Synthesis, Quantitative Structure–Activity Relationship and Biological Evaluation of 1,3,4-Oxadiazole Derivatives Possessing Diphenylamine Moiety as Potential Anticancer Agents

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Synthesis of 2,5-disubstituted-1,3,4-oxadiazole (2a–c), 3-substituted aminomethyl-5-substituted-1,3,4-oxadiazole-2(3*H*)-thione (4a–m) and 2-substituted thio-5-substituted-1,3,4-oxadiazole (5a, b) had been described. All the synthesized derivatives were screened for anticancer activity against HT29 and MCF7 cancer cell lines using Sulfo-Rodamine B (SRB) standard method. Most of the tested compounds exploited potent antiproliferative activity against HT29 cancer cell line rather than MCF7 cancer cell line. Compounds 2a–c, 4f and 5a exhibited potent cytotoxicity (IC_{50} 1.3–2.0 μM) and selectivity against HT29 cancer cell line. Quantitative structure–activity relationship (QSAR) study was applied to find a correlation between the experimental antiproliferative activities of the newly synthesized oxadiazole derivatives with their physicochemical parameter and topological index.

Key words 1,3,4-oxadiazole; Mannich base; anticancer; HT29 cell line; MCF7 cell line; quantitative structure–activity relationship study

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. Cancer is the second most common cause of death in the U.S., exceeded only by heart disease, accounting for nearly 1 of every 4 deaths.[1)] Some of the most common cancer types, such as breast cancer, cervical cancer, oral cancer and colorectal cancer have higher cure rates when detected early and treated according to best practices.[2)] Colorectal cancer is the third most common cancer in both men and women.[1)] Adjuvant chemotherapy (anticancer drugs in addition to surgery or radiation) for colon cancer is equally effective.[1)] Excluding cancers of the skin, breast cancer is the most frequently diagnosed cancer in women.[1,3,4)] Treatment may involve radiation therapy, chemotherapy (before or after surgery), hormone therapy or targeted therapy.[1)] Accordingly, continued research is needed to develop new antitumor agents.

1,3,4-Oxadiazoles are an important class of heterocyclic compounds and attracted great interest in medicinal chemistry. Substituted 1,3,4-oxadiazoles display a remarkable broad spectrum of biological activity such as antimicrobial,[5,6)] antitubercular,[7)] analgesic,[8,9)] anti-inflammatory,[9–12)] anticonvulsant[13)] and anticancer[14–18)] activities. 2-Mercapto-5-substituted-1,3,4-oxadiazole were found to exhibit antimicrobial,[6,19)] anti-human immunodeficiency virus (anti-HIV),[19)] antitubercular,[20)] pesticidal,[21,22)] tyrosinase inhibition[23)] and anticancer[24–27)] activities. Moreover, Mannich bases of 1,3,4-oxadiazole derivatives were reported to possess antimicrobial,[28)] analgesic, anti-inflammatory[29)] and anticancer activities.[30)]

Furthermore, diphenylamine moiety was proved to exert *in-vitro*[26)] and *in-vivo*[31,32)] anti-tumor activity in addition to inhibition of autophosphorylation of epidermal growth factor receptor (EGFR),[26)] FGF-R2[31)] and MEK[32–34)] tyrosine kinase enzymes.

In view of the above mentioned facts and as an attempt to obtain new potent antitumor agents, the present work describes the synthesis of new series of 2,5-disubstituted-1,3,4-oxadiazole, 2-substituted thio-5-substituted-1,3,4-oxadiazole and Mannich bases of 5-substituted-1,3,4-oxadiazole-2(3*H*)-thione possessing diphenylamine moiety and evaluation of the anti-tumor properties of the prepared compounds against human tumor cell lines (HT29 "colon" and MCF7 "breast" cancers). Quantitative structure–activity relationship (QSAR) analysis was performed to find a correlation between the physicochemical parameter and topological index of the studied compounds and their experimental activity. It is noteworthy to mention that, steric bulk and structure conformation of a molecule have been shown to be useful in pharmacokinetic profile prediction of the designed molecules. QSAR study was also performed for understanding and validating the antiproliferative activities.

Results and Discussion

Chemistry The targeted compounds **2a**–**c**, **4a**–**m** and **5a**, **b** were synthesized as depicted in Chart 1. The starting compound, *N*-phenylanthranilic acid hydrazide **1**[35)] derived from *N*-phenylanthranilic acid[36)] and intermediate compound 5-[2-(phenylamino)phenyl]-1,3,4-oxadiazole-2-thiol **3**,[37)] were prepared according to the previously reported procedures.

N-Phenylanthranilic acid hydrazide **1** was cyclized with (substituted) benzoic acid in the presence of phosphorus oxychloride to afford 2,5-disubstituted-1,3,4-oxadiazole derivatives **2a**–**c**. Structures of compounds **2a**–**c** were confirmed on the bases of elemental analysis and spectroscopic data (IR, ^{1}H-NMR and MS). IR spectra showed only one NH stretching band at 3421–3290 cm^{-1} and disappearance of carbonyl stretching band of the parent *N*-phenylanthranilic acid hydrazide. ^{1}H-NMR spectra showed protons of methoxy group at δ= 3.82 ppm in compound **2c** spectrum in addition to increased number of aromatic protons at δ=6.68–8.21 ppm and NH appeared at δ=8.30–8.90 ppm, exchanged with D_2O for compounds **2a**–**c**. Mass spectra showed molecular ion peak at 313, 347 and 343 for compounds **2a**, **2b** and **2c** respectively.

5-Substituted-1,3,4-oxadiazol-2-thiol **3** underwent *N*-amino-

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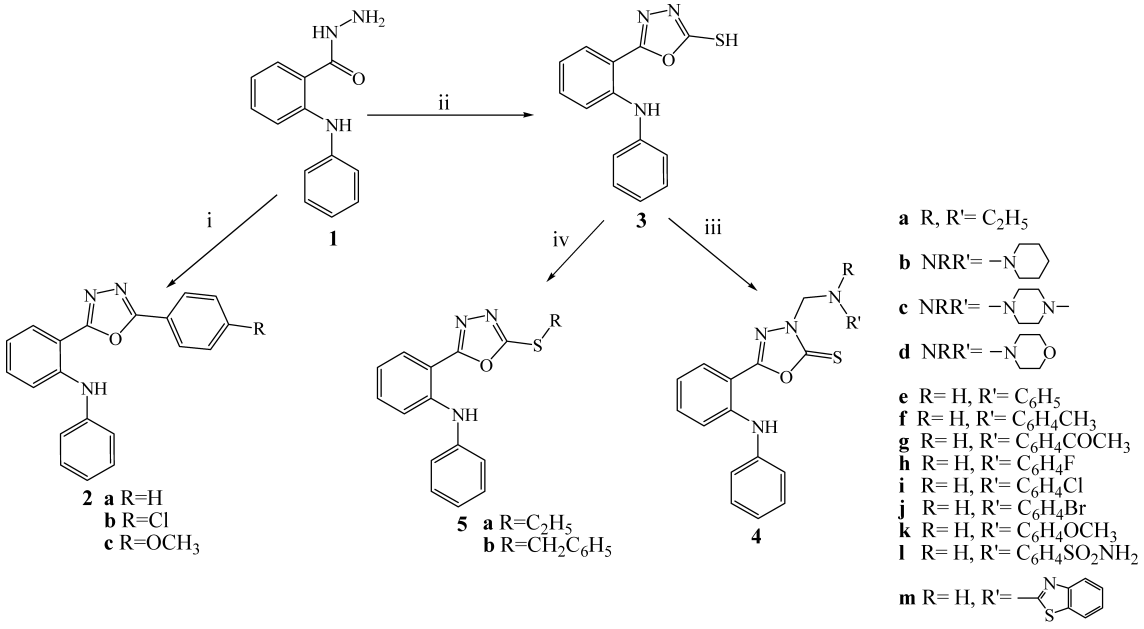

Reagents and solvents: (i) 4-(un)substituted benzoic acid–$POCl_3$; (ii) Cs_2–KOH–ethanol; (iii) CH_2O–NHRR′–ethanol; (iv) RX–K_2CO_3–acetone.

