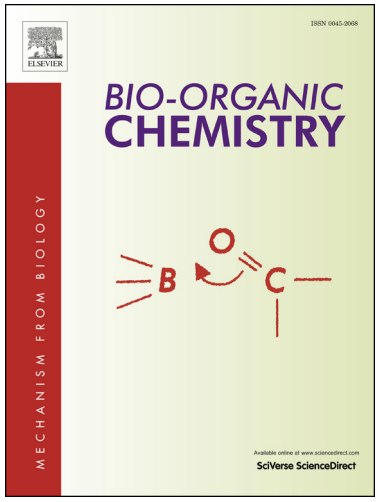

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PII: S0045-2068(15)00030-9
DOI: http://dx.doi.org/10.1016/j.bioorg.2015.04.003
Reference: YBIOO 1802

To appear in: *Bioorganic Chemistry*

Received Date: 24 December 2014

Please cite this article as: S.A. Shahzad, M. Yar, M. Bajda, L. Shahzadi, Z.A. Khan, S.A.R. Naqvi, S. Mutahir, N. Mahmood, K.M. Khan, Synthesis, thymidine phosphorylase inhibition and molecular modeling studies of 1,3,4-oxadiazole-2-thione derivatives, *Bioorganic Chemistry* (2015), doi: http://dx.doi.org/10.1016/j.bioorg.2015.04.003

Synthesis, thymidine phosphorylase inhibition and molecular modeling studies of 1,3,4-oxadiazole-2-thione derivatives

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Abstract: Thymidine phosphorylase (TP) inhibitors have attracted great attention due to their ability to suppress the tumors formation. In our ongoing research, a series of 1,3,4-oxadiazole-2-thione (**1-12**) has been synthesized under simple reaction conditions in good to excellent yields (86-98%) and their TP inhibition potential has also been evaluated. The majority of synthesized compounds showed moderate thymidine phosphorylase inhibitory activity with IC_{50} values ranging from 38.24 $\pm$ 1.28 to 258.43 $\pm$ 0.43 µM, and 7-deazaxanthine (7DX) was used as a reference compound (IC_{50} 38.68 $\pm$ 4.42). The TP activity was very much dependent on the C-5 substituents; among this series the compound **6** bearing 4-hydroxyphenyl group was found to be the most active with IC_{50} 38.24 $\pm$ 1.28 µM. Molecular docking studies revealed their binding mode.

Keywords: Thymidine phosphorylase, oxadiazole-2-thione, molecular modeling, anti-cancer

Introduction

Although angiogenesis is a very critical process in a repairing of tissues and organs it is a highly undesirable phenomenon during the tumor formation. It is believed that, tumor growth could be blocked by stopping angiogenesis.[1,2] Regarding the mechanism of the action of thymidine phosphorylase (TP), it triggers the reversible phosphorolysis of thymidine to produce thymine and 2□-deoxy-D-ribose 1-phosphate.[3,4] Subsequently, 2□-deoxy-D-ribose 1-phosphate shows dephosphorylation reaction and as a result 2□-deoxy-D-ribose is produced. It is possible that the 2□-deoxy-D-ribose stimulates the production of vascular endothelial growth factor (VEGF), which initiates a number of processes, for example, invites endothelial cells for secretion of *matrix metalloproteinases*, proliferation, and also migration to tumor tissue. All these actions help in the formation of new blood vessels, which could cause cancer metastasis.[5] This is the reason why anti-angiogenic substances are highly desirable.

The 2□-deoxy-D-ribose is considered a valuable target to suppress the tumor growth, and TP inhibitors are able to reduce the production of 2□-deoxy-D-ribose.[5,6] In such scenario, one can easily understand the advantages of TP inhibitors in the control of cancer and that is the reason recently a number of efforts have been reported on the development of TP inhibitors.[5,7,8] One of the leading candidate in this field is the 5-chloro-6-[1-(2-iminopyrrolidinyl) methyl] uracil hydrochloride (TPI). It is a pyrimidine based compound and the most active human TP inhibitor, whereas, 7-deazaxanthine (7DX) is the first known TP inhibitor as shown in Figure 1.[9-12]

