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Synthesis and antitumor evaluation of some new 1,3,4-oxadiazole-based heterocycles

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ABSTRACT

The synthetic strategies and characterization of some novel 1,3,4-oxadiazole derivatives carrying different pharmacophores and heterocyclic rings that are relevant to potential antitumor and cytotoxic activities are described. The antitumor activities of the newly synthesized compounds were evaluated according to the protocol of the National Cancer Institute (NCI) *in-vitro* disease-oriented human cells screening panel assay. The results revealed that five compounds, namely **2**, **7a**, **11a**, **12b**, and **17**; displayed promising *in-vitro* antitumor activity in the 4-cell lines assay. Incorporating a thiazole ring to 1,3,4-oxadiazole skeleton resulted in better antitumor activities than those displayed by the pyrazole and thiophene ring systems. Transformation of 1,3,4-oxadiazole **2** to *N*-(6-amino-7*H*-pyrazolo[5,1-*c*][1,2,4]triazol-3-yl)benzamide (**15**) diminished the antitumor activity.

1. Introduction

1,3,4-Oxadiazoles are an important class of heterocyclic compounds with a wide range of biological activities such as antiviral [1], antimicrobial [2], antineoplastic [3], fungicidal [4], anticancer [5–8], inhibition of tyrosinase [9] and cathepsin K [10]. They are also useful intermediates in organic synthesis [11] and widely employed as electron transporting and hole-blocking materials [12]. Further, 1,3,4-oxadiazole heterocycles are very good bioisosteres of amides and esters, which can contribute substantially in increasing pharmacological activity by participating in hydrogen bonding interactions with the receptors [13]. 2,4-Disubstituted 1,3,4-oxadiazoles have also attracted significant interest because of their applications in organic light-emitting diodes, photoluminescence, polymers, and material science [14,15]. In view of the great medicinal significance and material applications a number of synthetic routes have been developed for 1,3,4-oxadiazole. The majority of them are based on the cyclization of the diacylhydrazides or acylthiosemicarbazides and the oxidation of acylhydrazones [16] with a variety of reagents such as thionyl chloride, phosphorus oxychloride, or sulfuric acid, usually under harsh reaction conditions.

Cancer treatment has been a major endeavor of research and development in academia and pharmaceutical industry for the last many years as it is one of the leading causes of death [17]. Many of the available anticancer agents exhibit undesirable side effects such as reduced bioavailability, toxicity and drug-resistance [18–22]. Therefore, the search for novel and selective anticancer agents is urgently required due to problems associated with currently available anticancer drugs.

Encouraged by the afore-mentioned findings and in a continuation of an ongoing program aiming at finding new structural leads with potential chemotherapeutic activities [23–30]; it was rationalized to synthesize some novel 1,3,4-oxadiazoles-substituted heterocycles that would produce anticancer activity. The proposed candidates were supported with a variety of pharmacophoric groups which would impart various electronic and lipophilic properties. Synthesis of 1,3,4-oxadiazoles with an amide group was rationalized on the fact that many antitumor antibiotics such as bleomycin, and pyrazofurin incorporate in their structures amidic group [31].

2. Results and discussion

2.1. Chemistry

The suggested synthetic plans to obtain the target compounds are shown in Schemes 1 and 2. The versatile hitherto unreported