Chart 1

methylation reaction (Mannich reaction) on treating with paraformaldehyde and primary/secondary amines to afford the compounds **4a**–**m**. Attempts to perform *N*-aminomethylation *via* reaction with formaldehyde and primary/secondary amines in methanol[6] or ethanol[30] at room temperature gave the parent compound **3**, supported by the measured melting point and IR spectral data. Therefore reaction was performed using paraformaldehyde and primary/secondary amines in refluxing ethanol.[38,39] The proposed structures of compounds **4a**–**m** were confirmed by micro analysis and spectroscopic data. IR spectra showed NH bands at 3444–3232 cm^{-1} and C=S stretching band at 1296–1265 cm^{-1}. ^{1}H-NMR spectra of compounds **4a**–**m** showed singlet signal at δ=4.46–5.76 ppm corresponding to CH_2 protons. The ^{13}C-NMR spectra showed bands at δ=66.00, 163.00 and 176.54 ppm for compound **4a** and 67.12, 167.61 and 176.43 ppm for compound **4k** corresponding to CH_2, C-5 oxadiazole and C=S respectively, in addition to signals of the remaining aromatic Cs. Mass spectra showed molecular ion peak.

Further, the alkylation of **3** with alkyl halide in presence of anhydrous potassium carbonate yielded *S*-alkylated products **5a**, **b**. The structure assignment for the prepared compounds **5a**, **b** was deduced by elemental and spectral analysis. IR spectra showed NH band at 3302–3290 cm^{-1}. ^{1}H-NMR spectrum of compound **5a** showed the triplet and quartet signals of ethyl protons at the region δ = 1.54 and 3.33 ppm for CH_3 and CH_2 respectively, while that of **5b** showed the benzyl CH_2 protons at δ=4.54 ppm. The ^{13}C-NMR spectra for compound **5b** showed band at δ=36.73 ppm for benzylic CH_2, 162.00 ppm for C-5 oxadiazole and 165.46 ppm for C-2 oxadiazole, in addition to signals of the remaining aromatic Cs. Mass spectra showed molecular ion peak.

Antitumor Screening *In-vitro* antitumor activity for all newly synthesized compounds was performed utilizing *in-vitro* Sulfo-Rodamine B (SRB) standard method[40] against the tumor cell line HT29 (colon adenocarcinoma cell line) and MCF7 (breast cancer cell line) as they may be good predictors of clinically useful drugs. In this protocol, cell line was inoculated and incubated in plate for 24 h. Test compounds were then added with different concentrations (0.001, 0.01, 0.1, 1, 10, 100 μM) and incubated for 48 h. Surviving curves were plotted as a relation between concentration and the surviving fraction to calculate IC_{50} (concentration that reduce the surviving fraction to 50%) and R, the residual unaffected fraction (the resistance fraction)[41] using known drug Doxorubicin (Dox) as a positive control, Table 1.

From the observed antitumor activity data, Table 1, it has been noticed that most of the tested compounds exerted significant activity against HT29 "colon" cell line compared with MCF7 "breast" cell line and less percent of resistant fraction. Results indicated more sensitivity and less resistance of HT29 "colon" than MCF7 "breast" cell line towards tested compounds.

Considering the observed antitumor screening data against MCF7 "breast" cancer cell line, only compounds **2a**–**c** and **4c** revealed pharmacological activity (IC_{50}=3.6, 9.0, 9.5, 6.6 μM, respectively) but compound **2c** showed high percent of resistant fraction (34.4%).

However, all tested compounds showed activity against HT29 "colon" cancer cell line except compounds **4a**, **b**, **e** and **5b**. Compound **4f** had the most promising antitumor properties among all the tested analogs against HT29 "colon" cancer cell line (IC_{50}=1.3 μM). Additionally, compounds **2a**–**c** and **5a** revealed also promising antitumor properties against the same cell line (IC_{50}=1.5, 1.9, 1.7, 2.0 μM, respectively). Activity of rest of active compounds ranged from IC_{50}=2.4–6.0 μM.

Structure–activity relationship based on the observed antitumor properties of the synthesized compounds against HT29 "colon" cancer cell line, indicated that in 2,5-disubstituted-1,3,4-oxadiazole derivatives **2a**–**c**, substitution of the 4-position of phenyl ring with either chloro or methoxy group at 5-position slightly decreased activity but retain promising

Table 1. IC_{50} and R Fraction (Resistant Fraction) of Tested Compounds for Antitumor Screening against HT29 (Colon Adenocarcinoma Cell Line) and MCF7 (Breast Cancer Cell Line)

Cpd. No.	HT29	MCF7
	IC_{50} μM (R fraction) %	
2a	1.5 (0.0)	3.6 (0.2)
2b	1.9 (0.0)	9.0 (0.0)
2c	1.7 (0.0)	9.5 (34.4)
4a	12.7 (0.5)	12.0 (0.0)
4b	10.8 (0.0)	10.8 (0.0)
4c	2.4 (0.3)	6.6 (0.0)
4d	4.9 (0.0)	11.0 (0.0)
4e	10.1 (0.0)	54.3 (0.2)
4f	1.3 (0.0)	10.3 (0.6)
4g	3.6 (0.0)	64.7 (11.8)
4h	4.9 (0.0)	14.9 (5.3)
4i	2.7 (0.13)	22.6 (1.2)
4j	2.97 (0.8)	10.3 (2.6)
4k	3.6 (0.0)	153.7 (—)
4l	6.0 (0.1)	12.7 (0.6)
4m	4.1 (0.0)	14.0 (0.34)
5a	2.0 (4.0)	13.3 (39.4)
5b	22.0 (—)	22.0 (29.4)
Dox	0.25 (0.0)	0.13 (0.0)

activity. In case of Mannich reaction with primary amine that yielded Mannich bases of 1,3,4-oxadiazole-2(3*H*)-thione **4e**–**l**, only **4e** with unsubstituted phenyl ring was inactive while all substituted derivatives showed activity and the methyl substitution was the most active one. Replacement of phenyl ring with heterocycle such as benzothiazole produced also active compound **4m**. Mannich reaction with secondary amine that yielded Mannich bases, only *N*-methylpiperazine and morpholine derivatives **4c**, **d** were active while diethylamine or piperidine **4a**, **b** were inactive. *S*-Alkylated derivatives at 2-position of 1,3,4-oxadiazole **5a**, **b** showed promising activity of ethyl derivative **5a** while the benzyl derivative **5b** was inactive.

