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Design, synthesis, modelling studies and biological evaluation of 1,3,4-oxadiazole derivatives as potent anticancer agents targeting thymidine phosphorylase enzyme

Shalini Bajaj [1], Maushmi S. Kumar [1], Hussain Tinwala, Mayur YC [*]

Shobhaben Pratapbhai Patel School of Pharmacy Technology Management, SVKM's NMIMS, Mumbai, India

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ABSTRACT

A series of novel 1,3,4-oxadiazole derivatives with substituted phenyl ring were designed and synthesized with an objective of discovering newer anti-cancer agents targeting thymidine phosphorylase enzyme (TP). The 1,3,4-oxadiazole derivatives were synthesized by simple and convenient methods in the lab. Chemical structure of the all the synthesized compounds were characterized by IR, ^{1}H NMR and mass spectral methods and evaluated for cytotoxicity by MTT method against two breast cancer cell lines (MCF-7 and MDA-MB-231). Further, results of TP assay identified that 1,3,4-oxadiazole molecules displayed anti-cancer activity partially by inhibition of phosphorylation of thymidine. The TP assay identified **SB8** and **SB9** as potential inhibitors with anti-cancer activity against both the cell lines. The molecular docking studies recognized the orientation and binding interaction of molecule at the active site amino acid residues of TP (PDB: 1UOU). Acute toxicity studies of compound **SB8** at the dose of 5000 mg/kg has identified no signs of clinical toxicity was observed. The SARs study of synthesized derivatives revealed that the substitution of phenyl ring with electron withdrawing group at *ortho* position showed significant TP inhibitory activity compared to *para* substitution. The experimental data suggests that 1,3,4-oxadiazole with substituted phenyl can be taken as a lead for the design of efficient TP inhibitors and active compounds which can be taken up for further studies.

1. Introduction

Cancer still remains the second leading cause of mortality all over the globe. With increasing number of cancer incidences in developed as well developing countries, cancer is one of the major health research area being explored by researchers all over the globe. According to the American Cancer Society in 2019, around 1,762,450 new cancer cases and 606,880 cancer deaths were projected to occur in the United States alone [1]. At present, chemotherapy is one of the most important treatment available for treatment of cancer. The ultimate goal of chemotherapy is to kill the cancerous cells while safeguarding the surrounding healthy cells. The success of chemotherapy is hindered by obstacles such as toxicity of chemotherapeutics on the healthy cells, higher dose regime and developing resistance over existing chemotherapeutic drugs. The only feasible solution to all the above challenges is developing newer chemotherapeutic molecules with toxicity specific to cancerous cell at a lower dose. In this endeavour, nitrogen containing heterocyclic compounds have proved to be a boon for the development of newer chemotherapeutic molecules.

In recent years, studies pertaining to structural activity relationships (SARs) with target structures along with the underlying mechanisms involving 1,3,4-oxadiazole moiety have carved their niche owing to the versatility of the core ring which enables various substitutions at the carbon and nitrogen atoms of the ring thereby generating various mono- and di- substituted 1,3,4-oxadiazole derivatives. These derivatives have exhibited a wide array of pharmacological activities including antibiotic [2] anti-malarial [3,4], anti-viral [5,6], analgesic [7] anti-inflammatory [8] vasodilation [9] anti-cancer [10–12] and many others [13–15]. In the last decade, 1,3,4-oxadiazole has been used as a motif to develop newer chemotherapeutic molecules for improved and efficient chemotherapeutic application. Over recent years, newer hallmarks have been explored in search for a potential molecular target for treating cancer [16].

The 1,3,4-oxadiazole ring containing compound showed potent anti-

* Corresponding author at: Department of Pharmaceutical Chemistry, SPPSPTM, SVKM's NMIMS University, Vile Parle (West), Mumbai 400056, India.
E-mail address: mayuryc@hotmail.com (M. YC).
[1] Both the authors have contributed equally.

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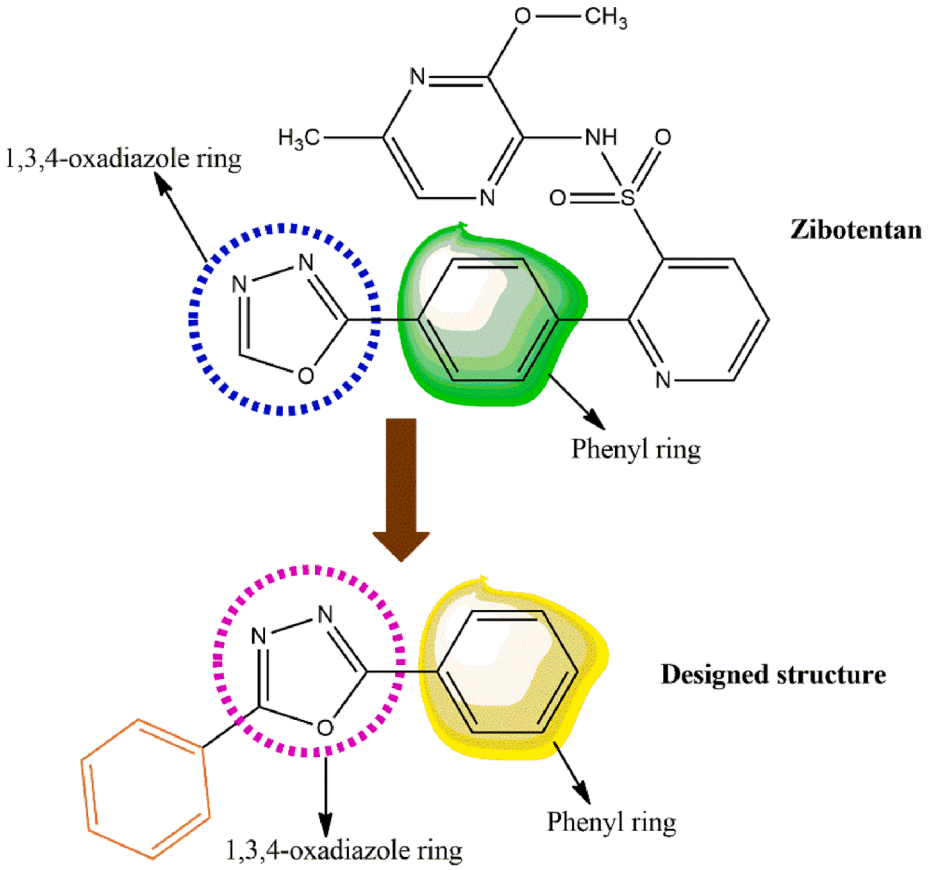


Fig. 1. Design approach of 1,3,4-oxadiazole derivatives.