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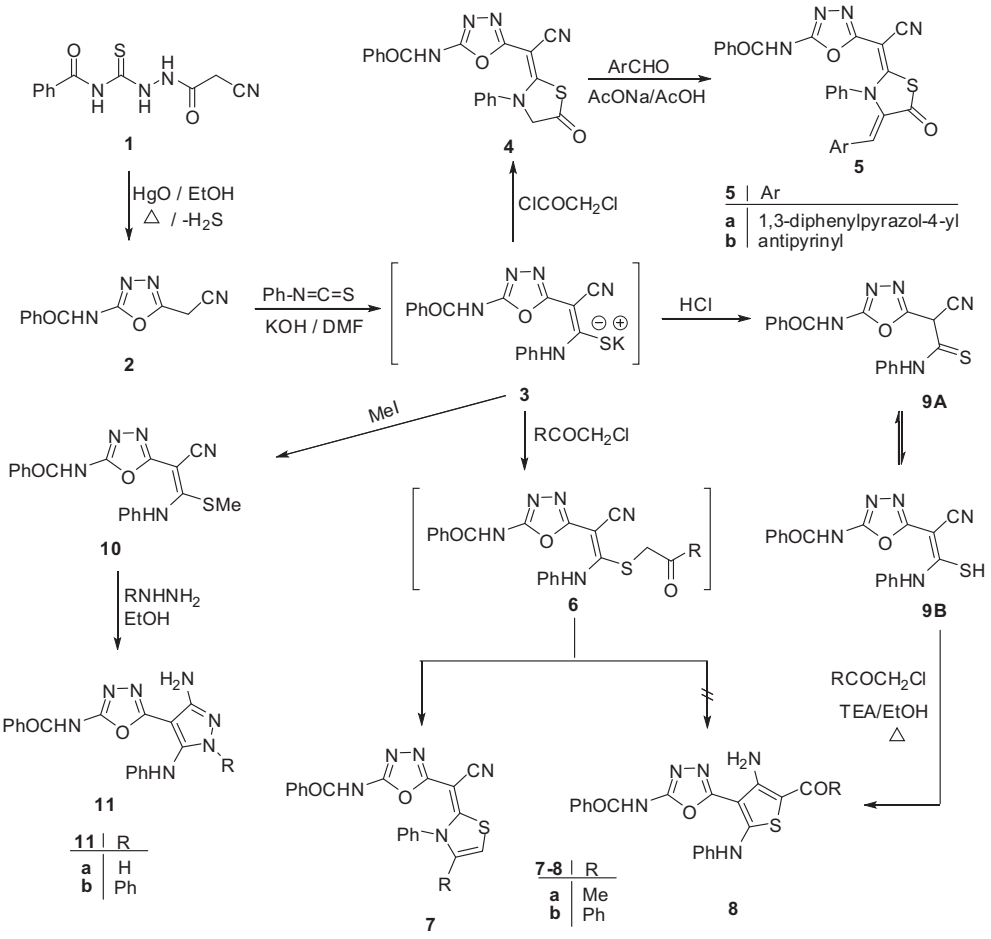


Scheme 1. Synthetic pathway to compounds **2**–**11**.

starting material, *N*-(5-(cyanomethyl)-1,3,4-oxadiazol-2-yl)benzamide (**2**) could be easily obtained *via* cyclodesulfurization of 4-benzoyl-1-cyanoacetylthiosemicarbazide (**1**) [32] upon boiling in ethanolic mercuric oxide solution.

The reactivity of hydrogen atoms at C-5 is the most outstanding chemical property of 5-cyanomethyl-1,3,4-oxadiazole **2** which undergoes the characteristic condensation and electrophilic substitution reactions.

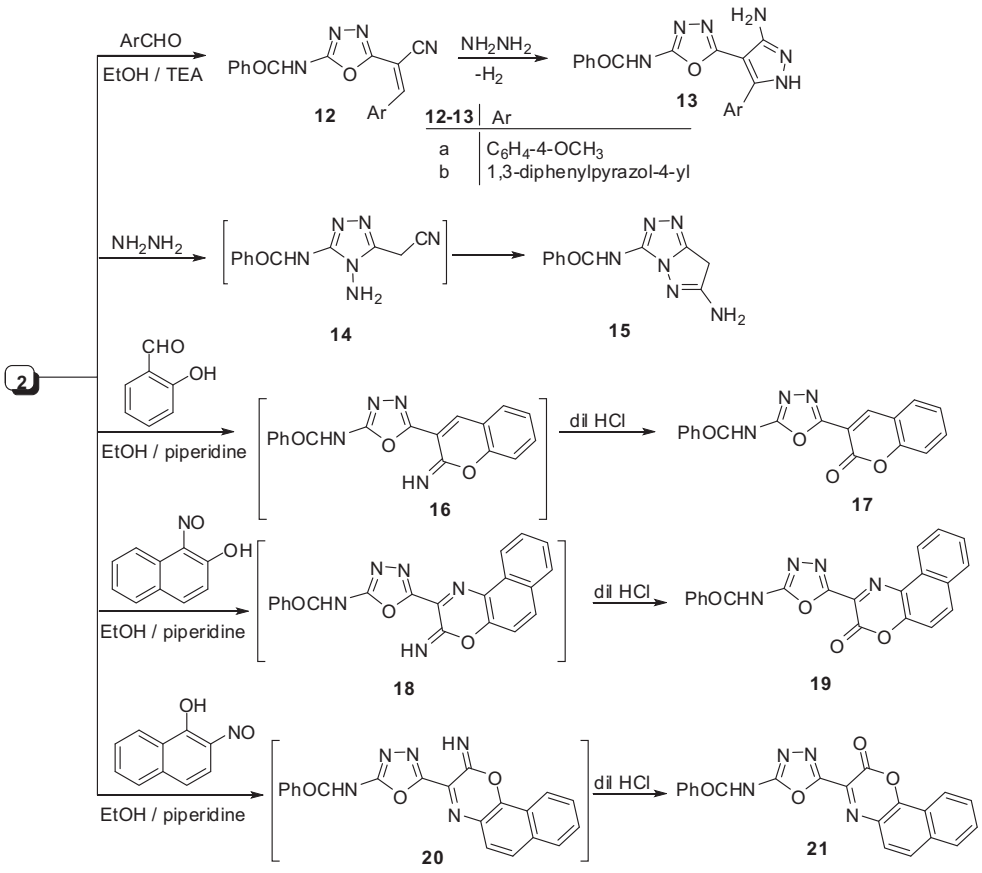
To show the synthetic potentiality of compound **2**, the reactivity of **2** with isothiocyantes was examined. Thus, treatment of compound **2** with phenyl isothiocyanate in potassium hydroxide solution afforded the non-isolable intermediate **3**, which was converted *in situ* to thiazolidin-5-one **4** upon treatment with chloroacetyl chloride. The structure of the latter product was established on the basis of elemental analysis, spectral data, and chemical transformation. The Claisen–Schmidt condensation of thiazolidin-5-one **4** with heterocyclic aldehydes, namely, 1,3-diphenyl-1*H*-pyrazole-4-carbaldehyde and/or 4-formylantipyrine, in glacial acetic acid containing anhydrous sodium acetate as a basic catalyst furnished the arylidene derivatives **5a**,**b**. The structure of latter products were confirmed based on elemental analysis and spectral data (see experimental).