QSAR Study In an attempt to correlate the antiproliferative activity with the structure conformation of the synthesized oxadiazole derivatives, QSAR study was undertaken. Descriptors of the molecular modeling software, Molecular Operating Environment (MOE version 2008.10.2),[42] were used. The QSAR model development was restricted to a maximum of three variables as one should select one parameter for five compounds data set (5 : 1 for compounds: descriptor). The structural descriptors used in the generation of these models include; Molar refractivity (mr), Weiner polarity number (weinerPol) and Interaction feild area (vsurf_S) as shown in Table 2. The best derived QSAR linear model for the 14 oxadiazole compounds (3 compounds were considered as outliers and 1 compound was used for evaluation) were presented by the following estimated equation:

$$\text{HT29 } IC_{50} = 12.85973 + 14.47783 \times \text{mr} - 3.40007 \times \text{weinerPol} - 0.05556 \times \text{vsurf\_S}$$

$$r^2 = 0.72546, \quad q^2 = 0.58158$$

The observed IC_{50} was plotted against their predicted values (MLR), Table 3, Fig. 1. The *Z* score method was adopted for the detection of outliers. *Z* score can be defined as absolute difference between the value of the model and the activity field, divided by the square root of the mean square error of the data set. Any compound which shows a value of *Z* score higher than 2.5, during generation of a particular QSAR model, is considered as outlier.[43]

Table 2. The Molecular Descriptor Values of the Studied Compounds

Cpd. No.	Descriptors		
	mr	weinerPol	Vsur_S
2a	9.5194	34.0000	527.7904
2b	10.0170	36.0000	587.3488
2c	10.1787	38.0000	535.3326
4a	10.3340	36.0000	548.4037
4b	10.6024	37.0000	599.2478
4c	10.8817	39.0000	649.9304
4d	10.3350	37.0000	601.9377
4e	10.9681	38.0000	574.9385
4f	11.4181	40.0000	617.9083
4g	11.9956	44.0000	621.7568
4h	11.0388	40.0000	580.0325
4i	11.4699	40.0000	642.4895
4k	11.6309	42.0000	684.5125
5b	10.7373	35.0000	534.0283

From the equation, antiproliferative activity was positively correlated with mr and negatively correlated with weinerPol and vsurf_S. The high coefficient value of mr and the comparatively lower value of weinerPol suggested that the increase in steric bulk and polarizability of the substituent and decrease in branching lead to enhancement of activity. In other words, increase in molar refractivity while keeping proper balance with branching of this substituent and structure conformation of the overall molecule is the key for activity. This was in good agreement with the obtained experimental data, where in case of compounds 2,5-disubstituted-1,3,4-oxadiazole **2a**–**c**, substitution of the phenyl ring showed slight increase in molar refractivity value accompanied by increase in weinerPol value leading to slight drop in activity. On the other hand, in case of Mannich bases with primary amine of 5-substituted-1,3,4-oxadiazole-2(3*H*)-thione, compounds **4f**–**i**, **k** showed higher mr value and increase in weinerPol value due to the substituent compared with parent unsubstituted inactive compound **4e** but with considerable balance between mr value and weinerPol value accounted for activity.

QSAR results have suggested that the steric parameter of the substituents is one of the most important determinants for the activities against colon cancers, with a major contribution coming from the molar refractivity of the substituents.[44]

Cross-Validations Test Cross-validations are the most commonly used techniques for internal validation, In the case of leave-one-out (LOO) cross-validation, each member of the original data set in turn is removed and developed new QSAR models in order to verify the internal predictive ability of the original QSAR model, *e.g.* q^2 (leave-one-out)=0.58158, it was calculated by the following Equation[43]:

$$q^2 = 1 - \frac{\sum(IC_{50}\,\text{Obs.} - IC_{50}\,\text{Pred.})^2}{\sum(IC_{50}\,\text{Obs.} - IC_{50}\,\text{average})^2}$$

Statistical Diagnosis Fraction of the variance (r^2): Represent the goodness of fit. The value of r^2 may vary between 0 and 1, when multiplied by 100 gives explained variance in

Table 3. The Observed Activities for HT29 Cell Line (Obs. IC_{50}) together with the Predicted Activities (Pred. IC_{50}) for the Tested Compounds Calculated Using Multi-linear Regression (MLR)

Cpd. No.	Obs. IC_{50}	MLR validation		
		Pred. IC_{50}	Residual	*Z*-Score
2a	1.5000	5.7521	−4.2521	1.4217
2b	1.9000	2.8461	−0.9461	0.3163
2c	1.7000	1.2773	0.4227	0.1413
4a	12.7000	9.5999	3.1001	1.0365
4b	10.8000	7.2613	3.5387	1.1831
4c	2.4000	1.6883	0.7117	0.2379
4d	4.9000	3.2407	1.6593	0.5548
4e	10.1000	10.5066	−0.4066	0.1359
4f	1.3000	7.8332	−6.5332	2.1844
4g	3.6000	2.3799	1.2201	0.4079
4h	4.9000	4.4463	0.4537	0.1517
4i	2.7000	7.2178	−4.5178	1.5105
4k	3.6000	0.4131	3.1869	1.0655
5b	22.0000	19.6375	2.3625	0.7899

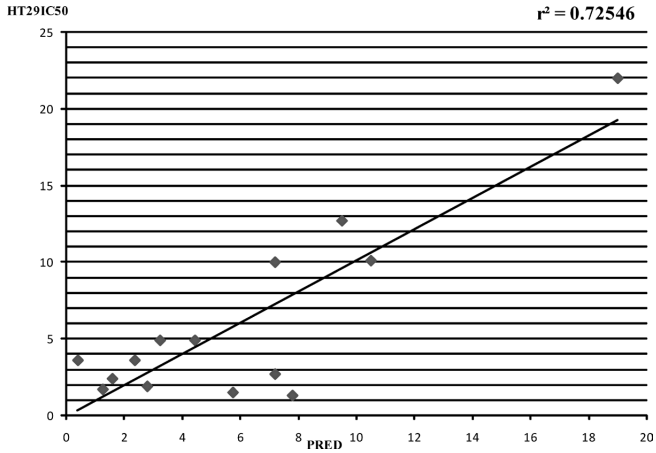

Fig. 1. Correlation of Observed and Predicted IC_{50} Using MLR (r^2=0.72546)

biological activity, where 1 means a perfect model explaining 100% of the variance in the data, and 0 means a model without any explanatory power. It has already been suggested that the only QSAR model having $r^2>0.6$ will be considered for validation.[45] The value of r^2 for this QSAR model is 0.72546.

Cross-Validation Test (q^2): A measure of quality of the QSAR model. According to the literature, a QSAR model must have $q^2>0.5$ for their predictive ability.[45] The value of q^2 for this QSAR model is 0.58158.

$r^2-q^2<0.3$: This difference between r^2 and q^2 for a QSAR model should never be exceeded by 0.3. A large difference between r^2 and q^2 suggests the following: over-fitted model, presence of outliers or presence of irrelevant variables in the data set.[44] The value of r^2-q^2 for this QSAR model is 0.14388.

Model Evaluation Compound **4m** was used for evaluation of the model. It showed low residual value (0.0146) as the predicted IC_{50} was 4.0854 μM compared with the observed IC_{50} 4.1000 μM.