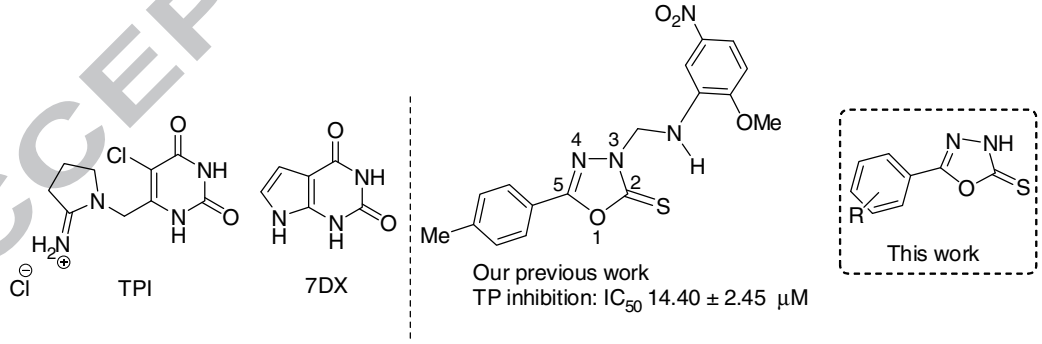


Figure 1. Chemical structure of known TP inhibitors; TPI, 7DX, our previously reported potent TP inhibitor and newly proposed TP inhibitors

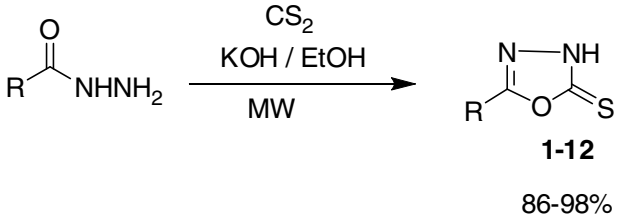
Oxadiazole motif is well known due to its huge importance in medicinal chemistry.[13-16] We have recently identified and reported the TP inhibitory potential of 1,3,4-oxadiazole-2-thiones

Mannich base derivatives,[17] derived from the compounds listed in the current paper. There it was observed that the oxadiazole motif played a crucial role in the inhibitory process along with the contributions from the various substituents around the molecules. In that effort we synthesized the Mannich base derivatives of 1,3,4-oxadiazole-2-thiones, which indeed required an extra synthetic step and additional chemicals. As part of our ongoing medicinal chemistry interests,[17-23] in current research, we have synthesized the simple 1,3,4-oxadiazole-2-thione derivatives[24,25] and analyzed their TP inhibition potential. We have also performed molecular modeling studies for all the synthesized compounds to rationalize their binding modes with the TP.

1. Results and Discussion

1.1. Chemistry

In our current research, a series of 1,3,4-oxadiazoline-2-thione derivatives **1-12**[17] bearing different level of C-5 substituents was prepared by condensing respective hydrazides with carbon disulfide in the presence of potassium hydroxide and ethanol on alumina as a solid support as shown in Scheme 1.[24] Neutral alumina oxide was used as solid support which does not affect the yield and speed of reaction[25] The reaction proceeded and completed efficiently under microwave irradiation within 7 minutes. The pure solid products were isolated as precipitates, which were washed with 50% aqueous ethanol and needed no further chromatographic techniques for purification. Also, it can be seen from percentage yield that different substituents influence the conversion rate and shorten the reaction time (please see Table 1). All final compounds were structurally characterized by IR, NMR, EIMS and elemental analysis.



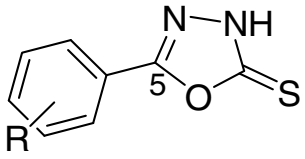
Scheme 1: Synthetic protocol of 1,3,4-oxadiazole-2-thione derivatives **1-12**.

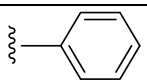
1.2. Thymidine phosphorylase inhibition activities