The non-isolable potassium salt **3** was exploited to synthesis some new thiazoline derivatives. Thus, treatment of **3** with α-chloroketones namely, chloroacetone and phenacyl chloride at room temperature afforded a single product, in each case, that was identified as 2,3-dihydrothiazolines **7a**,**b** on the basis of its spectral data. The structures of the latter products were confirmed by the appearance of a cyano absorption band around 2199 cm^{-1} in their IR spectra, and the presence of a characteristic singlet signal was due to the thiazoline H-5 proton at 5.15 ppm in their ^1H NMR spectra.

The appearance of the cyano function in the IR spectra of the reaction products supports structures **7a**,**b** and ruled out the possibility of the other structures **8a**,**b**. Formation of compounds **7a**,**b** was assumed to proceed *via* the initial alkylation of **3** to give the non-isolable thioether intermediate **6** followed by *in situ* heterocyclization through nucleophilic addition of the secondary amino group to the carbonyl group and elimination of water molecule (Scheme 1).

Treatment of the intermediate **3** with cold HCl afforded the thiocarbamoyl derivative **9**, which can exist in two tautomeric thione-thiol forms (**9A** and **9B**). The thiol form **9B** was verified by its ^1H NMR spectrum which displayed a downfield singlet signal at 14.12 ppm due to SH proton, besides the other expected signals. The reaction of the latter product with a variety of α-halocarbonyl compounds, as a key step for the synthesis of polysubstituted thiophene derivatives was examined. Thus, compound **9** reacted with each of chloroacetone and phenacyl chloride in refluxing ethanol containing a catalytic amount of triethylamine, to afford a single product, in each case, identified as the aminothiophene derivatives **8a**,**b** based on the elemental analysis and spectral data of the isolated products (see experimental).

The plausible mechanism for the formation of the aminothiophenes **8a**,**b** is attributed to the initial alkylation of **9B** with α-chloroketones to form the non-isolable thioether **6** which *in situ*



Scheme 2. Synthetic pathway to compounds **12**–**21**.

underwent intramolecular cyclization *via* the nucleophilic addition of the active methylene group to the cyano function and tautomerization according to the Thorpe–Ziegler reaction [33].

As an extension of our investigation, treatment of **3** with methyl iodide afforded the ketene *N*,*S*-acetal **10**. The novel ketene *N*,*S*-acetal **10** represents the versatile precursors for the synthesis of aminopyrazoles. Thus, treatment of the ketene *N*,*S*-acetal **10** with each of hydrazine hydrate and phenyl hydrazine in boiling ethanol afforded, in each case, a single product, that was identified as 3-aminopyrazole derivatives **11a**,**b** based on elemental analysis and spectral data (see experimental).

Pyrazolyl–oxadiazoles have recently received considerable attention because of their synthetic and pharmaceutical importance [34–37]. In the present work we explore the synthetic potentialities of **2** to obtain some novel pyrazolyloxadiazole derivatives. Thus, the Knoevenagel condensation of **2** with each of *p*-anisaldehyde and 1,3-diphenylpyrazole-4-carbaldehyde in refluxing ethanol containing a catalytic amount of triethylamine furnished the arylidene derivatives **12a**,**b**. Treatment of **12a**,**b** with hydrazine hydrate in ethanol, under reflux, furnished the pyrazolyloxadiazole derivatives **13a**,**b**.

Formation of compounds **13a**,**b** is supposed to proceed through the Michael addition of hydrazine hydrate to α,β-unsaturated nitrile **12** and *in situ* intramolecular 1,5-dipolar cyclization *via* the addition of amino group to a cyano function to give dihydropyrazole which underwent auto-oxidation to give the target pyrazoles.