Conclusion

According to anti-proliferative inhibitory activity of tested compounds against HT29 "colon" cell line and MCF7 "breast" cell line, it is apparent from the results that: HT29 "colon" cell line was more sensitive and less resistant than MCF7 "breast" cell line towards most of the tested compounds.

In case of 2,5-disubstituted-1,3,4-oxadiazole derivatives, substitution of the 4-position of phenyl ring slightly decreased activity.

In case of Mannich bases of 1,3,4-oxadiazole-2(3*H*)-thione produced from reaction with primary amine, only unsubstituted phenyl ring was inactive while all substituted derivatives showed activity and the methyl substitution was the most active one. Replacement of phenyl ring with heterocycle (benzothiazole) retained activity. Mannich bases of 1,3,4-oxadiazole-2(3*H*)-thione resulted from reaction with secondary amine showed that, *N*-methylpiperazine and morpholine derivatives gave active compounds while diethylamine or piperidine gave inactive compounds.

S-Alkylation of position 2 of 1,3,4-oxadiazole with ethyl group exhibeted promising activity while in case of benzyl removed activity.

Briefly, 1,3,4-oxadiazole derivatives serve as promising nucleus for subsequent modification in the search for novel anti-tumors. Furthermore, the result of the QSAR studies performed made clear that steric bulk and structure conformation of a molecule is the key for antiproliferative activity. Cytotoxic results of colon cell line screening and QSAR study of the synthesized compounds in this work make them a good trial to discover new anticancer agents. These results urge further investigations to seek for new derivatives containing oxadiazole heterocycle, diphenylamine moiety or both in future work.

Experimental

Chemistry Melting points were determined by open capillary tube method using Gallen Kamp melting point apparatus MFB-595-010M (Gallen Kamp, London, England) and were uncorrected. Microanalysis was carried out at The Regional Center for Mycology and Biotechnology, Al-Azhar University and the micro analytical center, Faculty of science, Cairo University. Infrared Spectra were recorded as potassium bromide discs on Schimadzu FT-IR 8400S spectrophotometer (Shimadzu, Kyoto, Japan) and expressed in wave number (cm^{-1}). The NMR spectra were recorded on a Varian Mercury

VX-300 NMR spectrometer. ^{1}H spectra were run at 300 MHz and ^{13}C spectra were run at 75.46 MHz in deuterated chloroform ($CDCl_3$) or dimethylsulphoxide (DMSO-d_6). Chemical Shifts are quoted in δ as parts per million (ppm) downfield from tetramethylsilane (TMS) as internal standard. Mass spectra were recorded using Hewlett Packard Varian (Varian, Polo, U.S.A.) and Shimadzu Gas Chromatograph Mass spectrometer-QP 1000 EX at 70 eV and Direct inlet unit of Shimadzu GC/MS-QP5050A at 70 eV. TLC were carried out using Art.DC-Plastikfolien, Kieselgel 60 F254 sheets (Merck, Darmstadt, Germany), the developing solvents were benzene–methanol (4 : 1) and the spots were visualized at 366, 254 nm by UV Vilber Lourmat 77202 (Vilber, Marne La Vallee, France).

General Procedure for Synthesis of 2-[2-(Phenylamino)-phenyl]-5-(un)substituted Phenyl-1,3,4-oxadiazole (2a–c) A mixture of acid hydrazides **1** (0.23 g, 1 mmol), 4-(un)-substituted benzoic acids (1 mmol) and phosphorus oxychloride (2.5 mmol) was refluxed at 100–110°C for 6 h. The excess solvent was distilled off under reduced pressure and the residue was quenched with ice cold water. The solid separated was filtered, washed and dried to afford oxadiazoles in 70–75% yield.

2-[2-(Phenylamino)phenyl]-5-phenyl-1,3,4-oxadiazole (**2a**): The crude product was crystallized from ethanol. Yield 70%, mp 192–194°C. ^{1}H-NMR (DMSO-d_6) δ: 7.11 (2H, d, J=8.4 Hz, H-2′,6′ Ar), 7.19 (1H, d, J=6.3 Hz, H-3 Ar), 7.28 (2H, t, H-5,4′ Ar), 7.37–7.60 (3H, m, H-4,3′,5′ Ar), 7.80–7.97 (4H, m, H-6,3″,4″,5″ Ar), 8.06 (2H, d, J=8.7 Hz, H-2″,6″ Ar), 8.90 (1H, s, NH exch. D_2O). IR (KBr) cm^{-1}: 3421 (NH), 3062 (CH Ar), 1650, 1635, 1589, 1523 (C=N, NH, C=C). MS m/z: 313 (M^+). *Anal.* Calcd for $C_{20}H_{15}N_3O$ (313.35): C, 76.66; H, 4.82; N, 13.41. Found: C, 76.83; H, 4.97; N, 13.47.

5-(4-Chlorophenyl)-2-[2-(phenylamino)phenyl]-1,3,4-oxadiazole (**2b**): The crude product was crystallized from ethanol. Yield 73%, mp 136–138°C. ^{1}H-NMR (DMSO-d_6) δ: 6.98 (3H, d, J=8.4 Hz, H-3,2′,6′ Ar), 7.15–7.29 (2H, m, H-5,4′ Ar), 7.39–7.45 (3H, m, H-4,3′,5′ Ar), 7.56 (2H, d, J=8.1 Hz, H-3″,5″ Ar), 7.93 (1H, d, J=8.4 Hz, H-6 Ar), 8.19 (2H, d, J=8.4 Hz, H-2″,6″ Ar), 8.30 (1H, s, NH exch. D_2O). IR (KBr) cm^{-1}: 3290 (NH), 3059 (CH Ar), 1654, 1635, 1593, 1519 (C=N, NH, C=C). MS m/z: 347 (M^+) and 349 (M^++2). *Anal.* Calcd for $C_{20}H_{14}ClN_3O$ (347.80): C, 69.07; H, 4.06; N, 12.08. Found: C, 69.29; H, 4.13; N, 12.42.

5-(4-Methoxyphenyl)-2-[2-(phenylamino)phenyl]-1,3,4-oxadiazole (**2c**): The crude product was crystallized from ethanol. Yield 75%, mp 171–174°C. ^{1}H-NMR (DMSO-d_6) δ: 3.82 (3H, s, OCH_3), 6.68 (3H, d, J=8.7 Hz, H-3,2′,6′ Ar), 6.88 (2H, t, H-5,4′ Ar), 7.04–7.41 (3H, m, H-4,3′,5′ Ar), 7.53 (2H, d, J=8.7 Hz, H-3″,5″ Ar), 7.93 (1H, d, J=8.7 Hz, H-6 Ar), 8.21 (2H, d, J=8.7 Hz, H-2″,6″ Ar), 8.70 (1H, s, NH exch. D_2O). IR (KBr) cm^{-1}: 3405 (NH), 3050 (CH Ar), 2920, 2850 (CH aleph), 1604, 1508 (C=N, NH, C=C). MS m/z: 343 (M^+). *Anal.* Calcd for $C_{21}H_{17}N_3O_2$ (343.38): C, 73.45; H, 4.99; N, 12.24. Found: C, 73.59; H, 5.08; N, 12.60.