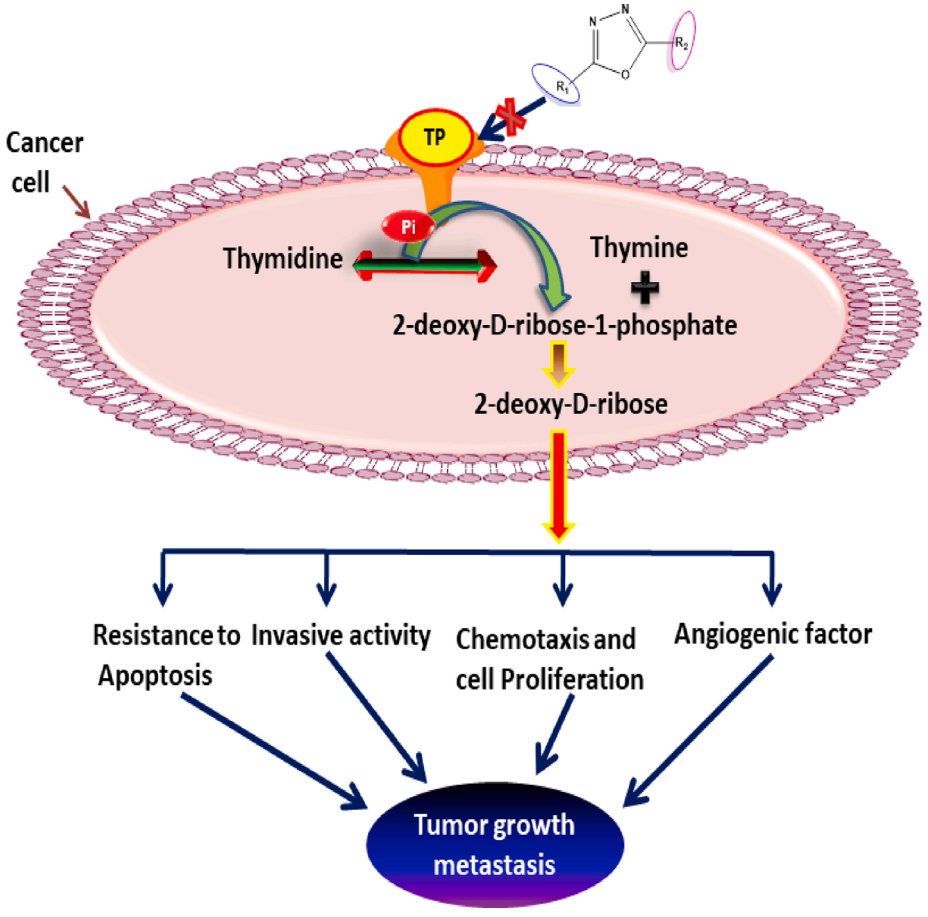
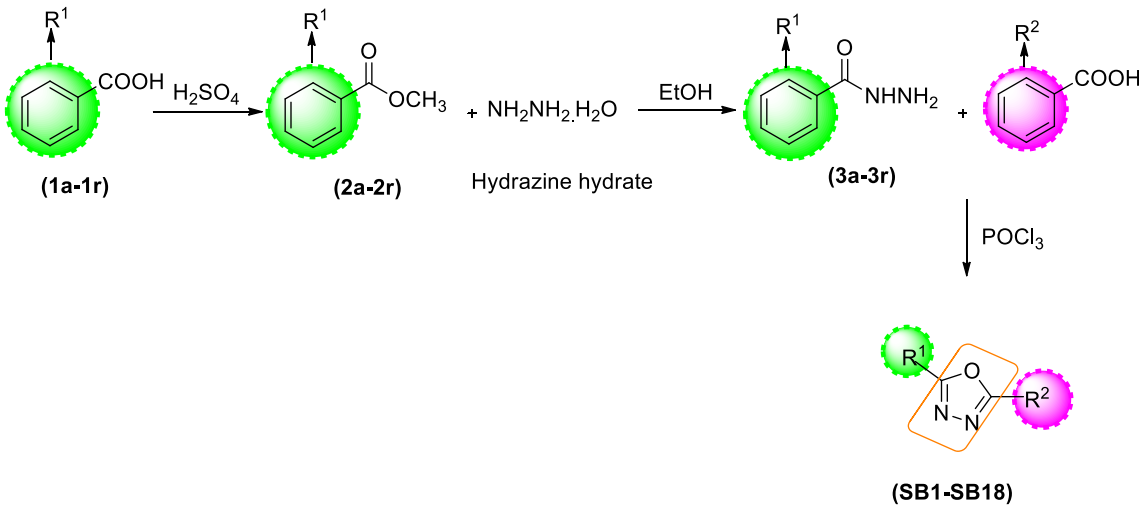
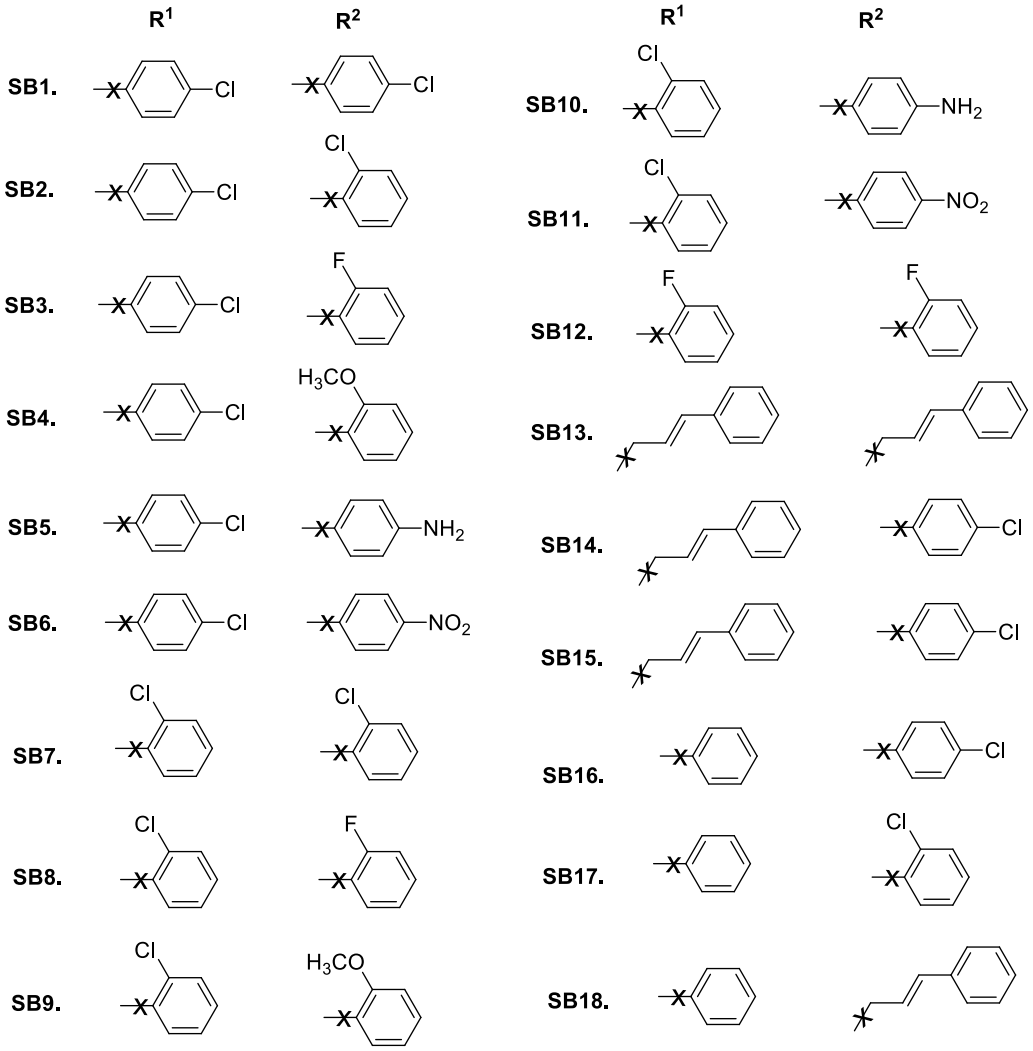


Fig. 2. Thymidine phosphorylase as a potential target for cancer treatment.



(SB1-SB18)



Scheme 1. Synthesis of 1,3,4-oxadiazole derivatives from different acids.

cancer activity. Zibotentan (ZD4054) is under clinical trial for its anti-cancer activity that contain 1,3,4-oxadiazole ring [17] (Fig. 1). Many researchers have reported different derivatives of 1,3,4-oxadiazole which showed potent anti-cancer activity against breast cancer. Target specific anti-cancer activity is one of the most encouraging strategies. In this research work, we mainly focus on 1,3,4-oxadiazole derivatives induced anti-cancer activity against breast cancer with inhibition of thymidine phosphorylase (TP) enzyme [18–21]. TP is overexpressed in many types of cancer specially in breast cancer [22,23]. The TP converts thymidine into thymine and 2-deoxy-α-D-ribose-1-phosphate (dRib-1-P) [24]. This metabolite of TP is involved in promoting tumor growth by enhancing angiogenesis induced metastasis and resistance to apoptosis which establishes TP as a potential target for cancer treatment (Fig. 2). Due to this, inhibition of TP activity may assist as probable therapeutic approach for preventing the growth of tumor cells and give insights in development of potent derivatives against cancer.

In view of the significant studies on anti-cancer activity of 1,3,4-oxadiazole derivatives, and in continuation of our previous work on its TP inhibitory activity we aim for newer 1,3,4-oxadiazole heterocyclic pharmacophores targeting cancer via inhibition of TP activity [25].

In this research work, we have attempted to synthesize a novel series of 1,3,4-oxadiazole derivatives, characterized and tested them for *in vitro* activity in human MCF-7 and MDA-MB-231 breast cancer cell lines for inhibition of TP activity. This study also highlights the molecular docking on TP receptor and active compounds were evaluated for *in vivo* acute toxicity studies and were correlated with predicated LD_{50} values [26,27]. We also compared the 1,3,4-oxadiazole structural modifications and have proven to give more cytotoxic drugs with TP inhibitory activity.

2. Results and discussion

2.1. Chemistry

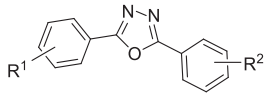
In the present study, **18 derivatives** of 1,3,4-oxadiazole have been synthesized as highlighted in Scheme 1. The intermediate (**3a-3r**) was prepared by the esterification of 4-chlorobenzoic acid, 2-chlorobenzoic acid, 2-fluorbenzoic acid, styryl and benzoic acid followed by reaction with hydrazine hydrate. The final compounds (**SB1-SB18**) were prepared by the reaction of **3a-3r** with different benzoic acid derivatives such as 4-chloro, 2-chloro, 2-fluro, 2-methoxy, 2-amino, 4-nitro and styryl in presence of phosphoryl chloride ($POCl_3$) as cyclizing agent (Scheme 1).

The depicted structures of all the synthesized derivatives were strengthened by the different spectroscopy analysis such as IR, ^{1}H NMR, ^{13}C NMR and mass spectral data. The physicochemical property of all the synthesized derivatives is depicted in Table 1.

IR spectral data of synthesized compounds **3a-3r** showed characteristic peaks at υ cm^{-1} 794, 1434 and 710 due to presence of C—Cl, C—F and styryl respectively. Compound **SB1** showed peak at 794 cm^{-1} due to C—Cl stretching of chlorine group. Compound **SB3** showed characteristic IR peaks at υ cm^{-1} 1449 and 825 due to stretching of aromatic C—F and C—Cl group. Compound **SB6** showed peak at 1366 υ cm^{-1} due to presence of nitro group.

The ^{1}H NMR spectra of compound **SB13** showed signals at δ 7.2–7.8 indicating the presence of phenyl ring aromatic protons. The mass spectrum of compound **SB1** displayed a molecular ion peak at *m/z* 443 and base peak at *m/z* 230, compound **SB3** showed molecular ion peak at *m/z* 411 and base peak at *m/z* 275 and compound **SB12** showed molecular ion peak at *m/z* 539.1 and base peak at *m/z* 259. The possible fragment of base peak of compound **SB1** and **SB12** are 1-chloro-4-methylbenzene, 1-fluoro-2-methylbenzene respectively. Similarly, all the synthesized derivatives showed their respective *m/z* value peaks according to the molecular mass of the compounds.

Table 1
Physiochemical properties of 1,3,4-oxadiazole molecules.