On the other hand, the solvent-free reaction of **2** with an excess of hydrazine hydrate furnished *N*-(6-amino-7*H*-pyrazolo[5,1-*c*][1,2,4]triazol-3-yl)benzamide (**15**) in a reasonable chemical yield.

It is worthwhile to mention that transformation of 1,3,4-oxadiazole ring to 1,2,4-triazole ring by hydrazine hydrate is in the line with reported results by Padmavathi and coworkers [38].

As an extension of our study, the behavior of **2** towards salicylaldehyde and nitrosonaphthols as a convenient synthetic route to some new coumarin and naphtho[1,2-*b*]oxazines incorporating 1,3,4-oxadiazole moiety was also investigated. Thus, condensation of **2** with salicylaldehyde in ethanolic piperidine solution furnished coumarin derivative **17** (Scheme 2).

In a similar manner, treatment of **2** with either 1-nitroso-2-naphthol or 2-nitroso-1-naphthol in boiling ethanolic piperidine solution afforded a single product, in each case, identified as *N*-(5-(3-oxo-3*H*-naphtho[2,1-*b*] [1,4]oxazin-2-yl)-1,3,4-oxadiazol-2-yl) benzamide (**19**) and *N*-(5-(2-oxo-2*H*-naphtho[1,2-*b*] [1,4]oxazin-3-yl)-1,3,4-oxadiazol-2-yl)benzamide (**21**), respectively.

The plausible mechanism for the formation of compounds **17**, **19**, and **21** may be attributed to the initial Knoevenagel condensation of the active methylene nitrile of **2** with each of the carbonyl group of salicylaldehyde and nitroso group of nitrosonaphthols followed by an intramolecular 1,6-dipolar cyclization *via* the addition of the phenolic OH group to the cyano function to give the intermediates **16**, **18**, and **20** those underwent acid catalyzed hydrolysis to afford the target compounds.

3. Pharmacology

3.1. Cytotoxicity and antitumor evaluation

Out of the newly synthesized compounds, seventeen analogs were selected to be evaluated for their *in-vitro* anticancer effect *via* the standard MTT method [39–41], against a panel of four human tumor cell lines namely; heptacelluar carcinoma HepG2, lung fibroblasts WI 38, kidney of a normal adult African green monkey VERO, and breast cancer MCF-7. The cell lines were obtained from

ATCC *via* the Holding company for biological products and vaccines (VACSERA), Cairo, Egypt. 5-Fluorouracil (5-Fu) was used as a standard anticancer drug for comparison. The results of cytotoxic activity is reported in Table 1.

MTT assay is a standard colorimetric assay for measuring cell growth. It is used to determine cytotoxicity of potential medicinal agents and other toxic materials. In brief, yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan by mitochondrial dehydrogenases of living cells. A suitable solvent is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength. When the amount of purple formazan produced by cells treated with an agent is compared with that produced by unreacted control cells, the effectiveness of the agent in causing death of cells can deduced, through the production of a dose–response curve.

The obtained results revealed that five of the tested compounds namely; **2**, **7a**, **11a**, **12b**, **17** exhibited variable degrees of inhibitory activity towards the four tested human tumor cell lines. As for activity against hepatocellular carcinoma HepG2, the highest cytotoxic activity was displayed by compounds **2** and **7a** which showed the percentage viability IC_{50} at 21.2 and 12.4 μg/ml, respectively. Remarkable inhibitory activity was also demonstrated by compounds **12b** and **17**.

The lung fibroblasts WI 38 cell line showed highest sensitivity towards the tested compounds, as its growth was found to be initiated by three compounds. The best activity was demonstrated by compounds **2**, **7a**, and **12b** which have IC_{50} at 24.4, 17.3, and 24.1 μg/ml, respectively. The remaining compounds exhibited less inhibitory activity with percentage inhibition range of 51–100 μg/ml.

On the other hand, Kidney carcinoma VERO was proved to be the least sensitive among the cell lines tested as it was affected by the only three test compounds. However, an outstanding growth inhibition was shown by compound **7a** with IC_{50} 15.8 μg/ml. The remaining two active compounds, namely **2** and **17** showed moderate activity against the same cell line with IC_{50} 30.3 and 34.5 μg/ml.

Further interpretation of the results revealed that compounds **2**, **7a**, **11a**, **12b**, **17** showed moderate anticancer activity against breast cancer MCF-7 with percentage inhibition range of 26–50 μg/ml. Compound **5a** showed no cytotoxicity against all four cell lines.

Table 1
Cytotoxicity (IC_{50}) of tested compounds on different cell lines.