General Procedure for Synthesis of 5-[2-(Phenylamino)-phenyl]-3-[(substituted amino)methyl]-1,3,4-oxadiazole-2(3*H*)-thione (4a–m) A mixture of 5-substituted-1,3,4-oxadiazole-2-thiol **3** (2.69 g, 10 mmol), paraformaldehyde (0.3 g, 10 mmol), and primary/secondary amine (10 mmol) was suspended in absolute ethanol (50 mL) and refluxed for 8 h. After the completion of the reaction, as established by TLC, the solvent was evaporated under reduced pressure. After cooling, the gummy oil was extracted with chloroform and dried over anhydrous sodium sulfate then chloroform was distilled under reduced pressure to provide the title compounds in 55–70% yield.

3-[(Diethylamino)methyl]-5-[2-(phenylamino)phenyl]-1,3,4-oxadiazole-2(3*H*)-thione (**4a**): The crude product was crystallized from ethanol. Yield 55%, mp 132–135°C. ^{1}H-NMR ($CDCl_3$) δ: 1.47 (6H, t, 2×$CH_2\underline{CH_3}$), 3.10 (4H, q, 2×$C\underline{H_2}CH_3$), 5.20 (2H, s, CH_2), 6.81 (2H, d, J=7.8 Hz, H-2′,6′ Ar), 6.86 (1H, d, J=6.9 Hz, H-3 Ar), 7.08 (2H, t, H-5,4′ Ar), 7.22–7.42 (3H, m, H-4,3′,5′ Ar), 7.90 (1H, d, J=6.3 Hz, H-6 Ar), 8.98 (1H, s, NH exch. D_2O). ^{13}C-NMR ($CDCl_3$) δ: 11.16 (2×$CH_2\underline{C}H_3$), 42.33 (2×$\underline{C}H_2CH_3$), 66.00 (NCH_2), 108.28 (C-4′ NHC_6H_5), 113.53 (C-5), 117.95 (C-3), 121.35 (C-2′,6′ NHC_6H_5), 122.93 (C-6), 127.88 (C-4), 129.22 (C-3′,5′ NHC_6H_5), 131.05 (C-1), 140.87 (C-1′ NHC_6H_5), 142.86 (C-2), 163.00 (C-5 oxadiazole), 176.54 (C=S). IR (KBr) cm^{-1}: 3296 (NH), 3035 (CH Ar), 2974, 2935, 2858 (CH aleph), 1640, 1610, 1593, 1550 (C=N, NH, C=C), 1280 (C=S). MS m/z: 354 (M^+). *Anal.* Calcd for $C_{19}H_{22}N_4OS$ (354.47): C, 64.38; H, 6.26; N, 15.81. Found: C, 63.96; H, 6.38; N, 16.17.

5-[2-(Phenylamino)phenyl]-3-[(piperidin-1-yl)methyl]-1,3,4-oxadiazole-2(3*H*)-thione (**4b**): The crude product was crystallized from ethanol. Yield 58%, mp 125–128°C. ^{1}H-NMR ($CDCl_3$) δ: 1.60–1.73 (2H, m, C-4 piperidine H), 1.83–1.93 (4H, m, C-3,5 piperidine H), 3.21 (4H, t, C-2,6 piperidine H), 5.00 (2H, s, CH_2), 6.82 (2H, d, J=6.3 Hz, H-2′,6′ Ar), 6.85 (1H, d, J=8.1 Hz, H-3 Ar), 7.12 (2H, t, H-5,4′ Ar), 7.23–7.37 (3H, m, H-4,3′,5′ Ar), 7.88 (1H, d, J=7.5 Hz, H-6 Ar), 8.73 (1H, s, NH exch. D_2O). IR (KBr) cm^{-1}: 3344 (NH), 3010 (CH Ar), 2951, 2924, 2843 (CH aleph), 1662, 1614, 1599 (C=N, NH, C=C), 1280 (C=S). MS m/z: 366 (M^+). *Anal.* Calcd for $C_{20}H_{22}N_4OS$ (366.48): C, 65.55; H, 6.05; N, 15.29. Found: C, 65.93; H, 6.12; N, 15.00.

3-[(4-Methylpiperazin-1-yl)methyl]-5-[2-(phenylamino)-phenyl]-1,3,4-oxadiazole-2(3*H*)-thione (**4c**): The crude product was crystallized from ethanol. Yield 55%, mp 77–78°C. ^{1}H-NMR ($CDCl_3$) δ: 2.42 (3H, s, CH_3), 2.76–2.86 (4H, m, piperazine H), 3.26–3.40 (4H, m, piperazine H), 5.37 (2H, s, CH_2), 6.84 (2H, d, J=6.3 Hz, H-2′,6′ Ar), 6.89 (1H, d, J=8.1 Hz, H-3 Ar), 7.09–7.18 (2H, m, H-5,4′ Ar), 7.23–7.38 (3H, m, H-4,3′,5′ Ar), 7.87 (1H, d, J=8.1 Hz, H-6 Ar), 8.51 (1H, s, NH exch. D_2O). IR (KBr) cm^{-1}: 3290 (NH), 3035 (CH Ar), 2950, 2924, 2854 (CH aleph), 1654, 1610, 1593 (C=N, NH, C=C), 1280 (C=S). MS m/z: 381 (M^+). *Anal.* Calcd for $C_{20}H_{23}N_5OS$ (381.49): C, 62.97; H, 6.08; N, 18.36. Found: C, 63.18; H, 6.19; N, 18.27.

3-[(Morpholin4-yl)methyl]-5-[2-(phenylamino)phenyl]-1,3,4-oxadiazole-2(3*H*)-thione (**4d**): The crude product was crystallized from ethanol. Yield 60%, mp 123–126°C. ^{1}H-NMR (DMSO-d_6) δ: 3.61–3.78 (4H, m, morpholine H), 4.26–4.46 (4H, m, morpholine H), 5.16 (2H, s, CH_2), 6.93 (2H, d, J=8.4 Hz, H-2′,6′ Ar), 6.97 (1H, d, J=6.6 Hz, H-3 Ar), 7.04–7.23 (2H, m, H-5,4′ Ar), 7.32–7.48 (3H, m, H-4,3′,5′ Ar), 7.73 (1H, d, J=6.6 Hz, H-6 Ar), 8.22 (1H, s, NH exch. D_2O). IR (KBr) cm^{-1}: 3344 (NH), 3066 (CH Ar), 2978, 2924, 2870 (CH aleph), 1662, 1600, 1593, 1570 (C=N, NH, C=C), 1296 (C=S). MS m/z: 368 (M^+). *Anal.* Calcd for $C_{19}H_{20}N_4O_2S$ (368.45): C, 61.94; H, 5.47; N, 15.21. Found: C, 61.80; H, 5.30;

N, 14.90.