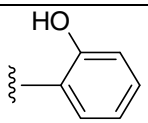
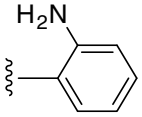
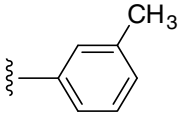
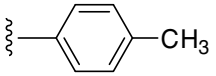
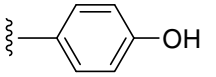
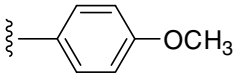
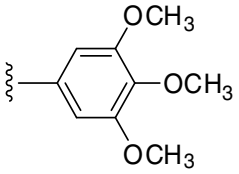
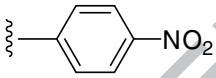
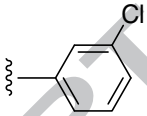
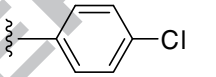
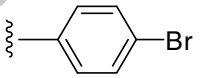
In this study, to develop and understand structure–activity relationship (SAR) twelve derivatives of oxadiazole-2-thione were synthesized bearing different degree of aryl substituents at C-5 position. Among the tested compounds, compound **6** having 4-hydroxyphenyl at C-5 position was found to be the most active with an IC_{50} value 38.24 ± 1.28μM (entry 6, Table 1), which was decreased to IC_{50} 68.37±1.23μM in the case of its analogue **2** having 2-hydroxyl group at C-5 (entry 2 and 6, Table 1). Similarly, compound **7**, having methoxy group at *para* position showed IC_{50} 72.43 ± 0.48μM while its trimethoxy analogue **8** exhibited slightly enhanced enzyme inhibition activity (IC_{50} 63.43 ± 0.92), this is may be due to combined greater inductive effect by three methoxy substituents.

Again very interesting results have been seen on the basis of position of substituents. For example, compound **10** and **11**, they both contain chloro substituents in phenyl ring but IC_{50} value of compound **11** having chloro group at *para* position was 63.97 ± 0.73μM, and the IC_{50} value dramatically increased to 258.43 ± 0.43μM in case of compound **10** having chloro group at *meta* position. The results also revealed the electron-withdrawing polar group like nitro completely hinders the enzyme inhibition activity and resulted as precipitates.

Table 1: Showing percentage yield and thymidine phosphorylase inhibition activity of synthesized oxadiazole derivatives



Comp.	R	Yield %	IC_{50} (μM)[a]
1		90	50.00 ± 0.78

2		89	68.37 ± 1.23
3		86	74.34 ± 0.73
4		96	91.78 ± 0.97
5		98	-
6		**91**	**38.24 ± 1.28**
7		94	72.43 ± 0.48
8		91	63.43 ± 0.92
9		93	PPT
10		95	258.43 ± 0.43
11		92	63.97 ± 0.73
12		93	63.97 ± 0.73
Standard	7-Deazaxanthine		**38.68± 4.42**

[a] Enzyme inhibition IC_{50} values are means of three independent experiments (mean ± SEM, n = 3)

1.3. Molecular modeling studies

Analysis of the binding mode for the novel derivatives **1-12** was performed according to the previously described method.[17] Thymidine phophorylase (TP) from *Escherichia coli* of high resolution (1.50 A; PDB code: 4EAD) was used for docking studies upon initial preparation.

This enzyme structure represents the most closed form, characteristic for the most complexes with inhibitors, bound with 3□-azido-2□-fluoro-dideoxyuridine (ONP). Docking validation was based on two reference compounds from crystal structures – ONP and TPI (5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]-uracil) and it was shown that docking runs were able to reproduce original arrangement of the ligand with low rmsd (root mean square deviation) value below 1. Further, 7-deazaxanthine (7DX) - assay reference compound – was docked to TP. It was observed that NH and CO groups formed hydrogen bonds with Lys190, Ser186 and Arg171 while the whole molecule created ππstacking with Tyr168 residue.

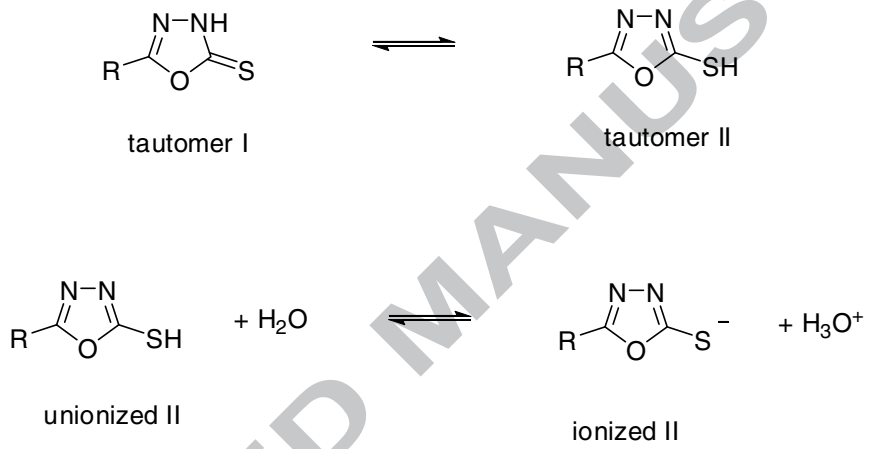


Figure 2. The novel compounds can occur in tautomeric forms (top). The ionization of lactim form (bottom).