Comp. No.	R^1	R^2	Mol. Formula	Mol Wt.	λ_{max} (nm)
SB1.	4-chloro	4-chloro	$C_{14}H_8Cl_2N_2O$	291	407.0, 262.0
SB2.	4-chloro	2-chloro	$C_{14}H_8Cl_2N_2O$	291	407.0, 259.0
SB3.	4-chloro	2-floro	$C_{14}H_8ClFN_2O$	275	407.0, 257.0
SB4.	4-chloro	2-methoxy	$C_{15}H_{11}ClN_2O_2$	287	408.0, 263.0
SB5.	4-chloro	4-amino	$C_{14}H_{10}ClN_3O$	271	409.0, 262.0
SB6.	4-chloro	4-nitro	$C_{14}H_8ClN_3O_3$	302	406.0, 259.0
SB7.	2-chloro	2-chloro	$C_{14}H_8Cl_2N_2O$	291	407.0, 258.0
SB8.	2-chloro	2-floro	$C_{14}H_8ClN_2FO$	275	408.0, 264.0
SB9.	2-chloro	2-methoxy	$C_{15}H_{11}ClN_2O_2$	287	408.0, 262.0
SB10.	2-chloro	4-amino	$C_{14}H_{10}ClN_3O$	271	407.0, 258.0
SB11.	2-chloro	4-nitro	$C_{14}H_8ClN_3O_3$	302	407.0, 261.0
SB12.	2-floro	2-floro	$C_{14}H_8F_2N_2O$	258	407.0, 259.0
SB13.	styryl	styryl	$C_{18}H_{14}N_2O$	274	406.0, 257.0
SB14.	styryl	4-chloro	$C_{16}H_{11}ClN_2O$	283	407.0, 259.0
SB15.	styryl	2-chloro	$C_{16}H_{11}ClN_2O$	283	407.0, 261.0
SB16.	H	4-chloro	$C_{14}H_8ClN_2O$	256	408.0, 262.0
SB17.	H	2-chloro	$C_{14}H_8ClN_2O$	256	406.0, 261.0
SB18.	H	styryl	$C_{16}H_{12}N_2O$	248	407.0, 262.0

2.2. Biological activity

2.2.1. Cell proliferation assay (MTT)

All the synthesized derivatives of 1,3,4-oxadiazole **(SB1-SB18)** were screened for *in vitro* cytotoxicity studies against human breast cancer cell lines MCF-7, MDA-MB-231 and VERO cell lines using MTT assay (Table 2). The cytotoxicity of all the synthesized derivatives against MCF-7 and MDA-MB-231 cell lines are shown in Fig. 3. The data from Table 2 and Fig. 3, some of the compounds have showed good cytotoxicity against MCF-7 and MDA-MB-231 cell lines. The results of anti-cancer activity are expressed in terms of compound concentration required to reduce the viability of the cells by 50% with respect to the control (IC_{50} μM). Compounds also found to be active against TP

Table 2
In vitro IC_{50}, CC_{50} and SI index of synthesized compounds (IC_{50} is the concentration at which 50% cell death occur and CC_{50} is the concentration at which 50% cells survive).

Comp. Code	IC_{50} (μM)		CC_{50} (μM)Vero cell lines	SI of MCF-7	SI of MDA-MD-231
	MCF-7	MDA-MB-231			
SB1.	18.89 ± 0.69	19.02 ± 0.27	55.01	2.90	2.89
SB2.	9.98 ± 0.54	5.98 ± 0.13	35.18	3.52	5.98
SB3.	4.50 ± 0.2	7.69 ± 1.92	33.52	7.44	4.36
SB4.	61.49 ± 1.2	49.01 ± 0.3	71.08	1.1	1.45
SB5.	8.86 ± 0.45	6.57 ± 1.49	27.12	3.06	8.86
SB6.	57.01 ± 1.3	41.40 ± 0.92	56.09	0.98	1.35
SB7.	67.91 ± 0.4	51.39 ± 0.2	59.71	0.98	1.16
SB8.	2.50 ± 0.35	4.88 ± 1.74	39.03	15.60	7.99
SB9.	1.85 ± 0.28	2.27 ± 0.73	26.69	14.42	11.75
SB10.	15.86 ± 1.27	13.68 ± 1.72	29.45	1.85	2.15
SB11.	12.28 ± 0.98	32.88 ± 1.21	45.02	3.66	1.36
SB12.	11.28 ± 0.38	12.24 ± 0.75	23.15	2.05	1.89
SB13.	58.01 ± 0.88	62.05 ± 0.24	48.08	0.82	0.77
SB14.	28.57 ± 1.29	20.07 ± 1.91	29.01	1.20	1.44
SB15.	25.54 ± 1.17	30.14 ± 1.44	77.45	1.01	2.57
SB16.	17.74 ± 0.68	21.98 ± 1.22	65.28	3.67	2.96
SB17.	18.82 ± 0.74	19.25 ± 0.92	72.16	3.83	3.74
SB18.	11.28 ± 1.02	10.67 ± 1.86	54.11	4.79	5.07
[a]**DOX**	0.21 ± 0.03	0.28 ± 0.04	ND	ND	ND

IC_{50} = The required concentration of compound for inhibition of cell proliferation by 50%.
ND = Not Determined.
[a] DOX = Doxorubicin, positive control compound.

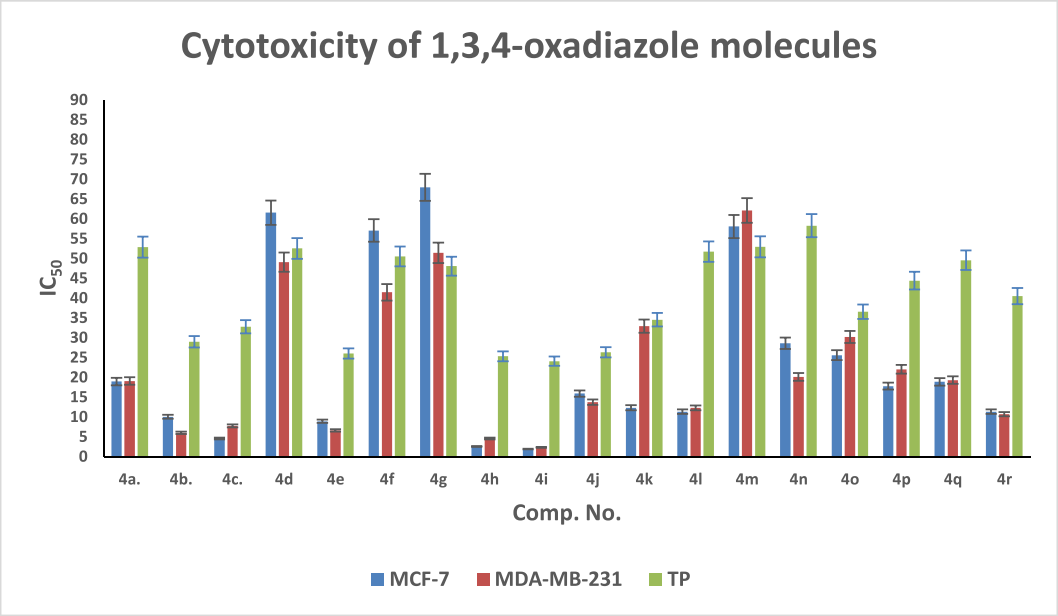


Fig. 3. Cytotoxicity and TP inhibitory profile of 1,3,4-oxadiazole derivatives.

Table 3
In vitro thymidine phosphorylase (TP) inhibitory assay (IC_{50}) results for synthesized compounds.

Comp. Code	IC_{50} (μM)
SB1.	52.81 ± 2.8
SB2.	28.91 ± 0.98
SB3.	32.71 ± 2.6
SB4.	52.49 ± 1.2
SB5.	25.96 ± 2.54
SB6.	50.47 ± 4.2
SB7.	48.01 ± 0.36
SB8.	25.27 ± 1.8
SB9.	24.03 ± 3.78
SB10.	26.28 ± 4.5
SB11.	34.49 ± 3.5
SB12.	51.66 ± 1.4
SB13.	52.90 ± 3.7
SB14.	58.22 ± 1.4
SB15.	36.48 ± 1.3
SB16.	44.34 ± 1.9
SB17.	49.5 ± 0.68
SB18.	40.45 ± 3.3
[b]**7-DX**	38.68 ± 4.42

[b] 7-DX = 7-Deazaxanthine, standard compound

enzyme. Compound **SB9**, **SB8** and **SB3** showed most potent anti-cancer activity against MCF-7 with IC_{50} value of 1.85 ± 0.28 μM, 2.50 ± 0.35 μM and 4.50 ± 0.2 μM respectively. Substitution of 2-chloro group at R^1 position and 2-methoxy at R^2 position of 1,3,4-oxadiazole ring showed potent anti-cancer activity. Compound **SB4, SB6, SB7 and SB14** showed better activity against MDA-MB-231 cell lines comparatively in MCF-7 cell line. Un-substituted phenyl ring attached to 1,3,4-oxadiazole ring through styryl showed less activity against MCF-7 cancer cell line. Compound **SB9, SB8** and **SB2** exhibited good activity against MDA-MB-231 cell line with IC_{50} value 2.27 ± 0.73 μM, 4.88 ± 1.74 μM and 5.98 ± 0.13 μM respectively with IC_{50} less than 8 μM. The selectivity index (SI) was calculated by dividing CC_{50} of VERO cell line by IC_{50} of both MCF-7 and MDA-MB0231 cell line SI= (CG_{50}) / (IC_{50}).

2.2.2. *Thymidine phosphorylase inhibitory assay*

Eighteen compounds were evaluated for TP inhibitory activity by taking 7-deazaxanthine (7-DX) as standard. TP inhibitory activity of all the compounds is shown in Table 3**.** The structure activity relationship for TP inhibitory activity revealed that substitution of 4-chloro phenyl at 2nd and 5th position of 1,3,4-oxadiazole ring have more potent activity. Compounds **SB9** and **SB8** showed highest IC_{50} value of 24.03 ± 3.78 and 25.27 ± 1.8 μM compared to IC_{50} value of standard drug (7-DX) 38.68 ± 4.42 μM.

2.2.2.1. Determination of the significance of correlation coefficient. Moreover, to determine the significance of IC_{50} in both cell lines and TP inhibitory activity for two most active compounds (**SB8 and SB9**), correlations for both cell lines and TP inhibitory IC_{50} activity were generated and presented in Fig. 4. Correlation between IC_{50} value of both cell lines and TP inhibitory activity for compound **SB8** showed regression coefficient R^2 = 0.8147 and for compound **SB9** regression coefficient R^2 = 0.7642 (Fig. 4). This indicates that 1,3,4-oxadiazole molecules with cytotoxicity also have potent TP inhibitory activity.