3-(Phenylaminomethyl)-5-[2-(phenylamino)phenyl]-1,3,4-oxadiazole-2(3*H*)-thione (**4e**): The crude product was crystallized from ethanol. Yield 60%, mp 107–108°C. ^{1}H-NMR ($CDCl_3$) δ: 5.13 (2H, s, CH_2), 6.54 (1H, s, NH exch. D_2O), 6.78–6.83 (5H, m, H-3,2′,6′,2″,6″ Ar), 7.08 (3H, t, H-5,4′,4″ Ar), 7.17–7.33 (5H, m, H-4,3′,5′,3″,5″ Ar), 7.80 (1H, d, J=6.3 Hz, H-6 Ar), 8.18 (1H, s, NH exch. D_2O). IR (KBr) cm^{-1}: 3425, 3344 (2 NH), 3032 (CH Ar), 2924, 2854 (CH aleph), 1650, 1610, 1597, 1573 (C=N, NH, C=C), 1280 (C=S). MS *m/z*: 374 (M^+). *Anal.* Calcd for $C_{21}H_{18}N_4OS$ (374.46): C, 67.36; H, 4.85; N, 14.96. Found: C, 67.20; H, 4.90; N, 14.80.

3-[(4-Methylphenyl)aminomethyl]-5-[2-(phenylamino)-phenyl]-1,3,4-oxadiazole-2(3*H*)-thione (**4f**): The crude product was crystallized from ethanol. Yield 65%, mp 98–99°C. ^{1}H-NMR ($CDCl_3$) δ: 2.37 (3H, s, CH_3), 5.10 (2H, s, CH_2), 6.83 (2H, d, J=7.8 Hz, H-2″,6″ Ar), 6.94 (2H, d, J=7.8 Hz, H-2′,6′ Ar), 7.02 (1H, d, J=7.8 Hz, H-3 Ar), 7.21–7.52 (5H, m, H-4,5,3′,4′,5′ Ar), 7.80 (2H, d, J=7.8 Hz, H-3″,5″ Ar), 8.12 (1H, d, J=7.8 Hz, H-6 Ar), 8.40 (2H, s, 2×NH exch. D_2O). IR (KBr) cm^{-1}: 3348 (2 NH), 3032 (CH Ar), 2951, 2924, 2862 (CH aleph), 1670, 1610, 1597, 1573 (C=N, NH, C=C), 1276 (C=S). MS *m/z*: 387 (M^+-1). *Anal.* Calcd for $C_{22}H_{20}N_4OS$ (388.49): C, 68.02; H, 5.19; N, 14.42. Found: C, 68.41; H, 5.26; N, 14.56.

3-[(4-Acetylphenyl)aminomethyl]-5-[2-(phenylamino)-phenyl]-1,3,4-oxadiazole-2(3*H*)-thione (**4g**): The crude product was crystallized from ethanol. Yield 63%, mp 189–190°C. ^{1}H-NMR ($CDCl_3$) δ: 2.37 (3H, s, CH_3), 5.59 (2H, s, CH_2), 6.72 (2H, d, J=7.8 Hz, H-2′,6′ Ar), 6.94 (1H, d, J=8.4 Hz, H-3 Ar), 6.97 (2H, d, J=9 Hz, H-2″,6″ Ar), 7.10 (2H, t, H-5,4′ Ar), 7.25 (2H, d, J=7.8 Hz, H-3″,5″ Ar), 7.32–7.37 (3H, m, H-4,3′,5′ Ar), 7.72 (1H, d, J=9 Hz, H-6 Ar), 7.89 (1H, s, NH exch. D_2O), 8.21 (1H, s, NH exch. D_2O). IR (KBr) cm^{-1}: 3444, 3336 (2 NH), 3055 (CH Ar), 2924, 2850 (CH aleph), 1740 (C=O), 1654, 1610, 1593, 1570 (C=N, NH, C=C), 1265 (C=S). MS *m/z*: 416 (M^+). *Anal.* Calcd for $C_{23}H_{20}N_4O_2S$ (416.50): C, 66.33; H, 4.84; N, 13.45. Found: C, 66.54; H, 4.92; N, 13.68.

3-[(4-Fluorophenyl)aminomethyl]-5-[2-(phenylamino)-phenyl]-1,3,4-oxadiazole-2(3*H*)-thione (**4h**): The crude product was crystallized from ethanol. Yield 60%, mp 101–104°C. ^{1}H-NMR ($CDCl_3$) δ: 4.46 (2H, s, CH_2), 6.84–6.87 (5H, m, H-3,2′,6′,2″,6″ Ar), 7.03 (2H, t, H-5,4′ Ar), 7.12–7.39 (3H, m, H-4,3′,5′ Ar), 7.85 (2H, d, J=8.4 Hz, H-3″,5″ Ar), 8.07 (1H, d, J=6.3 Hz, H-6 Ar), 8.20 (1H, s, NH exch. D_2O), 9.06 (1H, s, NH exch. D_2O). IR (KBr) cm^{-1}: 3421, 3344 (2 NH), 3047 (CH Ar), 2924, 2854 (CH aleph), 1662, 1610, 1593, 1570 (C=N, NH, C=C), 1292 (C=S). MS *m/z*: 392 (M^+). *Anal.* Calcd for $C_{21}H_{17}FN_4OS$ (392.45): C, 64.27; H, 4.37; N, 14.28. Found: C, 64.10; H, 4.40; N, 14.00.

3-[(4-Chlorophenyl)aminomethyl]-5-[2-(phenylamino)-phenyl]-1,3,4-oxadiazole-2(3*H*)-thione (**4i**): The crude product was crystallized from ethanol. Yield 62%, mp 110–112°C. ^{1}H-NMR (DMSO-d_6) δ: 5.36 (2H, s, CH_2), 6.89 (2H, d, J=7.8 Hz, H-2″,6″ Ar), 6.94 (2H, d, J=7.8 Hz, H-2′,6′ Ar), 7.08 (1H, d, J=9 Hz, H-3 Ar), 7.26 (2H, t, H-5,4′ Ar), 7.33–7.53 (3H, m, H-4,3′,5′ Ar), 7.71 (2H, d, J=6.3 Hz, H-3″,5″ Ar), 7.91 (1H, d, J=6.3 Hz, H-6 Ar), 8.22 (1H, s, NH exch. D_2O), 9.94 (1H, s, NH exch. D_2O). IR (KBr) cm^{-1}: 3344, 3275 (2 NH), 3035 (CH Ar), 2951, 2924 (CH aleph), 1656, 1597, 1573 (C=N, NH, C=C), 1276 (C=S). MS *m/z*: 408 (M^+). *Anal.* Calcd for $C_{21}H_{17}ClN_4OS$ (408.90): C, 61.68; H, 4.19; N, 13.70. Found: C, 61.80; H, 4.30; N, 13.79.

3-[(4-Bromophenyl)aminomethyl]-5-[2-(phenylamino)-phenyl]-1,3,4-oxadiazole-2(3*H*)-thione (**4j**): The crude product was crystallized from ethanol. Yield 60%, mp 117–118°C. ^{1}H-NMR ($CDCl_3$) δ: 5.10 (2H, s, CH_2), 5.73 (1H, s, NH exch. D_2O), 6.80 (2H, d, J=9.3 Hz, H-2″,6″ Ar), 7.03 (3H, d, J=7.8 Hz, H-3,2′,6′ Ar), 7.12 (2H, t, H-5,4′ Ar), 7.17–7.42 (3H, m, H-4,3′,5′ Ar), 7.76 (2H, d, J=8.4 Hz, H-3″,5″ Ar), 7.83 (1H, d, J=8.4 Hz, H-6 Ar), 8.00 (1H, s, NH exch. D_2O). IR (KBr) cm^{-1}: 3421, 3346 (2 NH), 3040 (CH Ar), 2970, 2924 (CH aleph), 1654, 1597, 1573 (C=N, NH, C=C), 1276 (C=S). MS *m/z*: 453 (M^+). *Anal.* Calcd for $C_{21}H_{17}BrN_4OS$ (453.35): C, 55.64; H, 3.78; N, 12.36. Found: C, 55.69; H, 3.85; N, 12.18.