Initial calculations revealed that all compounds **1-12** occurred in physiological conditions in ionized form II (Figure 2) and therefore such form was docked into thymidine phosphorylase. It was noted that the binding mode of novel compounds was highly dependent on the substituents in the phenyl ring (Figure 3). In case of the most active compound **6**, phenyl ring created CH-π interactions with Phe210. The hydroxyl group in position 4 formed hydrogen bond with Arg171. The oxadiazole moiety was engaged in two hydrogen bonds. The oxygen atom interacted with water molecule while nitrogen atom in position 4 created H-bond with hydroxyl group of Tyr168. The orientation of the inactive compound **5** was reversed. The phenyl ring created ππ stacking with Tyr168 while the oxygen atom from oxadiazole ring formed hydrogen bond with Ser186. Comparing both compounds **5** and **6**, it's worth to note that inhibitor **6** provided more interactions of greater importance within active center than inactive derivative **5**. This could

explain the observed differences in the activity. Moreover, other derivatives **1**-**4** and **7**-**12** presented variable binding mode. It seems that even the most potent compound **6** is not specific inhibitor of thymidine phosphorylase but it remains a good starting point for further optimization to obtain the highly active derivatives.

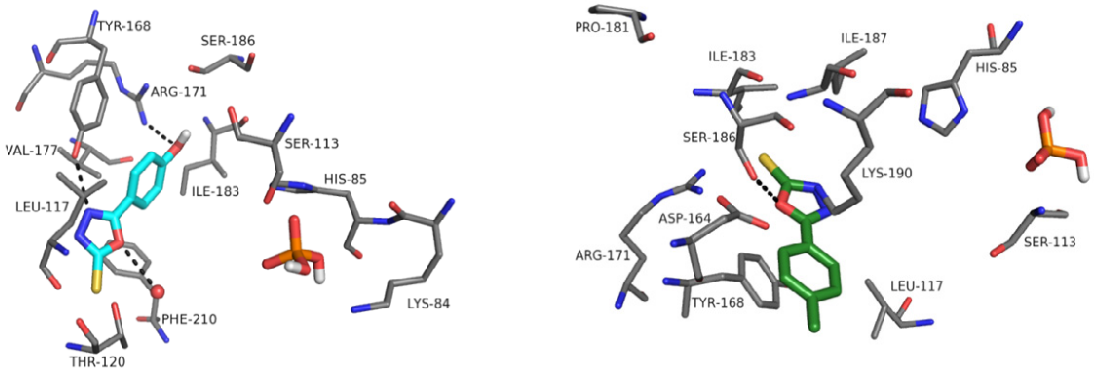


Figure 3. The binding mode of the compounds **6** (left) and **5** (right)

2. Conclusion

We synthesized 5-substituted-1,3,4-oxadiazole-2-thione derivatives via a procedure which is much more efficient and has the ability to tolerate a variety of substituents. Ease of separation of pure product and high yields are unique features of this used method. All the synthesized compounds were tested for their TP inhibition, most of the compounds exhibited moderate to good inhibition activity. Compound **6** has shown best inhibition results (IC_{50} 38.24 $\pm$ 1.28 µM) and molecular docking studies explained its binding pattern with the TP. We believe these TP inhibitors will find wide attraction in the field of anti-cancer drug development research.

3. Experimental

3.1. General Methods

The ultraviolet spectra were measured in chloroform on a Lambda 5 UV/Vis. spectrophotometer (Perkin-Elmer). IR spectra (KBr discs) were recorded on a Bruker FT-IR IFS48 spectrophotometer. EI mass spectra data were recorded with various MAT 711 (70eV) spectrophotometers and data are tabulated as m/z. ^{1}H-NMR spectra were recorded in CD_3COCD_3, CD_3OD and DMSO-d_6 using Bruker AC (300 MHz and 400 MHz, 500 MHz) spectrometers, respectively. Splitting patterns were as follows s (singlet), d (doublet), dt (doublet

of triplet), dd (double doublets), t (triplet), and m (multiplet). Chemical shifts are reported in δ (ppm) and coupling constants are given in Hz. The progress of all reactions was monitored by TLC, which was performed on 2.0 X 5.0 cm aluminum sheets precoated with silica gel $60F_{254}$ to a thickness of 0.25 mm (Merck). The chromatograms were visualized under ultraviolet light (254-366 nm) or iodine vapors. All the reagents were commercially available (Flulka, Aldrich, and Wako).