Comp. no.	IC_{50} (μg/ml)[a]			
	HepG2	WI-38	VERO	MCF-7
2	21.2	24.4	30.3	39.2
4	51.2	52.4	50.3	69.2
5a	250.2	340.5	256.6	410.6
5b	145.6	175.0	169.8	166.8
7a	12.4	17.3	15.8	25.8
8a	70.2	84.6	76.4	75.5
8b	90.7	89.6	100.6	100.5
9	100.2	84.6	82.4	77.5
10	125.4	98.7	132.4	107.3
11a	33.7	38.4	36.3	38.3
11b	98.7	90.5	98.8	99.1
12a	69.5	71.2	95.6	49.9
12b	38.1	24.1	39.6	37.4
15	65.6	65.0	69.8	66.8
17	35.6	35.8	34.5	32.6
19	189.8	180.7	189.2	221.8
21	111.4	90.9	95.4	96.9
5-Fu	8.6	3.2	6.5	2.3

[a] IC_{50} (μg/ml): 1–10 (very strong), 11–25 (strong), 26–50 (moderate), 51–100 (weak), 100–200 (very weak), Above 200 (non-cytotoxic).

3.2. Bleomycin-dependent DNA damage

The bleomycins are a family of glycopeptide antibiotics that are used routinely as antitumor agents. The bleomycin assay has been adopted for assessing the pro-oxidant effects of food antioxidants. The antitumor antibiotic bleomycin binds iron ions and DNA. The bleomycin–iron complex degrades DNA that, upon heating with thiobarbituric acid (TBA), yields a pink chromogen. Upon the addition of suitable reducing agents antioxidants compete with DNA and diminish chromogen formation [42].

To show the mechanism of action of our two potent compounds **2** and **7a**, their protective activity against DNA damage induced by the bleomycin–iron complex were examined. The results in Table 2 show that compounds **2** and **7a** have high protection against DNA damage induced by the bleomycin–iron complex, thus diminishing chromogen formation between the damaged DNA and TBA.

3.3. Structure activity relationship

By comparing the experimental cytotoxicity of the compounds reported in this study to their structures, the following structure activity relationships (SAR) were postulated.

- The presence of a basic skeleton 1,3,4-oxadiazole is necessary for the broad spectrum of cytotoxic activity towards different cell lines (HepG2, WI 38, VERO and MCF-7).
- Introducing a thiazolidin-5-one ring in position 2 to 1,3,4-oxadiazole reduced the activity towards HepG2 and diminished the activity towards WI38.
- Cyclization of thioamide **9** to 2,3-dihydrothiazole derivative **7a** enhances the antitumor activity against all cell lines.
- Conversion of ketene *N*,*S*-acetal **10** to 3-aminopyrazole derivative **11a** increases the cytotoxic activity.
- Transformation of 1,3,4-oxadiazole ring system to pyrazolo [5,1-*c*][1,2,4]triazole reduces the antitumor activity.
- Introducing of naphtho[2,1-*b*][1,4]oxazine in position 2 to 1,3,4-oxadiazole diminishes the activity against all cell lines.

4. Conclusion

The objective of the present study was to synthesize and investigate the anticancer activity of some novel 1,3,4-oxadiazoles with the hope of discovering new structure leads serving as anticancer agents. The results of the anticancer screening revealed that five compounds were found to exhibit variable degrees of anticancer activities against the four used cell lines. Compounds **2** and **7a** showed a considerable broad spectrum of anticancer activity against the four tested human tumor cell lines. In particular compound **7a** proved to the most active member in this study with special effectiveness against the human HepG2, VERO, and WI-38. Compound **2** was found to possess high activity against HepG2 and moderate activity against MCF-7. In addition, compounds **2** and **7a** showed high protection against DNA damage induced by the

Table 2
Results of the bleomycin-dependent DNA damage assay of compounds **2** and **7a**.

Sample	Absorbance[a]
2	0.006 ± 0.01
7a	0.004 ± 0.24
Ascorbic acid	0.0038 ± 0.32

[a] All experiments were performed three times. The data are expressed as the mean–standard error of the mean (S.E.M.).

bleomycin–iron complex, thus diminishing chromogen formation between the damaged DNA and TBA.

5. Experimental

All melting points were measured on a Gallenkamp electrothermal melting point apparatus. IR spectra were recorded for KBr disc on a Mattson 5000 FTIR spectrophotometer. NMR spectra were measured in $CDCl_3$ or DMSO-d_6 as solvent at 300 MHz (^{1}H NMR) and at 75 MHz (^{13}C NMR) on a Varian MercuryVX300 NMR spectrometer using TMS as an internal standard and chemical shifts are expressed as δ_{ppm}. Mass spectra were determined on GCMS-QP1000 EX spectrometer and JEOL JMS600 spectrometer at 70 eV. Elemental analyses were carried out in the Microanalytical Unit of the Faculty of Science, CairoUniversity. 4-Benzoyl-1-cyanoacetylthiosemicarbazide (**1**) was prepared according to literature procedure [32].