3-[(4-Methoxyphenyl)aminomethyl]-5-[2-(phenylamino)-phenyl]-1,3,4-oxadiazole-2(3*H*)-thione (**4k**): The crude product was crystallized from ethanol. Yield 67%, mp 168–170°C. ^{1}H-NMR ($CDCl_3$) δ: 3.83 (3H, s, OCH_3), 5.69 (2H, s, CH_2), 5.76 (2H, s, 2×NH), 6.95 (2H, d, J=6.3 Hz, H-2″,6″ Ar), 6.99 (3H, d, J=8.7 Hz, H-3,2′,6′ Ar), 7.21–7.43 (5H, m, H-4,5,3′,4′,5′ Ar), 7.52 (2H, d, J=7.8 Hz, H-3″,5″ Ar), 8.12 (1H, d, J=7.8 Hz, H-6 Ar). ^{13}C-NMR ($CDCl_3$) δ: 55.47 (OCH_3), 67.12 (NCH_2), 114.52 (C-2″,6″ $C_6H_4OCH_3$), 118.75 (C-3″,5″ $C_6H_4OCH_3$), 122.06 (C-4′ NHC_6H_5), 125.60 (C-5), 126.49 (C-2′,6′ NHC_6H_5), 127.24 (C-3), 128.18 (C-6), 129.19 (C-4), 129.35 (C-3′,5′ NHC_6H_5), 132.11 (C-1), 133.73 (C-1′ NHC_6H_5), 145.58 (C-1″ $C_6H_4OCH_3$), 145.84 (C-2), 158.87 (C-4″ $C_6H_4OCH_3$), 167.61 (C-5 oxadiazole), 176.43 (C=S). IR (KBr) cm^{-1}: 3444, 3421 (2 NH), 3020 (CH Ar), 2920, 2850 (CH aleph), 1678, 1593, 1570 (C=N, NH, C=C), 1288 (C=S). MS *m/z*: 404 (M^+). *Anal.* Calcd for $C_{22}H_{20}N_4O_2S$ (404.48): C, 65.33; H, 4.98; N, 13.85. Found: C, 65.81; H, 4.87; N, 14.08.

4-({5-[2-(Phenylamino)phenyl]-2-thioxo-1,3,4-oxadiazol-3(2*H*)-yl}methylamino)benzenesulfonamide (**4l**): The crude product was crystallized from ethanol. Yield 70%, mp 220–221°C. ^{1}H-NMR (DMSO-d_6) δ: 5.56 (2H, s, CH_2), 5.58 (1H, s, NH exch. D_2O), 6.94 (2H, d, J=7.8 Hz, H-2′,6′ Ar), 7.00 (1H, d, J=6.9 Hz, H-3 Ar), 7.14 (2H, d, J=8.1 Hz, H-2″,6″ Ar), 7.23–7.28 (2H, m, H-5,4′ Ar), 7.33–7.42 (3H, m, H-4,3′,5′ Ar), 7.57 (2H, d, J=8.7 Hz, H-3″,5″ Ar), 7.72 (1H, d, J=6.6 Hz, H-6 Ar), 7.80 (1H, t, NH exch. D_2O), 8.25 (2H, s, NH_2 exch. D_2O). IR (KBr) cm^{-1}: 3363, 3344, 3298, 3232 (NH_2, 2 NH), 3035 (CH Ar), 2924, 2850 (CH aleph), 1660, 1620, 1600, 1573 (C=N, NH, C=C), 1315, 1149 (SO_2), 1265 (C=S). MS *m/z*: 454 (M^++1). *Anal.* Calcd for $C_{21}H_{19}N_5O_3S_2$ (453.54): C, 55.61; H, 4.22; N, 15.44. Found: C, 55.92; H, 4.38; N, 15.64.

3-[(Benzo[*d*]thiazol-2-yl)aminomethyl]-5-[2-(phenylamino)-phenyl]-1,3,4-oxadiazole-2(3*H*)-thione (**4m**): The crude product was crystallized from ethanol. Yield 70%, mp 226–228°C. ^{1}H-NMR (DMSO-d_6) δ: 5.76 (2H, s, CH_2), 6.92 (2H, d, J=7.8 Hz, H-2′,6′ Ar), 7.03 (1H, d, J=7.8 Hz, H-3 Ar), 7.07–7.22 (2H, m, H-5,4′ Ar), 7.24–7.33 (3H, m, H-4,3′,5′ Ar), 7.37–7.41 (2H, m, H-5″,6″ Ar), 7.69 (1H, d, J=7.8 Hz, H-6 Ar), 7.74 (2H, d, J=7.8 Hz, H-4″,7″ Ar), 8.30 (1H, s, NH exch. D_2O), 9.18 (1H, s, NH exch. D_2O). IR (KBr) cm^{-1}: 3444, 3336, (2 NH), 3051 (CH Ar), 2924, 2854 (CH aleph), 1650, 1610, 1597, 1570 (C=N, NH, C=C), 1280 (C=S). MS *m/z*: 431 (M^+). *Anal.* Calcd for $C_{22}H_{17}N_5OS_2$ (431.53): C, 61.23; H, 3.97; N, 16.23. Found: C, 61.71; H, 4.12; N, 16.42.

General Procedure for Synthesis of 5-[2-(Phenylamino)-phenyl]-2-substituted Thio-1,3,4-oxadiazole (5a, b) 5-Sub-

stituted-1,3,4-oxadiazole-2-thiol **3** (2.69 g, 10 mmol), ethyl iodide or benzyl chloride (10 mmol) and anhydrous K_2CO_3 (2.8 g, 20 mmol) in dry acetone (30 mL) were refluxed with stirring for 14 h. After completion of the reaction, the reaction mixture was cooled and poured onto ice-water. The separated solid was filtered, washed with water and dried to afford the title compounds in 65–70% yield.

2-Ethylthio-5-[2-(phenylamino)phenyl]-1,3,4-oxadiazole (**5a**): The crude product was crystallized from ethanol. Yield 65%, mp 89–92°C. ^{1}H-NMR ($CDCl_3$) δ: 1.54 (3H, t, $CH_2\underline{CH_3}$), 3.33 (2H, q, $\underline{CH_2}CH_3$), 6.81 (2H, d, J=6.9 Hz, H-2′,6′ Ar), 6.86 (1H, d, J=6.6 Hz, H-3 Ar), 7.09 (2H, t, H-5,4′ Ar), 7.13–7.39 (3H, m, H-4,3′,5′ Ar), 7.81 (1H, d, J=8.4 Hz, H-6 Ar), 9.23 (1H, s, NH exch. D_2O). IR (KBr) cm^{-1}: 3290 (NH), 3035 (CH Ar), 2962, 2924, 2850 (CH aleph), 1610, 1597, 1577, 1550 (C=N, NH, C=C). MS *m/z*: 297 (M^+). *Anal*. Calcd for $C_{16}H_{15}N_3OS$ (297.37): C, 64.62; H, 5.08; N, 14.13. Found: C, 64.79; H, 5.18; N, 13.97.