3.2. General procedure for the synthesis of 5-substituted -1,3,4-oxadiazole-2-thione (1-12)

A mixture of respective hydrazide (10 mmol), potassium hydroxide (0.56 g, 10 mmol) and alumina were finely ground in a glove box with a mortar and pestle. Then carbon disulfide (1.2 mL, 20 mmol) was added to this mixture in a pyrex glass vial, which was placed in a screw-capped thick-walled Teflon® vessel. Microwave-irradiation (MW domestic type oven 900 W with a frequency 2450 MHz, Dawlance, Pakistan) was applied for 7 minutes. After the completion of reaction (TLC analysis), ethanol was added into reaction mixture and filtered. Filtrate was evaporated; distilled water was added to semi-solid material and acidified with hydrochloric acid to pH = 4. Precipitates so obtained were filtered and dried to afford off white solid compound 2a-r and then recrystallized from ethanol:water (50:50) mixture (Table 1).

5-phenyl-1,3,4-oxadiazole-2(3H)-thione (1)

Yield: 1.43 g, (80%). R_f = 0.61 (Ethyl acetate/hexane = 1:1).FTIR (KBr) ν_{max}: 3431 (NH), 1641 (C=N), 1328 (C=S), 1020 (C-O). EI-MS, (*m/z* %): 178 (M^+, 25), 118 (100), 105 (25), 103 (41), 77 (81), 51 (82). ^{1}H NMR (400 MHz, $CDCl_3$, drops of CD_3OD): 14.70 (bs, 1H, N*H*), 7.90 (d, 2H, δ, *J* = 7.3 Hz), 7.47-7.59 (m, 3H). Anal. calcd. for $C_8H_6N_2OS$: C, 53.92; H, 3.39; N, 15.72; found: C, 53.86; H, 3.41; N, 15.65.

5-(2'-Hydroxyphenyl-1,3,4-oxadiazole-2(3H)-thione (2)

Yield: 1.33 g (89%). M.p.: 200-201 °C. R_f = 0.67 (Ethyl acetate:hexane = 1:1). FT-IR (KBr, ν_{max}, cm^{-1}): 3364 (NH), 1618 (C=N), 1309 (C=S), 1051 (C-O-C). MS (*m/z*, %): 194 (M^+, 100), 134 (1), 121 (99), 119 (16), 93 (7), 65 (8). ^{1}H NMR (400 MHz, Acetone-d_6, δ, ppm): 14.33 (bs, 1H, NH), 8.84 (br s, 1H, OH), 7.61 (m, 1H, H-4´), 7.08 (d, 1H, H-3´), 7.07 (dd, 1H, *J* = 8.1 Hz, *J* = 6.4 Hz, H-5´), 7.01 (1H, *J* = 7.7 Hz, *J* = 5.0, J = 1.5 Hz, H-6´). Anal. calcd. for $C_8H_6N_2O_2S$: C, 49.47; H, 3.11; N, 14.42. Found: C, 49.49; H, 3.15; N, 14.46%.

5-(2′-Amino phenyl)-1,3,4-oxadiazole-2(3H)-thione (3):

Yield: 1.31 g (86 %). M.p.: 156-157 °C. R_f = 0.68 (Ethyl acetate:hexane = 1:1). FT-IR (KBr, ν_{max}, cm^{-1}): 1054 (C-O-C), 1616 (C=N), 3585 (NH). MS (*m/z*, %): 193 (M^+, 17), 177 (100), 133 (47), 120 (15), 118 (33), 92 (15), 76 (10). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 14.63 (bs, 1H, NH), 7.89 (dd, 1H, *J* = 7.0 Hz, 1.5 Hz, H-6´), 7.88 (bs, 2H, NH_2), 7.61 (dd, 1H, *J* = 7.2 Hz, 1.7 Hz, H-3´), 7.55 (dd , 1H, *J* = 7.0 Hz, 1.9 Hz, H-4´), 7.48 (t, 1H, *J* = 7.0 Hz, H-5´). Anal. calcd. for $C_8H_7N_3OS$: C, 49.73; H, 3.65; N, 21.75. Found: C, 49.71; H, 3.63; N, 21.74%.