2.3. *Structure-activity correlation of synthesized compounds with TP inhibitory activity*

The anti-cancer activity of all the synthesized derivatives of 1,3,4-oxadiazole against MCF7, MDA-MD-231 breast cancer cell lines and TP inhibitory activity explores the following assumptions about the structure–activity relationships (SARs) (Fig. 5)**:**

(i) Phenyl ring at 2nd and 5th position of 1,3,4-oxadiazole ring is very important for anti-cancer activity.
(ii) Compound with substituted *ortho* chloro group at both 2nd and 5th position phenyl ring of 1,3,4-oxadiazole ring showed more potent anti-cancer activity in MCF-7 cell line.
(iii) Compound with substituted *ortho* chloro group at 2nd and *ortho* methoxy group at 5th position phenyl ring of 1,3,4-oxadiazole ring highest activity in MDA-MB-231 cell line.
(iv) Compound with substitution of 2-chloro and 2-floro phenyl at 2nd position and 2-floro, 3-chloro and 2-methoxy group at 5th position showed more potent anti-cancer along with TP inhibitory activity.
(v) The replacement of the 2-chlorophenyl with 2-florophenyl at R^1 position showed a remarkable reduction in the inhibitory activity.

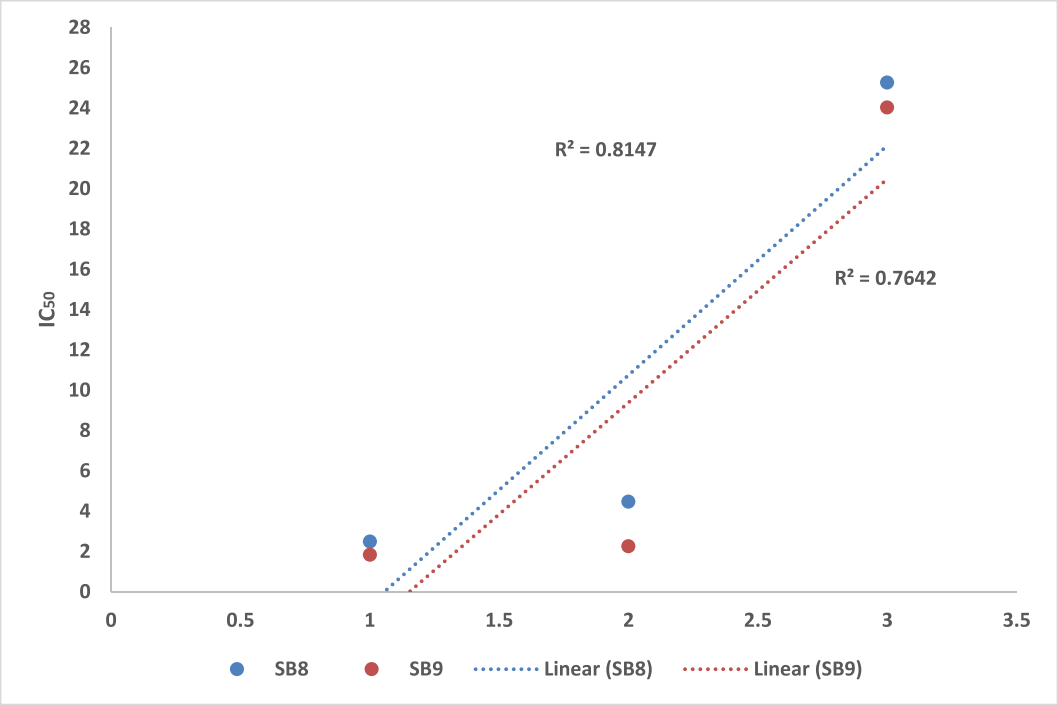


Fig. 4. Correlation between IC_{50} of MCF-7, MDA-MB-231 cell line and TP inhibitory activity of compound SB8 and SB9.

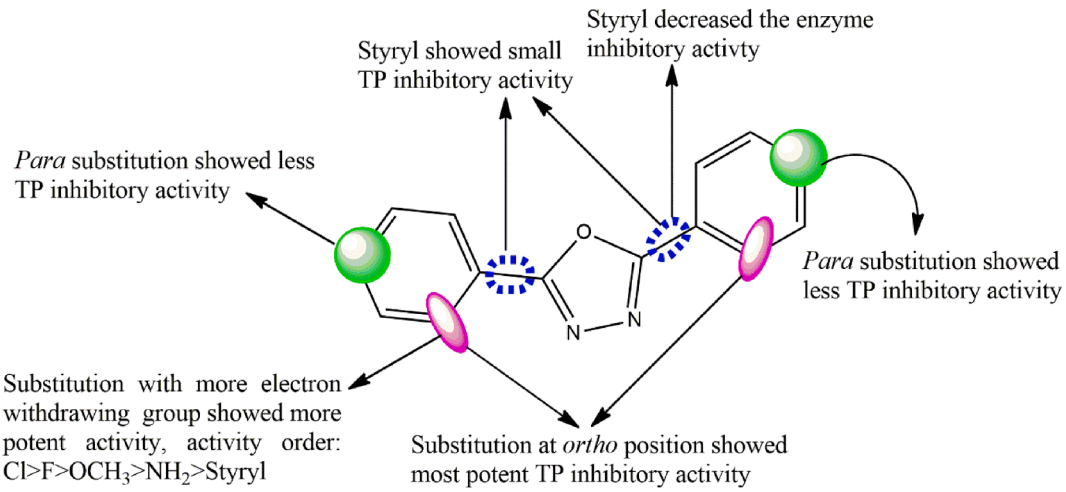


Fig. 5. Structure-activity relationship of the newly synthesized 1,3,4-oxadiazole derivatives as TP inhibitors and anti-cancer agents.

Table 4
Effect of **SB8** compound on body weight of rat.

Group	Dose (mg/kg)	Weekly Body Weight (g) (Mean ± SD)		
		Day 0	Day 7	Day 14
A	Control	155 ± 7	177 ± 12	197 ± 20
B	300	170 ± 9	193 ± 16	213 ± 22
C	2000	180 ± 10	197 ± 18	222 ± 12
D	5000	183 ± 11	208 ± 19	228 ± 16

(vi) Styryl group attached between phenyl and 1,3,4-oxadiazole showed minute activity against both MCF-7 and MDA-MB-231 cell lines and TP enzyme.
(vii) The derivative of 2-methoxy phenyl have less activity compared to 2-chlorophenyl and 2-florophenyl derivatives.
(viii) Among the different *ortho* substituents the order of activity was found to be F > Cl > OCH_3.
(ix) While analyzing the derivatives with substituents at *para* position of benzene ring, it was evident that the order of activity of different substituents was Cl > NH_2 > NO_2.
(x) Among the compounds containing 2-fluoro substituents at R^1, 4-chloro substituent at R^2 position showed better activity as compared to 2-chloro substituent at R^2 position compounds.
(xi) Among the compounds containing 4-chloro substituent at R^1 and different substituents at R^2 position exhibited cytotoxic potential in following position 2F > $4NH_2$ > 2Cl > $2OCH_3$ > 4Cl > $4NO_2$.

2.4. Acute oral toxicity

Compound **SB8** was selected for acute oral toxicity study as the toxicity prediction tool- Protox server identified its LD_{50} as 5000 mg/kg.

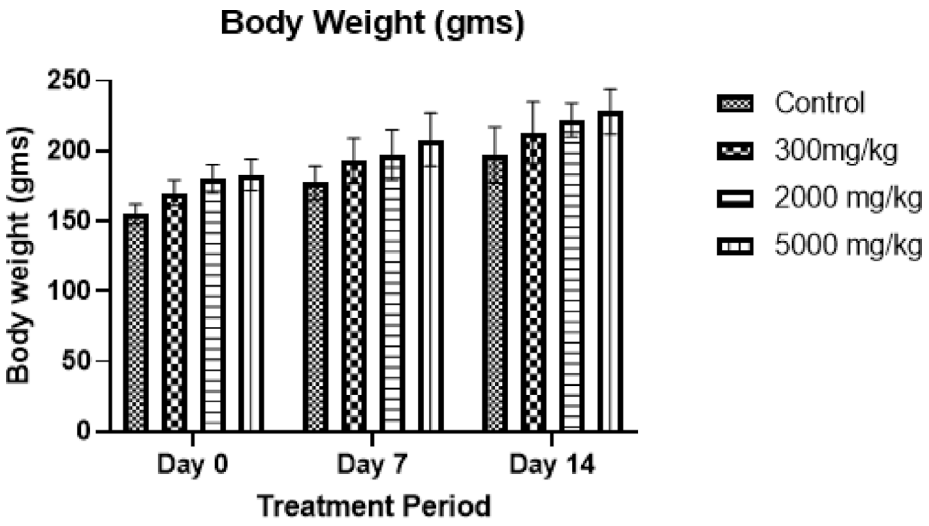


Fig. 6. Effect of **SB8** compound on body weight. All values are expressed as Mean ± SD.

