H, Ar–H), 12.96 (s, 1H, NH), 13.78 (br. s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 50.5 (CH_2), 127.2 (C-3′ and C-5′), 128.8 (C-2″, C-4″and C-6″), 128.9 (C-3″ and C-5″), 129.3 (C-1′, C-4′ and C-1″), 141.5 (C-2′ and C-6′), 141.7 (C-2), 151.6 (C-9), 166.8 (C-7), 175.8 (C-5); Anal. calcd. for $C_{17}H_{13}N_5OS$: C, 60.88; H, 3.91; N, 20.88. Found: C, 59.56; H, 4.02; N, 21.22.

4.2. Biological characterization

4.2.1. In vitro thymidine phosphorylase enzyme assay

A spectrophotometric assay method, originally developed by Krenitsky (Krenitsky et al., 1979) [27], 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:

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

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

All the experiments were carried out in triplicate.

4.2.2. Enzyme inhibition kinetics study

The TP inhibitory activity of compounds was evaluated at varying concentrations of thymidine (1000, 500, 300, 200, 100 μM). The saturating concentration of inorganic phosphate was fixed at 25 mM. The conversion of thymidine to thymine was monitored. All the experiments were conducted in triplicate.

4.2.3. VEGF assay

To determine the ability of TP inhibitors to prevent VEGF expression in MDA-MB-231 cells, a colorimetric enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions (Abfrontier) [31].

The cells were treated with different doses (10–200 μM) of compounds or DMSO and incubated at 37 °C for 24 h. The cell supernatant was then collected and it was subjected to centrifugation (5000 g for 5 min) to remove cells and debris. Each sample was diluted with dilution buffer to obtain a concentration suitable for measurement. Subsequently, 0.1 ml of different VEGF standard solutions and appropriately diluted samples were placed into the precoated 96-well plate and incubated at 37 °C for 90 min. In the next step, the contents from each plate were discarded and 0.1 ml of biotinylated anti-human VEGF antibody working solution was added and incubated for another 60 min. The plates were thoroughly washed with 0.01 M TBS and 0.1 ml of Avidin–Biotin–Peroxidase Complex working solution was introduced. After incubation at 37 °C for 30 min, 90 μl of prepared TMB colour developing solution was poured to each well and incubated at 37 °C for 20–25 min. Thereafter, 0.1 ml of prepared TMB stop solution was added to each well, leading to a colour change from blue to yellow and absorbance at 450 nm was measured.

4.2.4. Gelatine zymography

Gelatine zymography was preformed as described previously with a slight modification (Liotta and Stetler-Stevenson 1990) [35]. MDA-MB-231 cells were seeded onto six-well plates in RPMI with 10% FBS and allowed to propagate up to 80% confluence. The cells were then maintained in serum-free medium for at least 24 h prior to designated treatments with compounds and PMA (80 nM). After a 24-h incubation at 37 °C, the conditioned medium was collected and centrifuged at 8000 g for 8 min at 4 °C to remove cells and debris. The protein contents of the conditioned media were determined and the volume of each sample having equal amount of protein was adjusted by adding PBS. The resulting samples were mixed with 3× loading buffer and subjected to electrophoresis on a 7.5% SDS-PAGE gel containing 0.1% (w/v) gelatine using 1× Tris-Glycin SDS running buffer at 100 V for 90–120 min. Subsequently, the gels were washed in renaturing buffer (2.5% Triton X-100) for 30 min to remove SDS and equilibrated in 1× zymogram developing buffer (50 mM tris, 10 mM $CaCl_2$, 0.15 M NaCl, pH-7.5) for 30 min. The gels were then incubated in fresh developing buffer at 37 °C for 24 h to allow gelatine digestion. The gelatinolytic activity of MMP-9 was visualized by staining the gels with 1% Coomassie blue R-250 and distained in 50% methanol, 10% acetic acid, 40% water (v/v) until clear bands suggestive of gelatine digestion were observed.

4.2.5. Densitometric and statistical analysis

The intensity of each band was quantified using Image Gauge 4.0 software. The numerical data were presented as means ± SD of replicates. Statistical significance between treatment and control groups was analysed using ANOVA test. A value of $p < 0.05$ was considered statistically significant.

Acknowledgements

This work is supported by the National Medical Research Council grant, Singapore (R-148-000-102-275) and Academic Research Funds of National University of Singapore. The authors

are also thankful to Gokaraju Rangaraju Educational Society for providing essential laboratory facilities and support for this project.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2013.06.051. These data include MOL files and InChiKeys of the most important compounds described in this article.