5.1. Synthesis of N-(5-(cyanomethyl)-1,3,4-oxadiazol-2-yl) benzamide (*2*)

A mixture of 4-benzoyl-1-cyanoacetylthiosemicarbazide **1** (2.62 g, 0.01 mol) and excess yellow mercuric oxide (3.25 g, 0.015 mol) in ethanol (30 mL) was refluxed for 4 h. The reaction mixture was allowed to cool to room temperature (to allow the sedimentation of the black mercuric sulfide), was filtered and the mercuric sulfide was washed with hot ethanol. The filtrate and alcoholic washing were combined, treated with water until a permanent turbidity existed, and allowed to stand overnight. The product was separated, filtered off, dried, and crystallized from ethanol to give compound **2**.

Pale yellow powder; Yield 85%; mp 210–211 °C; IR (KBr) υ_{max}/cm^{-1} = 3320 (NH), 2263 (CN), 1695 (CO), 1631 (C=N). ^{1}H NMR (DMSO-d_6): δ_{ppm} = 4.68 (s, 2H, CH_2CN), 7.55–8.05 (m, 5H, Ar–H), 12.15 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ_{ppm} = 16.1, 115.0, 128.8, 129.9, 132.7, 133.5, 155.9, 159.2, 165.6. MS (EI, 70 ev) *m*/*z* (%): 228 (M^+, 16.5), 227 (25.5), 159 (0.3), 137 (2.2), 122 (8.2), 95 (100), 89 (5.4), 77 (4.9), 76 (33.3), 65 (1.2). Anal. Calcd. for $C_{11}H_8N_4O_2$ (228.21): C, 57.89; H, 3.53; N, 24.55%; Found: C, 57.92; H, 3.57; N, 24.58%.

5.2. Synthesis of N-(5-(cyano(5-oxo-3-phenylthiazolidin-2-yl) methyl)-1,3,4-oxadiazol-2-yl)benzamide (*4*)

A mixture of compound **2** (2.28 g, 0.01 mol), KOH (0.56 g, 0.01 mol), and phenyl isothiocyanate (1.35 g, 0.01 mol) was stirred in DMF (20 ml) at 0–5 °C for 10 h. Simultaneously chloroacetyl chloride (1.13 g, 0.01 mol) was added dropwise, and stirring was continued for 4 h. The reaction mixture was poured onto crushed ice. The formed precipitate was filtered off, dried, and recrystallized from ethanol to afford compound **4**.

Pale yellow crystals; yield 86%; m.p. 140–141 °C. IR (KBr): ν/cm^{-1} = 3448 (NH), 2221 (CN), 1736 (thiazolidin-5-one), 1666 (CO), 1631 (C=C), 1596 (C=N). ^{1}H NMR (DMSO-d_6): δ_{ppm} = 4.24 (s, 2H, SCH_2), 7.24–8.01 (m, 10H, Ar–H), 12.01 (s, 1H, NH). MS (EI, 70 ev) *m*/*z* (%) = 403 (M^+, 7.0), 340 (10.1), 297 (8.8), 226 (5.3), 193 (14.0), 149 (97.4), 105 (100), 77 (63.6). Anal. Calcd. for $C_{20}H_{13}N_5O_3S$ (403.41): C, 59.55; H, 3.25; N, 17.36%; Found: C, 59.53; H, 3.21; N, 17.32%.

5.3. Reaction of thiazolidin-5-one derivative 4 with aromatic aldehydes

To a solution of compound **4** (0.41 g, 0.001 mol) and anhydrous sodium acetate (0.12 g, 0.0015 mol) in glacial acetic acid (10 mL), was added an appropriate aromatic aldehydes (0.001 mol). The reaction mixture was heated for 6 h, where upon the solid product partially crystallized out. The reaction mixture was left to cool and the separated solid product was filtered off, washed with water, dried, and recrystallized from ethanol to give compounds **5a**,**b**.