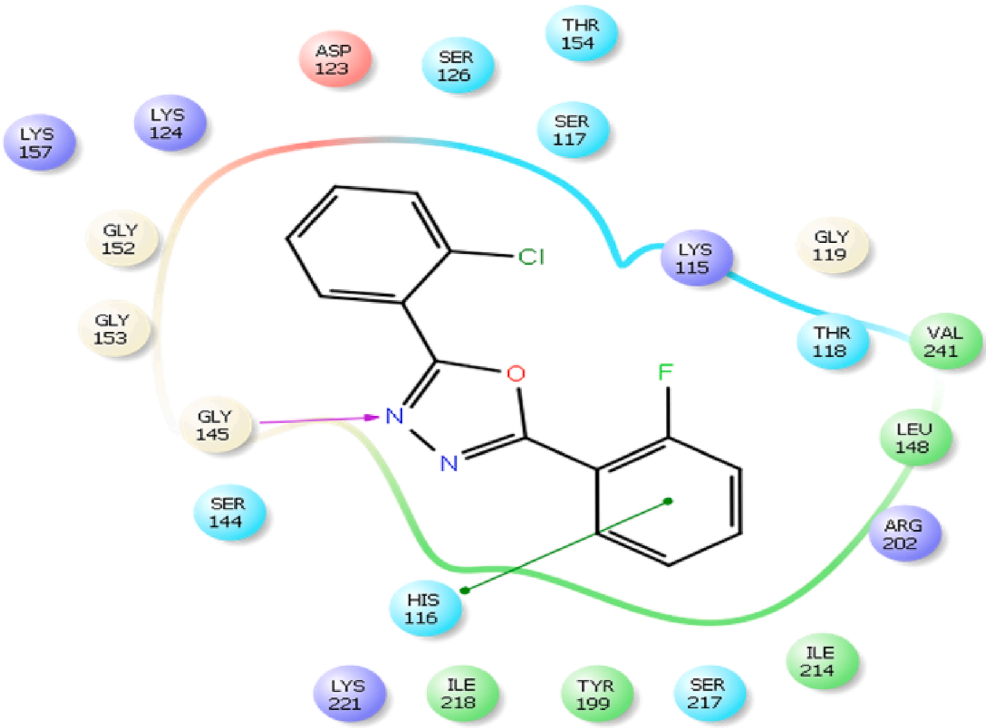


Fig. 7. Predicted binding mode of Compound **SB8**.

Rats were kept for overnight fasting before the day of dosing and for 3 h after dosing. On the first day of dosing, rats were monitored every hour up to 12 h. for mortality, clinical signs and behavioural changes. And thereafter, they were observed twice a day for 14 days and body weight was recorded daily (Table 4). No clinical sign of toxicity was observed during the period of 14 days under observation among the groups of control and tested compound. No statistically significant reduction in body weight in Fig. 6 were observed. Body weight changes if any are an indicator of adverse side effects, as the animals that survive cannot lose more than 10% of the initial body weight. There was no mortality recorded in all the treated groups, and all the treated experimental rats survived. Present study suggests that compound **SB8** is safe for oral administration of up to 5000 mg/kg in single dose to albino female Wistar rats and also in correlation with predicted LD_{50} values by Protox server.

2.5. Statistical analysis

Data obtained from acute oral studies was analysed by two-way analysis of variance (ANOVA) using GraphPad Prism 8 statistical software package to analyse the degree of variance among groups, and "p" values less than, or equal to 0.05 (Bonferroni's test) was considered significant.

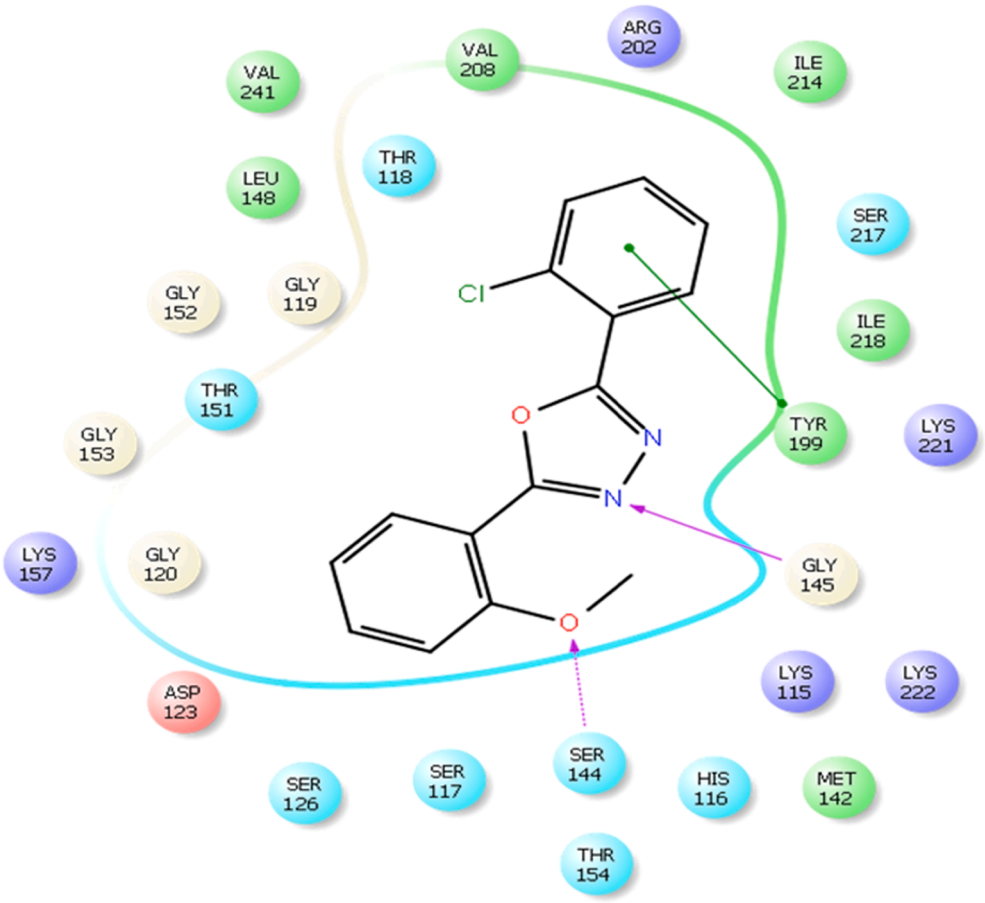


Fig. 8. Predicted binding mode of compound **SB9**.

2.6. Molecular docking

Molecular docking studies paves way for the designing of 1,3,4-oxadiazole derivatives by using the Schrödinger (Maestro 11.2) software. The crystal structure of human thymidine phosphorylase is a complex with a small molecule inhibitor (PDB ID: 1UOU, Resolution: 2.11 Å) was derived from protein data bank and was used for the study after refinement. Results suggested that compound **(SB1-SB18)** depicted specific Vander Waals (vdW) interactions with surrounding hydrophobic residues LEU148, VAL241, VAL208, TYR199, VAL125, ILE218, HIS166, GLY145, SER144 and form hydrogen bonds through the pyrimidine type of nitrogen of the oxadiazole ring. Of the results one of the best scoring pose of compounds **(SB8** and **SB9)** from the docking studies is shown in Fig. 7 and Fig. 8 with the residues interacting with ligand within the binding site. From the results, we found that compounds **(SB3** and **SB8)** exhibited highest docking score (−7.29). Most of the compounds showed binding affinity to human thymidine phosphorylase to lesser or greater extent depicted in Table 5.

Table 5
Docking study of 1,3,4-oxadiazole derivatives.

Comp. code	Docking score	Glide score
SB1.	−7.29	−59.466
SB2.	−7.29	−59.466
SB3.	−6.719	−61.224
SB4.	−6.599	−63.064
SB5.	−6.496	−48.244
SB6.	−6.32	−57.918
SB7.	−6.251	−57.198
SB8.	−6.111	−55.353
SB9.	−6.04	−52.92
SB10.	−5.941	−53.836
SB11.	−5.93	−51.296
SB12.	−5.748	−35.58
SB13.	−5.665	−56.042
SB14.	−5.627	−41.785
SB15.	−5.567	−47.148
SB16.	−5.437	−41.845
SB17.	−5.095	−50.125
SB18.	−5.035	−17.623

3. Conclusion

In summary, a series of newer 1,3,4-oxadiazole derivatives were designed and synthesized in good yields by convenient method and characterized by using different spectroscopy methods such as IR, NMR and MASS. Evaluation of all the derivatives were done against MCF-7 and MDA-MB-231 breast cancer cell lines and all the derivatives showed significant activity. Among them compound **SB9** with 2-chloro at R^1 and 4-methoxy group at R^2 position of 1,3,4-oxadiazole scaffold have shown potent cytotoxicity ($IC_{50} = 1.85 \pm 0.28$ μM) against MCF-7 cancer cell line and compound **SB8** have shown potent cytotoxicity against MDA-MB-231 cell lines with $IC_{50} = 2.27 \pm 0.73$ μM. All the synthesized compounds were screened for target specific activity against TP enzyme, among them compounds **SB8, SB9** and **SB3** have shown potent TP inhibitory activity.

The experimental data and SAR study revealed that electron withdrawing groups at R^1 and R^2 position of phenyl ring have better cytotoxicity and TP inhibitory activity. These studies indicate that the 1,3,4-oxadiazole scaffold substituted with phenyl ring can be considered as a lead molecule for designing of potent cytotoxic agents with TP inhibitory activity. Additionally, molecular-docking studies revealed that

nitrogen of 1,3,4-oxadiazole ring and both of phenyl ring played an important role in binding to the active site residues of TP. Compounds **SB8** and **SB9** showed the best binding interaction with amino acid residues HIS116, GLY145, TYR199 and SER144 of TP.

The *in vivo* acute rat toxicity study of most active compound clearly suggested that 1,3,4-oxadiazole derivatives with substituted phenyl ring could be safe and effective heterocycle for discovering future potent anti-cancer agents targeting thymidine phosphorylase enzyme.