References

[1] J. Folkman, E. Merler, C. Abernathy, G. Williams, Isolation of a tumor factor responsible or angiogenesis, J. Exp. Med. 133 (1971) 275–288.
[2] L. Hlatky, P. Hahnfeldt, J. Folkman, Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us, J. Natl. Cancer Inst. 94 (2002) 883–893.
[3] N. Weidner, J.P. Semple, W.R. Welch, J. Folkman, Tumor angiogenesis and metastasis – correlation in invasive breast carcinoma, N. Engl. J. Med. 324 (1991) 1–8.
[4] M. Tertil, A. Jozkowicz, J. Dulak, Oxidative stress in tumor angiogenesis – therapeutic targets, Curr. Pharm. Des. 16 (2010) 3877–3894.
[5] B.I. Terman, K.V. Stoletov, VEGF and tumor angiogenesis, Einstein J. Biol. Med. 18 (2001) 59–66.
[6] J. Folkman, Y. Shing, Angiogenesis, J. Biol. Chem. 267 (1992) 10931–10934.
[7] J. Folkman, What is the role of thymidine phosphorylase in tumor angiogenesis? J. Natl. Cancer Inst. 88 (1996) 1091–1092.
[8] A. Bronckaers, F. Gago, J. Balzarini, S. Liekens, The dual role of thymidine phosphorylase in cancer development and chemotherapy, Med. Res. Rev. 29 (2009) 903–953.
[9] I.V. Bijnsdorp, F. Capriotti, F.A.E. Kruyt, N. Losekoot, M. Fukushima, A.W. Griffioen, V.L. Thijssen, G.J. Peters, Thymidine phosphorylase in cancer cells stimulates human endothelial cell migration and invasion by the secretion of angiogenic factors, Br. J. Cancer 104 (2011) 1185–1192.
[10] G.J. Peters, I.V. Bijnsdorp, M. Fukushima, Thymidine phoshorylase as a target for antiangiogenesis treatment, Nucleic Acids Symp. Ser. 52 (2008) 629.
[11] M.J. Peìrez-Peìrez, E.M. Priego, A.I. Hernández, M.J. Camarasa, J. Balzarini, S. Liekens, Thymidine phosphorylase inhibitors: recent developments and potential therapeutic applications, Mini. Rev. Med. Chem. 5 (2005) 1113–1123.
[12] S. Matsushita, T. Nitanda, T. Furukawa, T. Sumizawa, A. Tani, K. Nishimoto, S. Akiba, K. Miyadera, M. Fukushima, Y. Yamada, H. Yoshida, T. Kanzaki, S.I. Akiyama, The effect of a thymidine phosphorylase inhibitor on angiogenesis and apoptosis in tumors, Cancer Res. 59 (1999) 1911–1916.
[13] H. Lu, R.S. Klein, E.L. Schwartz, Antiangiogenic and antitumor activity of 6-(2-aminoethyl)amino-5-chlorouracil, a novel small-molecule inhibitor of thymidine phosphorylase, in combination with the vascular endothelial growth factor-trap, Clin. Cancer Res. 15 (2009) 5136–5144.
[14] J. Balzarini, A.E. Gamboa, R. Esnouf, S. Liekens, J. Neyts, E. De Clercq, M.J. Camarasa, M.J. Peìrez-Peìrez, 7-Deazaxanthine, a novel prototype inhibitor of thymidine phosphorylase, FEBS Lett. 438 (1998) 91–95.
[15] S. Liekens, F. Bilsen, E. De Clercq, E.M. Priego, M.J. Camarasa, M.J. Pérez-Pérez, J. Balzarini, Anti-angiogenic activity of a novel multi-substrate analogue inhibitor of thymidine phosphorylase, FEBS Lett. 510 (2002) 83–88.
[16] K. Pomeisl, K. Horská, R. Pohl, J. Blažek, M. Krečmerová, Syntheses of 1-[2-(phosphonomethoxy)alkyl]thymine monophosphates and an evaluation of their inhibitory activity toward human thymidine phosphorylase, Nucleosides Nucleotides Nucleic Acids 31 (2012) 159–171.
[17] S.A. Diab, C. De Schutter, M. Muzard, R. Plantier-Royon, E. Pfund, T. Lequeux, Fluorophosphonylated nucleoside derivatives as new series of thymidine phosphorylase multisubstrate inhibitors, J. Med. Chem. 55 (2012) 2758–2768.
[18] T. Kita, H. Takahashi, Y. Hashimoto, Thymidine phosphorylase inhibitors with a homophthalimide skeleton, Biol. Pharm. Bull. 24 (2001) 860–862.