5.3.1. N-(5-(Cyano-4–-((1,3-diphenyl-1H-pyrazol-4-yl)methylene-5-oxo-3-phenylthiazolidin-2-ylidene)methyl)-1,3,4-oxadiazol-2-yl) benzamide (*5a*)

Yellow powder; yield 69%; m.p. 120–121 °C. IR (KBr): ν/cm^{-1} = 3259 (NH), 2195 (CN), 1700 (thiazolidin-5-one), 1671 (CO), 1602 (C=C), 1550 (C=N). ^{1}H NMR (DMSO-d_6): δ_{ppm} = 7.45–8.02 (m, 20H, Ar–H), 9.32 (s, 1H, pyrazole-H-5), 9.99 (s, 1H, CH=), 11.95 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ_{ppm} = 90.2, 100.8, 115.6, 119.8, 122.7, 128.3, 128.8, 129.1, 129.2, 129.7, 130.3, 131.8, 135.4, 139.2, 147.9, 150.1, 153.2, 164.8, 171.2, 185.2. MS (EI, 70 ev) *m*/*z* (%) = 633 (M^+, 6.8), 327 (2.3), 269 (2.5), 247 (100), 246 (70.2), 244 (17.8), 230 (3.2), 219 (7.9), 218 (21.9), 190 (3.9), 170 (7.4), 120 (8.6), 97 (13.4), 82 (9.2), 73 (77.9). Anal. Calcd. for $C_{36}H_{23}N_7O_3S$ (633.68): C, 68.23; H, 3.66; N, 15.47%; Found: C, 68.18; H, 3.64; N, 15.41%.

5.3.2. N-(5-(Cyano(-4–-((1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)methylene)-5-oxo-3-phenylthiazolidin-2-ylidene)methyl)-1,3,4-oxadiazol-2-yl)benzamide (*5b*)

Orange powder; yield 62%; m.p. 184–185 °C. IR (KBr): ν/cm^{-1} = 3403 (NH), 2208 (CN), 1697 (thiazolidin-5-one), 1674 (CO), 1590 (C=C). ^{1}H NMR (DMSO-d_6): δ_{ppm} = 2.36 (s, 3H, CH_3), 3.32 (s, 3H, CH_3), 7.25–7.46 (m, 15 H, Ar–H), 9.89 (s, 1H, CH=), 12.02 (s, 1H, NH). MS (EI, 70 ev) *m*/*z* (%) = 601 (M^+, 10), 510 (0.1), 451 (0.2), 401 (0.9), 339.8 (2.6), 297.7 (0.6), 268 (34.4), 256 (2.6), 236 (19.3), 212 (1.5), 194 (6.2), 188 (11.4), 150 (4.6), 135 (19.6), 105 (7.5), 102.8 (27.7), 95 (100.0), 88 (37.6), 76 (75.4), 69 (11.0), 60 (6.6). Anal. Calcd. for $C_{32}H_{23}N_7O_4S$ (601.63): C, 63.88; H, 3.85; N, 16.30%; Found: C, 63.93; H, 3.89; N, 16.37%.

5.4. Synthesis of 2,3-dihydrothiazoline derivates *7a*,*b*

To a stirred solution of potassium hydroxide (60 mg, 1 mmol) in dimethylformamide (15 mL), compound **2** (0.23 g, 1 mmol) was added. After stirring for 30 min, phenyl isothiocyanate (0.135 g, 1 mmol) was added to the resulting mixture. The stirring was continued for a further 6 h, then the appropriate α-chloroketones namely chloroacetone or phenacyl chloride (1 mmol) was added and the reaction mixture was further stirred overnight then diluted with cold water (5 mL). The solid product was filtered off, washed with water, dried, and finally recrystallized from DMF/EtOH to afford the corresponding 2,3-dihydrothiazoline derivatives **7a**,**b**.

5.4.1. N-(5-(Cyano(4-methyl-3-phenylthiazol-2(3H)-ylidene) methyl)-1,3,4-oxadiazol-2-yl)benzamide (*7a*)

Yellow powder; yield 81%; m.p. 175–176 °C. IR (KBr): ν/cm^{-1} = 3418 (NH), 2199 (CN), 1685 (CO), 1658 (C=C), 1600 (C=N). ^{1}H NMR ($CDCl_3$): δ_{ppm} = 1.95 (s, 3H, CH_3), 5.15 (s, 1H, CH, thiazole), 7.27–8.11 (m, 10H, Ar–H), 10.02 (s, 1H, NH). MS (EI, 70 ev) *m*/*z* (%) = 401 (M^+, 7.9), 376 (6.1), 346 (2.9), 334 (11.4), 308 (0.6), 287 (7.1), 264 (2.1), 256 (15.0), 239.5 (5.3), 229.6 (40.9), 214 (11.0), 183 (5.9), 152 (3.8), 135 (12.0), 105 (3.0), 95 (100.0), 76 (51.0), 73 (15.3), 65 (2.5). Anal. Calcd. for $C_{21}H_{15}N_5O_2S$ (401.44): C, 62.83; H, 3.77; N, 17.45%; Found: C, 62.91; H, 3.73; N, 17.42%.