4. Experimental section

4.1. Materials and methods

All the reagents and solvents used in this research work were of analytical grade and obtained from Sigma-Aldrich, S.D. Fine chemicals and E. Merck India Ltd. All the reaction was carried out in anhydrous condition. Progress of the reactions was monitored with the help of thin layer chromatography (silica gel G coated), using ethyl acetate: chloroform as mobile phase. The results were visualized under UV light cabinet. Open glass capillaries melting point (M.P) apparatus was used for the measurement of M.P of all synthesized compounds (℃) and all the values were used uncorrected. Synthesized compounds were purified either by recrystallization in ethanol or by column chromatography, column packed with silica gel of 230–400 mesh. To further confirm the purity of the compounds, high pressure liquid chromatography (HPLC) and CHN analysis studies were carried out on Shimadzu. LC 2010 CHT HPLC High-Performance Liquid Chromatography System using C18 column and water: acetonitrile as mobile phase while maintaining the flow rate at 1.0 mL/min and 20 μl injection volume. FTIR spectra was recorded in the range of 4000–400 cm^{-1} on Perkin Elmer RX1 Fourier transform spectrophotometer using KBr pellets. NMR (^{1}H NMR, ^{13}C NMR) spectra was obtained using Bruker 500 MHz system using $CDCl_3$ as solvent. The spectra were recorded in δ (ppm) employing tetramethyl silane (TMS) as internal standard. The ^{1}H NMR spectrum (splitting) was abbreviated are as follows: s (singlet), d (doublet), t (triplet), q (quartet), q (pentet), m (multiplet). Chemical information such as molecular weight of synthesized derivatives was determined by mass spectroscopy (MS) using methanol as solvent. MS/MS spectra of selected fragment were obtained for confirmation of drug structure (Supplementary Information provided).

4.2. Chemistry

4.2.1. General procedure for the synthesis of esters of substituted benzoic acids (2a-2r)

To a solution of substituted aromatic benzoic acid (0.04 mol) in anhydrous ethanol (1.2 mol), conc. sulphuric acid (2.0 mol) was added drop wise with continuous stirring and refluxed at 70–80 °C for 9–10 h. After cooling, the reaction mixture was poured on crushed ice. Ester in liquid/solid form was obtained after neutralizing the mixture with saturated $NaHCO_3$ solution and extracted in ethyl acetate and pass-through sodium sulphate [28].

4.2.2. General procedure for synthesis of aromatic hydrazide (3a-3r)

To a solution of substituted aromatic ester (1 mol) in ethanol, mixture of hydrazine hydrate (1.2 mol) in anhydrous ethanol was added dropwise and refluxed at 60–70 °C for 12–18 h. Upon completion of the reaction, excess of hydrazine hydrate was evaporated under vacuum and the resulting mixture was allowed to cool, to yield the hydrazide derivatives [28].

4.2.3. General procedure for synthesis of 2,5-disubstituted 1,3,4-oxadiazole derivatives (SB1-SB18).

Substituted aromatic hydrazide (1 mol) was heated to reflux along with substituted aromatic benzoic acids (0.9 mol) and phosphorous oxychloride (1.2 mol) for 8 h. After completion of reaction, the reaction mixture was allowed to cool to RT and poured over crushed ice. The crude solid product was obtained by filtration and dried under vacuum. The crude solid product so obtained was purified either by recrystallization in ethanol or by column chromatography as suited [29].

4.3. Structural analysis of the compounds

4.3.1. 2,5-bis(4-chlorophenyl)-1,3,4-oxadiazole (SB1)

Yield: 72%, M.P 252–255 °C, R_f = 0.7 (ethyl acetate: chloroform, 8:2), FTIR ($CHCl_3$, υ/(cm^{-1}): 3249 (N—H), 1640 (Ar C═C), 1266 (C═N), 1080 (C—O), 2342 (Ar C—H), 794 (C—Cl). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.55 (d, 4H, J = 7.54 Hz, Ar H), 8.1 (d, 4H, J = 8.09 Hz, Ar H). ^{13}CNMR (500 MHz, $CHCl_3$): δ (ppm), 122.8 (ArC), 128.8 (ArC), 135.7 (C—Cl), 164.6 (N═C—O), 129.2 (ArC). ESI Mass (*m/z*): 291 $(M+H)^+$. Anal. Calc. for $C_{14}H_8Cl_2N_2O$: C, 57.76; H, 2.77; Cl, 24.36; N, 9.62; O, 5.50. Found: C, 58.21; H, 2.99; Cl, 25.13; N, 10.25; O, 4.77.

4.3.2. 2-(2-chlorophenyl)-5-(4-chlorophenyl)-1,3,4-oxadiazole (SB2)

Yield: 69%. M.P 248–250 °C, R_f = 0.8 (ethyl acetate: chloroform, 9:1), FTIR ($CHCl_3$, υ/(cm^{-1}): 1639 (Ar C═C), 1265 (C═N), 1087 (C—O), 2342 (Ar C—H), 794 (C—Cl). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.3 (d, 1H, J = 7.34 Hz), 7.5 (d, 1H, J = 7.46 Hz), 7.6 (d, 3H, J = 7.51 Hz), 8.0 (d, 3H, J = 8.10 Hz). ^{13}CNMR (500 MHz, $CHCl_3$): δ (ppm), 126.4 (ArC), 128.8 (ArC), 133.2 (C—Cl), 131.2 (C—Cl), 164.6 (N═C—O), 129.2 (ArC), 122.8 (ArC). ESI Mass (*m/z*): 290.9 $(M+H)^+$. Anal. Calc. for $C_{14}H_8Cl_2N_2O$: C, 57.76; H, 2.77; Cl, 24.36; N, 9.62; O, 5.50. Found: C, 56.43; H, 3.29; Cl, 24.13; N, 9.95; O, 5.99.

4.3.3. 2-(4-chlorophenyl)-5-(2-fluorophenyl)-1,3,4-oxadiazole (SB3)

Yield: 68%, M.P 263–265 °C, R_f = 0.6 (ethyl acetate: chloroform, 9:1), FTIR ($CHCl_3$, υ/(cm^{-1}): 1544 (Ar C═C), 1280 (C═N), 1087 (C—O), 2361 (Ar C—H), 825 (C—Cl), 1449 (C-F). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.3 (d, 1H, J = 7.32 Hz), 7.4 (d, 1H, J = 7.56 Hz), 7.5 (d, 1H, J = 8.13 Hz), 7.6 (d, 1H, J = 7.36 Hz), 8.2 (d, 2H, J = 7.58 Hz). ^{13}CNMR (500 MHz, $CHCl_3$): δ (ppm), 114.3 (ArC), 131.9 (ArC), 160.4 (C-F), 135.7 (C—Cl), 164.6 (N═C—O), 127.6 (ArC), 130.7 (ArC). ESI Mass (*m/z*): 275 $(M+H)^+$. Anal. Calc. for $C_{14}H_8ClFN_2O$: C, 61.22; H, 2.94; Cl, 12.91; F, 6.92; N, 10.20; O, 5.82. Found: C, 62.47; H, 2.11; Cl, 13.14; F, 5.99; N, 11.19; O, 5.11.

4.3.4. 2-(4-chlorophenyl)-5-(2-methoxyphenyl)-1,3,4-oxadiazole (SB4)

Yield: 70%, M.P 274–276 °C, R_f = 0.6 (ethyl acetate: chloroform, 8:2), FTIR ($CHCl_3$, υ/(cm^{-1}): 1588 (Ar C═C), 1466 (C═N), 1232 (C—O—C), 2978 (Ar C—H), 794 (C—Cl). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.2 (d, 1H, J = 8.10 Hz), 7.3 (d, 1H, J = 7.6 Hz), 7.8 (d, 2H, J = 8.13 Hz), 7.9 (d, 2H, J = 8.13 Hz), 8.0 (s, 3H). ^{13}CNMR (500 MHz, $CHCl_3$): δ (ppm), 119.9 (ArC), 129.2 (ArC), 135.7 (C—Cl), 164.6 (N═C—O), 127.3 (ArC), 112.6 (ArC), 131.3 (ArC), 159 (C—O), 122.8 (C—C). ESI Mass (*m/z*): 287 $(M+H)^+$. Anal. Calc. for $C_{15}H_{11}ClN_2O_2$: C, 62.84; H, 3.87; Cl, 12.37; N, 9.77; O, 11.16. Found: C, 61.87; H, 4.55; Cl, 12.89; N, 9.79; O, 12.17.

4.3.5. 4-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl) aniline (SB5)

Yield: 64%, M.P 282–284 °C, R_f = 0.7 (ethyl acetate: chloroform 8:2), FTIR ($CHCl_3$, υ/(cm^{-1}): 1534 (Ar C═C), 1467 (C═N), 1255 (C—O—C), 3022 (Ar C—H), 781 (C—Cl). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 6.7 (d, 2H, J = 8.2 Hz), 7.7 (d, 2H, J = 8.26 Hz), 7.8 (d, 2H, J = 8.17 Hz). ^{13}CNMR (500 MHz, $CHCl_3$): δ (ppm), 131.3 (ArC), 128.8 (ArC), 129.2 (ArC), 114.1 (ArC), 131.3 (ArC), 149.1 (C—N), 135.7 (C—Cl), 164.6 (N═C—O), 122.8 (C—C). ESI Mass (*m/z*): 272 $(M+H)^+$. Anal. Calc. for $C_{14}H_{10}ClN_3O$: C, 61.89; H, 3.71; Cl, 13.05; N, 15.47; O, 5.89. Found: C, 62.79; H, 2.59; Cl, 13.89; N, 16.66; O, 6.17.

4.3.6. 2-(4-chlorophenyl)-5-(4-nitrophenyl)-1,3,4-oxadiazole (SB6)

Yield: 67%, M.P 278–280 °C, R_f = 0.8 (ethyl acetate: chloroform, 9:1), FTIR ($CHCl_3$, υ/(cm^{-1}): 1566 (Ar C═C), 1325 (C═N), 1189

(C—O—C), 3053 (Ar C—H), 1366 (N—O), 742 (C—Cl). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.9 (m, 4H), 8.0 (d, 2H, $J = 8.41$ Hz), 8.2 (d, 2H, $J = 8.78$ Hz). ^{13}C NMR (500 MHz, $CHCl_3$): δ (ppm), 124.1 (ArC), 129.2 (ArC), 128.8 (ArC), 130.1 (ArC), 148.0 (C—N), 135.7 (C—Cl), 164.6 (N═C—O), 122.8 (C—C). ESI Mass (*m/z*): 302 $(M+H)^+$. Anal. Calc. for $C_{14}H_8ClN_3O_3$: C, 55.74; H, 2.67; Cl, 11.75; N, 13.93; O, 15.91. Found: C, 54.47; H, 3.81; Cl, 12.11; N, 12.79; O, 16.17.