[19] S. Liekens, A.I. Hernández, D. Ribatti, E. De Clercq, M.J. Camarasa, M.J. Peìrez-Peìrez, J. Balzarini, The nucleoside derivative 5'-O-trityl-inosine (KIN59) suppresses thymidine phosphorylase-triggered angiogenesis via a noncompetitive mechanism of action, J. Biol. Chem. 279 (2004) 29598–29605.
[20] S. Liekens, A. Bronckaers, A.I. Hernández, E.M. Priego, E. Casanova, M.J. Camarasa, M.J. Peìrez-Peìrez, J. Balzarini, 5′-O-Tritylated nucleoside derivatives: inhibition of thymidine phosphorylase and angiogenesis, Mol. Pharmacol. 70 (2006) 501–509.
[21] C. Cole, P. Reigan, A. Gbaj, P.N. Edwards, K.T. Douglas, I.J. Stratford, S. Freeman, M. Jaffar, Potential tumor-selective nitroimidazolylmethyluracil prodrug derivatives: inhibitors of the angiogenic enzyme thymidine phosphorylase, J. Med. Chem. 46 (2003) 207–209.
[22] P. Reigan, A. Gbaj, E. Chinje, I.J. Stratford, K.T. Douglas, S. Freeman, Synthesis and enzymatic evaluation of xanthine oxidase-activated prodrugs based on inhibitors of thymidine phosphorylase, Bioorg. Med. Chem. Lett. 14 (2004) 5247–5250.
[23] A.V. Dolzhenko, A.V. Dolzhenko, W.K. Chui, 1,2,4-Triazolo[1,5-*a*][1,3,5]triazines (5-azapurines): synthesis and biological activity, Heterocycles 68 (2006) 1723–1759.
[24] R. Bokaldere, A. Liepina, s-Triazolo[1,5-*a*]-s-triazinonethiones. Synthesis and reactions, Khim. Geterotsikl. Soedin. 2 (1973) 276–280.
[25] A.V. Dolzhenko, A.V. Dolzhenko, W.K. Chui, Synthesis of 5,7-diamino1,2,4 triazolo[1,2-a1,3,5 triazines via annulation of 1,3,5-triazine ring onto 3(5)-amino-1,2,4-triazoles, Heterocycles 71 (2007) 429–436.
[26] A.V. Dolzhenko, A.V. Dolzhenko, G. Pastorin, W.K. Chui, An aqueous medium synthesis and tautomerism study of 3(5)-amino-1,2,4-triazoles, Tetrahedron Lett. 50 (2009) 2124–2128.
[27] T.A. Krenitsky, S.R.M. Bushby, U.S. Patent, 4,178,212, Burroughs Welcome Co., Research Triangle Park, NC, 1979, pp. 1–8, 1979.
[28] P.N. Craig, Interdependence between physical parameters and selection of substituent groups for correlation studies, J. Med. Chem. 14 (1971) 680–684.
[29] J. Balzarini, B. Degrevea, A. Esteban-Gamboa, R. Esnouf, E. De Clercq, Y. Engelborghs, M.J. Camarasa, M.J. Peìrez-Peìrez, Kinetic analysis of novel multisubstrate analogue inhibitors of thymidine phosphorylase, FEBS Lett. 483 (2000) 181–185.
[30] A. Hoeben, B. Landuyt, M.S. Highley, H. Wildiers, A.T. Van Oosterom, E.A. De Bruijn, Vascular endothelial growth factor and angiogenesis, Pharmacol. Rev. 56 (2004) 549–580.
[31] Human VEGF ELISA: Sandwich Enzyme-Linked Immunosorbent Assay for Quantitative Detection of human VEGF. Catalog No. LF-EK50413 (1 kit).
[32] G. Finkenzeller, A. Sparacio, A. Technau, D. Marmeìl, G. Siemeister, Sp1 recognition sites in the proximal promoter of the human vascular endothelial growth factor gene are essential for platelet-derived growth factor-induced gene expression, Oncogene 15 (1997) 669–676.
[33] M. Toi, K. Inada, S. Hoshina, H. Suzuki, S. Kondo, T. Tominaga, Vascular endothelial growth factor and platelet-derived endothelial cell growth factor are frequently coexpressed in highly vascularized human, Clin. Cancer Res. 1 (1995) 961–964.
[34] T. Kurizaki, M. Toi, T. Tominaga, Relationship between matrix metalloproteinase expression and tumor angiogenesis in human breast carcinoma, Oncol. Rep. 5 (1998) 673–677.
[35] L.A. Liotta, W.G. Stetler-Stevenson, Metalloproteinases and cancer invasion, Semin. Cancer Biol. 1 (1990) 99–106.