2-Benzylthio-5-[2-(phenylamino)phenyl]-1,3,4-oxadiazole (**5b**): The crude product was crystallized from ethanol. Yield 70%, mp 113–115°C. ^{1}H-NMR ($CDCl_3$) δ: 4.54 (2H, s, CH_2), 6.83 (2H, d, J=7.8 Hz, H-2′,6′ Ar), 6.86 (1H, d, J=6.6 Hz, H-3 Ar), 7.15 (2H, t, H-5,4′ Ar), 7.27–7.38 (3H, m, H-4,3′,5′ Ar), 7.40–7.51 (5H, m, H-2″,3″,4″,5″,6″ Ar), 7.78 (1H, d, J=8.7 Hz, H-6 Ar), 9.21 (1H, s, NH exch. D_2O). ^{13}C-NMR ($CDCl_3$) δ: 36.73 (SCH_2), 106.90 (C-4′ NHC_6H_5), 113.85 (C-5), 117.72 (C-3), 122.44 (C-2′,6′ NHC_6H_5), 123.61 (C-1), 128.04 (C-4″ $CH_2C_6H_5$), 128.13 (C-6), 128.75 (C-2″,6″ $CH_2C_6H_5$), 129.01 (C-3″,5″ $CH_2C_6H_5$), 129.29 (C-3′,5′ NHC_6H_5), 132.26 (C-4), 135.48 (C-1′ NHC_6H_5), 140.52 (C-1″ $CH_2C_6H_5$), 144.13 (C-2), 162.00 (C-5 oxadiazole), 165.46 (C-2 oxadiazole). IR (KBr) cm^{-1}: 3302 (NH), 3039 (CH Ar), 2920, 2840 (CH aleph), 1640, 1600, 1581, 1546 (C=N, NH, C=C). MS *m/z*: 359 (M^+). *Anal*. Calcd for $C_{21}H_{17}N_3OS$ (359.44): C, 70.17; H, 4.77; N, 11.69. Found: C, 70.30; H, 4.60; N, 11.78.

Antitumor Screening All newly synthesized compounds were tested against the tumor cell line HT29 (Colon adenocarcinoma cell line) and MCF7 (Breast cancer cell line) at Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University using the Sulfo-Rhodamine B stain (SRB) assay by the method of Skehan *et al.*[40]

Procedure Cells were plated in 96-multiwell plate (10^4 cells/well) for 24 h before treatment with the compounds to allow attachment of cell to the wall of the plate. Test compounds were dissolved in dimethylsulfoxide (DMSO) and diluted to the appropriate volume. Different concentrations of the compound under test (0.001, 0.01, 0.1, 1, 10, 100 μM) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h at 37°C and in atmosphere of 5% CO_2. After 48 h, cells were fixed, washed and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Excess stain was washed with acetic acid and attached stain was recovered with Tris ethylenediaminetetraacetic acid (Tris EDTA) buffer. Color intensity was measured in an enzyme-linked immunosorbent assay (ELISA) reader. The relation between the surviving fraction and drug concentration was plotted to get the survival curve of each tumor cell line after the specified compound. IC_{50} and R fraction of the tested compounds were illustrated in Table 1.

The dose response curve of compounds was analyzed using E_{max} model.

$$\%\ \text{Cell viability} = (100 - \text{R}) \times \left\{1 - \frac{[D]^m}{K_d^m + [D]^m}\right\} + \text{R}$$

Where R is the residual unaffected fraction (the resistance fraction), [D] is the drug concentration used, K_d is the drug concentration that produces a 50% reduction of the maximum inhibition rate and m is a Hill-type coefficient. IC_{50} was defined as the drug concentration required to reduce fluorescence to 50% of that of the control (*i.e.*, K_d=IC_{50} when R=0 and E_{max}=100−R).[41]

QSAR. Computational Method All the computational works were performed on Molecular Operating Environment software (MOE version 2008.10.2).[42] The structures of 18 compounds used as training set were sketched using molecular builder of MOE and each structure was subjected to energy minimization up to 0.01 kcal/mol Å using the MMFF94x force field. Optimization methods were used followed by conformational search of each energy-minimized structure. The most stable conformer of each structure was selected and saved into database to generate the common descriptors. QuaSAR descriptor module of MOE was used to calculate descriptors for each molecule. The probability density functions used are Gaussian. The root mean square deviation (RMSD) tolerance was set to 0.5 Å. Regression analysis was performed using HT29 IC_{50} as dependent factor and the calculated descriptors as predictable variables.

In this study, the pool of descriptors was optimized using principal components analysis (PCA). The optimization started with the reduction in the number of molecular descriptors by the determination of the highly inter-correlated descriptor pairs and only one from each pair was selected; then the descriptors with insignificant variance through the data set were also rejected. QSAR model was then constructed after ensuring reasonable correlation of antiproliferative activity with the individual descriptors and minimum inter-correlation among the descriptors used in the derived model. The quality of the model was assessed using the statistical parameter r^2 and q^2.

Molecular Descriptors **mr**; molar refractivity (Physical Properties): including implicit hydrogens and calculated by using the Lorentz–Lorenz equation,[46] where n is the refractive index, MW is the molecular weight, and d is the density of the substance.

$$\text{mr} = \frac{n^2 - 1 \times \text{MW}}{n^2 + 2 \times d}$$

mr is primarily a measure of the bulk and polarizability of substituent with no dependence on conformation.

weinerPol; Wiener polarity number (Adjacency and Distance Matrix Descriptors): half the sum of all the distance matrix entries).

vsurf_S; Interaction field surface area (Surface Area, Volume and Shape Descriptors): depend on the structure connectivity and conformation (dimensions are measured in Å). The vsurf_ descriptors are similar to the VolSurf descriptors and have been shown to be useful in pharmacokinetic property prediction, Table 2.

Validation and Cross-Validation of the Model Cross-validation statistical technique has been applied to estimate the quality with regard to predictive ability of the generated

model. This is the most common validation technique, where a number of modified data sets are created by deleting, in each case, one or a smaller group of objects from the data in such a way that each object is taken away once and only once. For each reduced data set, the model is calculated, and responses for the deleted objects are predicted from the model. The simplest and most general cross-validation procedure is the leave-one-out technique (LOO technique), where each object of the data set is taken away, one at a time. In this case, given n objects, n reduced models are developed with a value of q^2 found to be 0.58158.

The observed activities (Obs. IC_{50}) together with the predicted activities (Pred. IC_{50}) for the tested compounds calculated using multi-linear regression (MLR) are listed in Table 3. All compounds showed very good results with Z-scores not exceed the value of 2.5 indicating excellent predictive ability of the model.

The observed IC_{50} is plotted against their predicted values (calculated by MLR method) with a value of r^2 found to be 0.72546, Fig. 1.

Outliers Compounds **4j**, **4l**, **5a** were considered as outliers as they were unable to fit in a QSAR model. Separating these outliers from the main data set and formulating another QSAR can resolve the problem. Outliers may be acting by a different mechanism.

Model Evaluation By evaluation of the model and its trial on compound **4m** the predicted IC_{50} value was 4.0854 with residual value 0.0146 compared to the actual value IC_{50}=4.1000.

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