5-(3′-Methylphenyl-1,3,4-oxadiazole-2(3H)-thione (4):

Yield: 1.44 g (96%). M.p.: 148-149 °C. R_f = 0.69 (Ethyl acetate:hexane = 1:2). FT-IR (KBr, ν_{max}, cm^{-1}): 3400 (NH), 1635 (C=N), 1319 (C=S), 1022 (C-O). MS (*m/z*, %): 192 (M^+, 49), 132 (100), 116 (10), 104 (17), 91 (59), 77 (17), 65 (34), 63 (20), 51 (24). ^{1}H NMR (500 MHz, $CDCl_3$, drops of CD_3OD, δ, ppm): 14.45 (bs, 1H, NH), 7.71 (s, 1H, H-2′), 7.67 (d, 1H, *J* = 6.1 Hz, H-2′), 7.39 (bs, 2H, H- 3´/4′), 2.4 (s, CH_3). Anal. calcd. for $C_9H_8N_2OS$: C, 56.23; H, 4.19; N, 14.57. Found: C, 56.28; H, 4.17; N, 14.62%.

5-(4′-Methylphenyl-1,3,4-oxadiazole-2(3H)-thione (5):

Yield: 1.47 g (98%). M.p.: 159-160 °C. R_f = 0.69 (Ethyl acetate:hexane = 1:2). FT-IR (KBr, ν_{max}, cm^{-1}): 3409 (NH), 1636 (C=N), 1333 (C=S), 1016 (C-O). MS (*m/z*, %): 192 (M^+, 52), 132 (100), 119 (20), 117 (10), 104 (12), 102 (2), 91 (45), 65 (16). ^{1}H NMR (400 MHz, $CDCl_3$, drops of CD_3OD, δ, ppm): 14.53 (bs, 1H, NH), 7.74 (d, 2H, *J* =8.1 Hz H-2´/6´), 7.30 (d, 2H, *J* = 8.1 Hz, H-3´/5´), 2.38 (s, 3H, CH_3). Anal. calcd. for $C_9H_8N_2OS$: C, 56.23; H, 4.19; N, 14.57. Found: C, 56.28; H, 4.14; N, 14.52%.

5(4′-Hydroxyphenyl)-1,3,4-oxadiazole-2(3H)-thione (6):

Yield: 1.36 g (91%). M.p.: 233-235 °C. ^{1}H NMR (400 MHz, $CDCl_3$, drops of CD_3OD, δ, ppm): 11.4 (s, 1H, SH), 7.18 (d, 2H, *J* =8.46 Hz, Ar-H), 7.08 (d, 2H, *J* = 8.46 Hz, Ar-H), 5.12 (s, 1H, OH). Anal. calcd. for $C_8H_6N_2O_2S$: C, 49.47; H, 3.11; N, 14.42. Found: C, 49.52; H, 3.18; N, 14.47%.

5-(4′-Methoxyphenyl)-1,3,4-oxadiazole-2(3H)-thione (7):

Yield: 1.41 g (94%). M.p.: 190-191 °C. R_f = 0.65 (Ethyl acetate:hexane = 1:1). FT-IR (KBr, v_{max}, cm^{-1}): 3399 (NH), 1659 (C=N), 1333 (C=S), 1019 (C-O). MS (*m/z*, %): 208 (M^+, 100), 148 (54), 135 (12), 133 (88), 107 (3), 105 (20), 92 (10), 77 (13), 64 (14), 51 (18). ^{1}H NMR (500 MHz, $CDCl_3$, drops of CD_3OD, δ, ppm):14.23 (br s, 1H, *NH*), 7.84(d, 2H, *J* = 8.8 Hz, H-2´/6´), 7.05 (d, 2H, *J* =8.8 Hz, H-3´/5´), 3.61 (s, 3H, OCH_3). Anal. calcd. for $C_9H_8N_2O_2S$: C, 51.91; H, 3.87; N, 13.45. Found: C, 51.96; H, 3.83; N, 13.49%.