4.3.7. 2,5-bis(2-chlorophenyl)-1,3,4-oxadiazole (SB7)

Yield: 76%, M.P 254–256 °C, $R_f = 0.7$ (ethyl acetate: chloroform, 9:1), FTIR ($CHCl_3$, υ/(cm^{-1})): 1665 (Ar C═C), 1139 (C═N), 933 (C—O), 2341 (Ar C—H), 725 (C—Cl), 1449 (C-F). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.4 (d, 2H, $J = 7.45$ Hz), 7.5 (d, 2H, $J = 7.49$ Hz), 7.6 (d, 2H, $J = 7.58$ Hz), 8.2 (d, 2H, $J = 8.12$ Hz). ^{13}CNMR (500 MHz, $CHCl_3$): δ (ppm), 131.2 (ArC), 130.6 (ArC), 130.4 (ArC), 126.4 (ArC), 133.2 (C—Cl), 164.6 (N═C—O), 128.8 (C—C). ESI Mass (*m/z*): 291 $(M+H)^+$. Anal. Calc. for $C_{14}H_8Cl_2N_2O$: C, 55.76; H, 2.77; Cl, 24.36; N, 9.62; O, 5.50. Found: C, 56.80; H, 1.98; Cl, 24.89; N, 8.74; O, 6.10.

4.3.8. 2-(2-chlorophenyl)-5-(2-fluorophenyl)-1,3,4-oxadiazole (SB8)

Yield: 64%, M.P 263–265 °C, $R_f = 0.6$ (ethyl acetate: chloroform, 9:1), FTIR ($CHCl_3$, υ/(cm^{-1})): 1583 (Ar C═C), 1632 (C═N), 1028 (C—O—C), 2987 (Ar C—H), 704 (C—Cl), 1058 (C-F). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.3 (d, 1H, $J = 7.88$ Hz), 7.4 (m, 1H, $J = 7.63$ Hz), 7.5 (d, 1H, $J = 7.61$ Hz), 7.8 (d, 1H, $J = 7.61$ Hz), 7.9 (d, 1H, $J = 7.88$ Hz). ^{13}CNMR (500 MHz, $CHCl_3$): δ (ppm), 126.4 (ArC), 131.2 (ArC), 130.4 (ArC), 127.6 (ArC), 133.2 (C—Cl), 160.4 (C-F), 164.6 (N═C—O), 128.0 (C—C). ESI Mass (*m/z*): 275 $(M+H)^+$. Anal. Calc. for $C_{14}H_8ClFN_2O$: C, 61.22; H, 2.94; Cl, 12.91; F, 6.92; N, 10.20; O, 5.82. Found: C, 60.89; H, 3.00; Cl, 12.93; F, 6.94; N, 12.44; O, 5.87.

4.3.9. 2-(2-chlorophenyl)-5-(2-methoxyphenyl)-1,3,4-oxadiazole (SB9)

Yield: 57%, M.P 270–272 °C, $R_f = 0.7$ (ethyl acetate: chloroform, 8:2), FTIR ($CHCl_3$, υ/(cm^{-1})): 1499 (Ar C═C), 1652 (C═N), 1014 (C—O—C), 2898 (Ar C—H), 711 (C—Cl). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 3.8 (s, 3H), 7.2 (m, 2H), 7.4 (d, 1H, $J = 7.89$ Hz), 7.5 (d, 1H, $J = 7.59$ Hz), 7.9 (m), 8.0 (d, 1H, $J = 7.59$ Hz). ^{13}CNMR (500 MHz, $CHCl_3$): δ (ppm), 127.3 (ArC), 126.4 (ArC), 130.6 (ArC), 131.3 (ArC), 119.9 (ArC), 133.2 (C—Cl),164.6 (N═C—O), 128.0 (C—C), 112.6 (C—C), 159.7 (C—O), 55.9 (Al—C). ESI Mass (*m/z*): 287 $(M+H)^+$. Anal. Calc. for $C_{15}H_{11}ClN_2O_2$: C, 62.84; H, 3.87; Cl, 12.37; N, 9.77; O, 11.16. Found: C, 62.99; H, 3.81, Cl, 12.37; N, 9.82; O, 11.21.

4.3.10. 4-(5-(2-chlorophenyl)-1,3,4-oxadiazol-2-yl) aniline (SB10)

Yield: 59%, M.P 284–286 °C, $R_f = 0.7$ (ethyl acetate: chloroform, 9:1), FTIR ($CHCl_3$, υ/(cm^{-1})): 1522 (Ar C═C), 1616 (C═N), 1012 (C—O—C), 2833 (Ar C—H), 752 (C—Cl). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 6.7 (d, 2H, $J = 8.26$ Hz), 7.3 (d, 1H, $J = 7.93$ Hz), 7.4 (d, 1H, $J = 7.58$ Hz), 7.7 (d, 2H, $J = 8.26$ Hz), 7.8 (d, 1H, $J = 7.93$ Hz), 8.0 (d, 1H, $J = 7.58$ Hz). ^{13}CNMR (500 MHz, $CHCl_3$): δ (ppm), 114.1 (ArC), 130.6 (ArC), 126.4 (ArC), 131.3 (ArC), 133.2 (C—Cl), 149. 1 (C—N), 164.6 (N═C—O), 128.0 (C—C), 128.8 (C—C). ESI Mass (*m/z*): 272 $(M+H)^+$. Anal. Calc. for $C_{14}H_{10}ClN_3O$: C, 61.89; H, 3.71; Cl, 13.05; N, 15.47; O, 5.89. Found: C, 62.01, H, 3.69; Cl, 12.06; N, 15.48; O, 5.78.

4.3.11. 2-(2-chlorophenyl)-5-(4-nitrophenyl)-1,3,4-oxadiazole (SB11)

Yield: 64%, M.P 274–276 °C, $R_f = 0.6$ (ethyl acetate: chloroform, 9:1), FTIR ($CHCl_3$, υ/(cm^{-1})): 1583 (Ar C═C), 1631 (C═N), 1101 (C—O—C), 3883 (Ar C—H), 794 (C—Cl). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.4 (d, 1H, $J = 7.63$ Hz), 7.5 (d, 1H, $J = 8.09$ Hz), 7.8 (d, 1H, $J = 7.59$ Hz), 7.9 (d, 2H, $J = 8.72$ Hz), 8.0 (d, 1H, $J = 8.09$ Hz), 8.2 (d, 2H, $J = 8.72$ Hz). ^{13}CNMR (500 MHz, $CHCl_3$): δ (ppm), 126.4 (ArC), 131.2 (ArC), 130.4 (ArC), 124.1 (ArC), 133.2 (C—Cl), 148.0 (C—N), 164.6 (N═C—O), 128.0 (C—C), 122.8 (C—C). ESI Mass (*m/z*): 302 $(M+H)^+$. Anal. Calc. for $C_{14}H_8ClN_3O_3$: C, 55.74; H, 2.67; Cl, 11.75; N, 13.93; O, 15.91. Found: C, 56.16, H, 1.89; Cl, 13.06; N, 14.80; O, 16.71.

4.3.12. 2,5-bis(2-fluorophenyl)-1,3,4-oxadiazole (SB12)

Yield: 68%, M.P 264–266 °C, $R_f = 0.8$ (ethyl acetate: chloroform, 8:2), FTIR ($CHCl_3$, υ/(cm^{-1})): 2954(C—H), 1587 (Ar C═C), 1434 (C-F), 1115 (C═N), 1092 (C—O), 1HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.3 (2H, $J = 7.3$ Hz), 7.4 (2H, $J = 7.6$ Hz), 7.6 (2H, $J = 7.9$ Hz), 8.2 (2H, $J = 7.1$ Hz). ^{13}CNMR (500 MHz, $CHCl_3$): δ (ppm), 127.4 (ArC), 114.2 (ArC), 131.9 (ArC), 160.4 (C-F), 164.6 (N═C—O), 130.7 (C—C). ESI Mass (*m/z*): 259 $(M+H)^+$. Anal. Calc. for $C_{14}H_8F_2N_2O$: C, 65.12; H, 3.12; F, 14.71; N, 10.85; O, 6.20. Found: C, 66.28, H, 4.20; F, 15.06; N, 13.89; O, 7.80.

4.3.13. 2,5-di((E)-styryl)-1,3,4-oxadiazole (SB13)

Yield: 78%, M.P 263–265 °C, $R_f = 0.6$ (ethyl acetate: chloroform, 8:1), FTIR ($CHCl_3$, υ/(cm^{-1})): 1548 (Ar C═C), 1678, 1654 (Al C═C), 1682 (C═N), 1098 (C—O—C), 3892 (Ar C—H). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.2 (d, 4H, $J = 8.11$ Hz), 7.3 (d, 2H, $J = 15.45$ Hz), 7.4 (m, 6H), 7.6 (d, 2H, $J = 15.45$ Hz). ^{13}CNMR (500 MHz, $CHCl_3$): δ (ppm), 128.7 (ArC), 127.2 (ArC), 161.9 (N═C—O), 118.0 (C—C), 133.4 (AlC). ESI Mass (*m/z*): 275.1 $(M+H)^+$. Anal. Calc. for $C_{18}H_{14}N_2O$: C, 78.81; H, 5.14; N, 10.21; O, 5.83. Found: C, 79.91, H, 4.92; N, 11.25; O, 6.16.