5-(3',4',5'-Trimethoxyphenyl)-1,3,4-oxadiazole-2(3H)-thione (8):

Yield: 1.36 g (91%). M.p.: 175-176 °C. R_f = 0.59 (Ethyl acetate:hexane = 1:1). FT-IR (KBr, v_{max}, cm^{-1}): 3171 (NH), 1579 (C=N), 1331 (C=S), 1041 (C-O-C). MS (*m/z*, %): 268 (100), 208 (32), 193 (70), 178 (12), 167 (6), 152 (7), 135 (13). ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 14.81 (br s, 1H, *NH*), 7.09 (s, 2H, H-2´,6´), 3.84 (s, 6H, OCH_3-3´/5´), 3.72 (s, 3H, OCH_3-4´). Anal. calcd. for $C_{11}H_{12}N_2O_4S$: C, 49.24; H, 4.51; N, 10.44. Found: C, 49.28; H, 4.56; N, 10.47%.

5-(4'-Nitrophenyl)-1,3,4-oxadiazole-2(3H)-thione (9):

Yield: 1.39 g (93%). M.p.: >250 °C (Decompose). R_f = 0.64 (Ethyl acetate:hexane = 1:1). FT-IR (KBr, v_{max}, cm^{-1}): 3366 (NH), 1632 (C=N), 1328 (C=S), 1086 (C-O-C). MS (*m/z*, %): 223 (M^+, 79), 163 (100), 150 (4), 133 (21), 117 (28), 105 (4), 104 (12), 102 (9), 76 (34). ^{1}H NMR (400 MHz, $CDCl_3$, drops of CD_3OD, δ, ppm): 14.73 (bs, 1H, NH), 8.40 (d, 2H, *J* = 8.7 Hz, H-3′,5′), 8.17 (d, 2H, *J* = 8.7 Hz, H- 2′,6′). Anal. calcd. for $C_8H_5N_3O_3S$: C, 43.05; H, 2.26; N, 18.83.Found: C, 43.01; H, 2.22; N, 18.86%.

5-(3'-Chlorophenyl-1,3,4-oxadiazole-2(3H)-thione (10):

Yield: 1.42 g (95%). M.p.: 178-179 °C. R_f = 0.69 (Ethyl acetate:hexane = 1:1). FT-IR (KBr, v_{max}, cm^{-1}): 1063 (C-O-C), 1608 (C=N), 3594 (NH). MS (*m/z*, %): 214 (M^+, 48), 212 (M^+, 100), 179 (3), 155 (6), 154 (37), 153 (11), 152 (100), 141 (5), 139 (17), 137 (12), 117 (7), 102 (8), 76 (7). ^{1}H-NMR (500 MHz, DMSO-d_6): 14.49 (bs, 1H, *NH*), 7.85 (d, 1H, *J* = 1.6 Hz, H-6´), 7.83 (d, *J* = 7.8 Hz, H- 2´), 7.70 (dd, 1H, *J* =7.9 Hz, *J* = 1.2 Hz, H-4´), 7.60 (t, 1H, *J* = 7.9 Hz, *J* = 7.9 Hz, H-3´). Anal. calcd. for $C_8H_5ClN_2OS$: C, 45.18; H, 2.37; N, 13.17. Found: C, 45.23; H, 2.42; N, 13.15%.

5-(4'-Chlorophenyl-1,3,4-oxadiazole-2(3H)-thione (11):

Yield: 1.38 g (92%). M.p.: 173-174 °C. R_f = 0.69 (Ethyl acetate:hexane = 1:1). FT-IR (KBr, ν_{max}, cm^{-1}): 1023 (C-O-C), 1669 (C=N), 3348 (NH). MS (*m/z*, %): 214 (M^+,35), 212 (M^+, 100), 179 (3),154 (30), 152 (82), 141 (4), 139 (14), 137 (14), 117 (5), 102 (8), 76 (7). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 14.52 (bs, 1H, *NH*), 7.88 (d, 2H, *J* = 8.6 Hz, H-2′/6′), 7.65 (d, 2H, *J* = 8.6 Hz, H-3′/5′). Anal. calcd. for $C_8H_5ClN_2OS$: C, 45.18; H, 2.37; N, 13.17. Found: C, 45.15; H, 2.39; N, 13.14%.