4.3.14. (E)-2-(4-chlorophenyl)-5-styryl-1,3,4-oxadiazole (SB14)

Yield: 72%, M.P 278–280 °C, $R_f = 0.6$ (ethyl acetate: chloroform, 9:1), FTIR ($CHCl_3$, υ/(cm^{-1})): 1501 (Ar C═C), 1672, 1622 (Al C═C), 1600 (C═N), 1121 (C—O—C), 3873 (Ar C—H), 744 (C—Cl), 1648 (C═C). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.2 (d, 2H, $J = 8.00$ Hz), 7.3 (d, 1H, $J = 15.46$ Hz), 7.4 (m, 3H), 7.6 (d, 1H, $J = 15.46$ Hz), 7.9 (m, 4H). ESI Mass (*m/z*): 283 $(M+H)^+$. Anal. Calc. for $C_{16}H_{11}ClN_2O$: C, 76.97; H, 3.92; Cl, 12.54; N, 9.91; O, 5.66. Found: C, 75.83, H, 4.81; Cl, 13.06; N, 10.22; O, 6.23.

4.3.15. (E)-2-(2-chlorophenyl)-5-styryl-1,3,4-oxadiazole (SB15)

Yield: 69%, M.P 276–278 °C, $R_f = 0.7$ (ethyl acetate: chloroform, 9:1), FTIR ($CHCl_3$, υ/(cm^{-1})): 1642 (Ar C═C), 1632 (C═N), 1087 (C—O—C), 3901 (Ar C—H), 765 (C—Cl), 1648 (C═C). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.2 (d, 2H, $J = 8.0$ Hz), 7.3 (m, 2H), 7.4 (d, 1H, $J = 7.65$ Hz), 7.6 (m, 2H), 7.7 (d, 1H, $J = 7.62$ Hz), 7.9 (d, 1H, $J = 7.89$ Hz). ESI Mass (*m/z*): 283 $(M+H)^+$. Anal. Calc. for $C_{16}H_{11}ClN_2O$: C, 67.97; H, 3.92; Cl, 12.54; N, 9.91; O, 5.56. Found: C, 68.72, H, 2.85; Cl, 12.80; N, 10.52; O, 6.42.

4.3.16. 2-(4-chlorophenyl)-5-phenyl-1,3,4-oxadiazole (SB16)

Yield: 73%, M.P 239–241 °C, $R_f = 0.8$ (ethyl acetate: chloroform, 9:1), FTIR ($CHCl_3$, υ/(cm^{-1})): 1552 (Ar C═C), 1642 (C═N), 1063 (C—O—C), 3822 (Ar C—H), 723 (C—Cl). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.4 (d, 2H, $J = 7.80$ Hz), 7.5 (d, 1H, $J = 7.45$ Hz), 7.9 (m, 4H). ESI Mass (*m/z*): 257 $(M+H)^+$. Calc. for $C_{14}H_9ClN_2O$: C, 65.51; H, 3.53; Cl, 13.81; N, 10.91; O, 6.23. Found: C, 66.30, H, 3.89; Cl, 12.87; N, 11.22; O, 7.10.

4.3.17. 2-(2-chlorophenyl)-5-phenyl-1,3,4-oxadiazole (SB17)

Yield: 61%, M.P 246–248 °C, $R_f = 0.6$ (ethyl acetate: chloroform, 9:1), FTIR ($CHCl_3$, υ/(cm^{-1})): 1541 (Ar C═C), 1648 (C═N), 1043 (C—O—C), 3899 (Ar C—H), 742 (C—Cl). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.3 (d, 1H, $J = 7.85$ Hz), 7.4 (d, 2H, $J = 7.$ 08 Hz), 7.5 (d, 1H, $J = 7.45$ Hz), 7.6 (d, 1H, $J = 7.65$ Hz), 7.8 (d, 1H, $J = 7.65$ Hz), 7.9 (m, 3H). ESI Mass (*m/z*): 257 $(M+H)^+$. Calc. for $C_{14}H_9ClN_2O$: C, 65.51; H, 3.53; Cl, 13.81; N, 10.91; O, 6.23. Found: C, 65.90, H, 3.72; Cl, 13.10; N, 11.61; O, 5.74.

4.3.18. (E)-2-phenyl-5-styryl-1,3,4-oxadiazole (SB18)

Yield: 75%, M.P 278–280 °C, $R_f = 0.6$ (ethyl acetate: chloroform, 9:1), FTIR ($CHCl_3$, υ/(cm^{-1})): 1616 (Ar C═C), 1698 (C═N), 1074 (C—O—C), 3942 (Ar C—H), 1644 (C═C). ^{1}HNMR (500 MHz, $CHCl_3$): δ (ppm), 7.2 (d, 2H, $J = 8.00$ Hz), 7.3 (d, 1H, $J = 15.45$ Hz), 7.4 (d, 2H, $J = 7.33$ Hz), 7.5 (m, 2H), 7.6 (d, 2H, $J = 7.82$ Hz), 7.9 (d, 2H, $J = 7.82$

Hz). ESI Mass (*m/z*): 249 $(M+H)^+$. Calc. for $C_{16}H_{12}N_2O$: C, 77.40; H, 4.87; N, 11.28; O, 6.64. Found: C, 78.30, H, 5.82; N, 12.61; O, 6.72.

4.4. Biological evaluation

4.4.1. In vitro cell proliferation assay

Cancer cell lines such as MCF-7, MDA-MB-231 and VERO cell line (African Green monkey kidney) were purchased from National Centre for Cell Sciences (NCCS) (Pune, India). *In vitro* cytotoxic activity of all the synthesized 2,5-disubstituted 1,3,4-oxadiazole derivatives was evaluated using colorimetric MTT assay also used for calculating cell viability. Doxorubicin was used as a standard inhibitor. Briefly, cells were grown in DMEM media supplemented with foetal bovine serum (FBS) 10% and penicillin–streptomycin (50 U/ml, 50 mg/ml) at 37 °C, CO_2 (5%) and air (95%). Logarithmically, growing cells were seeded using 96-well plate at different concentrations of the test compounds ranging from 0.01 to 100 μM. After 24 h of seeding, the cells were observed under microscope and treated with varying drug concentration along with DMSO (vehicle control). Each dilution of test compound was added in triplicate. Following 48-h incubation with the compounds, cells were incubated with MTT reagent (5 mg/ml) for 4 h, and then 100 μl of DMSO was added to dissolve formazan crystals. The absorbance was then measured at 540 nm and 630 nm (background scan) using EPOCH 2 BioTek microplate reader. The results of anti-cancer activity were expressed as IC_{50} values in μM range and are presented in Table 2. IC_{50} is defined as the compound concentration required to reduce the viability of the cells by 50% with respect to the control [30]. CC_{50} value of VERO cell line and selectivity index also presented in Table 2.

4.4.2. Thymidine phosphorylase inhibitory assay

Synthesized compounds were evaluated for their thymidine phosphorylase inhibition activity by employing 7-deazaxanthine as standard. TP inhibitory assay results are shown in Table 3. The procedure for TP inhibitory assay was followed from Krenitsky *et al.,* (1979) with modifications and the absorbance was measured at 290 nm spectrophotometrically. Total reaction mixture of volume 200 μl was placed in wells, which consisted of 145 μl potassium phosphate buffer (pH 7.4), 30 μl of *E. coli* TP enzyme (Sigma T2807 500u) at concentration 0.002 U and 20 μl thymidine 5' mono phosphate solution (0.05 U concentration) as substrate. Test material 5 μl was added to this mixture and incubated for 20 min at 25 °C and taking reading in microplate reader at 290 nm. The wells containing reaction mixture devoid of substrate was considered blank and the absorbance of these blank wells was subtracted from wells containing reaction mixture with substrate to avoid the background absorbance. Microplate reader took the readings continuously after 10, 20, 30, 40, 50, and 60 mins after incubation. All assays were performed in triplicate [31,32].

4.4.3. Acute oral toxicity (LD_{50})

Animal toxicity study of the most active compounds **SB8** was conducted as per OECD (Organization for Economic Co-operation and Development) guidelines 420, test guideline 401, Acute Oral Toxicity - Fixed Dose procedure and institutional guidelines provided by regulatory committee of animal research [33]. Additionally, Acute oral toxicity was directed on twenty albinos female Wistar rats for 8–12 weeks old (150-200gm) and were maintained at 25 ± 2 °C in conditioned room with 50–60% of humidity and free access to food and water was given. These rats were randomly divided into four group (A, B, C, D) of five rats each. Rats were kept for fasting overnight (12 h) prior to dosing and weights were recorded periodically. Subsequent of the period of fasting, rats of group B, C and D were weighted and the test compounds were administered. The compound given in 300, 2000 and 5000 mg in to three groups of rats and rats of group A was kept for untreated control and given vehicle only. Compound was suspended in Tween 80 (2.0%) in normal saline and all the treatment given orally. After the compound was administered, food was not provided for further 3–4 h. All the rats were monitored once in a while for every one hour up to 12 h on first day and subsequently two times a day for checking mortality, behavioural changes, signs and symptoms of toxicity for 14 days. Weight of all the twenty rats were taken at the interval 7 days from the day zero to fourteen days and study was conducted two times for each dose.

4.5. Molecular docking

All the synthesized molecules were subjected to molecular docking studies to recognise the binding interactions within the active binding site residues of TP receptor (PDB ID: 1UOU). Molecular docking was done by using Glide module of Schrodinger software. Active site binding residues of TP receptor such as SER117, SER217, HIS116, LYS221, ARG202, TYR199, THR118, ARG146, LEU148, THR151, GLY152 and ILE214 are responsible for binding [26]. The ligands were prepared by computation of charges and setting number of torsions. The refinement of receptor was proceeded for addition of polar hydrogen, removal of water molecules and bind ligand present in the crystal structure. Receptor grid was generated around the binding site of previously attached ligand. The result of docking is expressed in terms of Glide score and binding energy (kcal/mol) and a lower scoring conformation is ranked higher [34].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2021.104873.

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