5-(4′-Bromophenyl-1,3,4-oxadiazole-2(3H)-thione (12):
Yield: 1.39 g (93%). M.p.: 230-231 °C. R_f = 0.66 (Ethyl acetate:hexane = 1:2). FT-IR (KBr, ν_{max}, cm^{-1}): 1073 (C-O-C), 1633 (C=N), 3280 (NH). MS (*m/z*, %): 258 (M^+, 60), 256 (M^+, 59), 198 (47), 196 (46), 185 (9), 184 (69), 183 (15), 181 (6), 157 (7), 155 (8), 117 (6), 76 (7), 50 (8). ^{1}H NMR (500 MHz, $CDCl_3$, drops of CD_3OD, δ, ppm): 14.59 (bs, 1H, NH), 7.8 (d, 2H, *J* = 8.4 Hz, H-2′/6′), 7.7 (d, 2H, *J* = 8.4 Hz, H-3′/5′). Anal. calcd. for $C_8H_5BrN_2OS$: C, 34.37; H, 1.96; N, 10.90. Found: C, 34.32; H, 1.98; N, 10.96%.

3.3. Docking studies

Corina online tool[29] was applied to creating three-dimensional structure of compounds. Protonation states were predicted by Marvin online tool[30] Gasteiger-Marsili charges were assigned by Sybyl-X 1.1[31] following check of atom types and protonation states of the ligands. Finally, analyzed structures were saved in the mol2 format.

Escherichia coli TP from 4EAD crystal structure was prepared in two steps. Initially, sulfate ion was replaced by phosphate in its dihydrogen form, the N- and C-terminal amino acids were set as charged and hydrogen atoms were added to the protein, water and ligands using Sybyl-X 1.1. Then, all histidine residues were protonated at Ne, ligand molecules except phosphate removed, and binding site defined as all amino acid residues within 10 Å from ONP using Hermes 1.5[32] The presence of water molecules within 5 Å from ONP was also taken into account. They were set as toggle.

Docking was performed using Gold 5.1 program.[33] A standard set of genetic algorithm with population size 100 and number of operations 100 000 was applied. As a result, 20 poses for each ligand were obtained and sorted according to GoldScore values. Results were visualized by PyMOL.[34]

3.4. Procedure for Thymidine Phosphorylase Inhibition

TP/PD-ECGF (*E. coli* TP (Sigma T6632) activity was determined by measuring the absorbance at 290 nm spectrophotometrically. The original method reported by Krenitsky[35] was modified. Briefly, a total reaction mixture of 200 µl contained 145 µl of potassium phosphate buffer (pH 7.4), 30 µl of enzyme (*E. coli* TP (Sigma T6632) at concentration 0.05 and 0.002 U, respectively, were incubated with 5 µl of test materials for 10 min at 25 °C in microplate reader. After incubation, pre read at 290 nm was taken to deduce the absorbance of substrate molecules. Substrate (20 µl, 1.5 mM) was dissolved in potassium phosphate buffer and was immediately added to plate and continuously read after 10, 20, and 30 min in micro-plate reader. All assays were performed in triplicate. The plate reader used was SpectraMax Plus 384 while 96-wells plate was used and the value of blank was subtracted from experimental wells to eliminate the back ground absorbance.

Acknowledgments

The authors are grateful to the Higher Education Commission (HEC) Pakistan for providing financial support under TTS program. Dr Ather Farooq Khan for his assistance in this research work.

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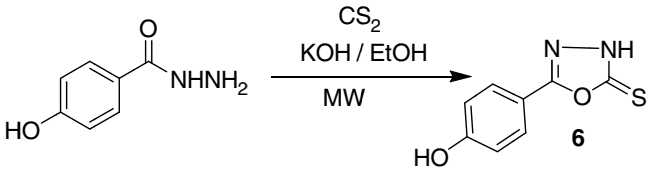
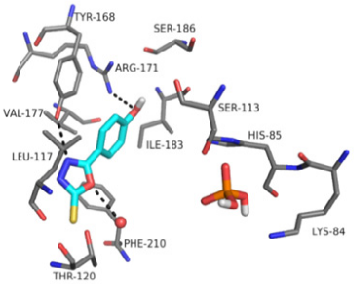
Graphical abstract





The binding mode of the compounds **6**

Highlights

- Efficient synthesis of 1,3,4-oxadiazole-2-thione derivatives
- Thymidine phosphorylase inhibition studies
- Molecular docking studies for binding mode investigations
- Compound **6** bearing 4-hydroxyphenyl group was found to be the most active