5.4.2. N-(5-(Cyano(3,4-diphenylthiazol-2(3H)-ylidene)methyl)-1,3,4-oxadiazol-2-yl)benzamide (*7b*)

Orange powder; yield 85%; m.p. 224–225 °C. IR (KBr): ν/cm^{-1} = 3405 (NH), 2198 (CN), 1689 (CO), 1645 (C=C), 1600 (C=N). ^{1}H NMR ($CDCl_3$): δ_{ppm} = 5.16 (s, 1H, CH, thiazole), 7.19–8.13 (m, 15H, Ar–H), 10.26 (s, 1H, NH). MS (EI, 70 ev) *m*/*z* (%) = 463 (M^+, 17.0), 376

(6.1), 346 (2.9), 334 (11.4), 308 (0.6), 287 (7.1), 264 (2.1), 256 (15.0), 239.5 (5.3), 229.6 (40.9), 214 (11.0), 183 (5.9), 152 (3.8), 135 (12.0), 105 (3.0), 95 (100.0), 76 (51.0), 73 (15.3), 65 (2.5). Anal. Calcd. for $C_{26}H_{17}N_5O_2S$ (463.51): C, 67.37; H, 3.70; N, 15.11%; Found: C, 67.41; H, 3.71; N, 15.22%.

5.5. Synthesis of the thioacetanilide derivative **9**

To a stirred solution of potassium hydroxide (0.11 g, 2 mmol) in dimethylformamide (20 mL), compound **2** (0.46 g, 2 mmol) was added. After stirring for 30 min, phenyl isothiocyanate (0.27 g, 2 mmol) was added to the resulting mixture. Stirring was continued for 6 h, then poured over crushed ice containing hydrochloric acid. The solid product that formed was filtered off, washed with water, dried, and finally recrystallized from ethanol to afford compound **9**.

Yellow crystals; yield 83%; m.p. 160–161 °C. IR (KBr): ν/cm^{-1} = 3477 (NH), 3334 (NH–Ph), 2196 (CN), 1700 (CO), 1621 (C=C), 1590 (C=N). ^{1}H NMR (DMSO-d_6): δ_{ppm} = 7.09–8.02 (m, 10H, Ar–H), 9.98 (s, 1H, NHPh), 12.17 (s, 1H, NHCO), 14.12 (s, 1H, SH). MS (EI, 70 ev) m/z (%) = 363 (M^+, 10.0), 327 (1.0), 299 (1.5), 256 (10.0), 239.8 (4.4), 216 (6.0), 186 (12.8), 167 (4.3), 147 (21.6), 135 (16.0), 122 (6.3), 103 (44.4), 95 (100), 88 (13.8), 76 (72.5), 63 (5.9), 57 (29.0), 55 (16.6). Anal. Calcd. for $C_{18}H_{13}N_5O_2S$ (363.39): C, 59.49; H, 3.61; N, 19.27%; Found: C, 59.46; H, 3.59; N, 19.19%.

5.6. Reaction of thioacetanilide derivative **9** with α-haloketones

To a solution of **9** (0.73 g, 2 mmol) in ethanol (20 mL), the appropriate α-chloroketone namely; chloroacetone or phenacyl chloride (2 mmol) was added followed by few drops of triethylamine. The mixture was refluxed for 2 h, then allowed to cool to room temperature. The formed solid was filtered off, washed with ethanol, and recrystallized from EtOH/DMF to afford the corresponding thiophene derivatives **8a**,**b**.

5.6.1. N-(5-(5-acetyl-4-amino-2-(phenylamino)thiophen-3-yl) 1,3,4-oxadiazol-2-yl)benzamide (**8a**)

Yellow powder; yield 67%; m.p. 138–139 °C. IR (KBr): ν/cm^{-1} = 3417–3334 (NH_2), 3207, 3185 (2NH), 1697 (CO), 1658 (CONh), 1580 (C=C). ^{1}H NMR ($CDCl_3$) δ_{ppm} = 2.25 (s, 3H, CH_3), 6.47 (s, 2H, NH_2), 6.99–7.56 (m, 10H, Ar–H), 9.81 (bs, 1H, NH), 11.96 (s, 1H, NH amidic). MS (EI, 70 ev) m/z (%) = 419 (M^+, 1.7), 344 (1.5), 285 (1.7), 253 (100), 225 (2.2), 208 (4.3), 181 (12.6), 117 (8.3), 105 (6.5), 99 (14.2), 91 (11.9), 84 (18.9), 73 (33.0), 69 (15.5). Anal. Calcd. for $C_{21}H_{17}N_5O_3S$ (419.46): C, 60.13; H, 4.09; N, 16.70%; Found: C, 60.18; H, 4.12; N, 16.67%.

5.6.2. N-(5-(4-amino-5-benzoyl-2-(phenylamino)thiophen-3-yl) 1,3,4-oxadiazol-2-yl)benzamide (**8b**)

Pale yellow powder; yield 74%; m.p. 270–271 °C. IR (KBr): ν/cm^{-1} = 3463–3411 (NH_2), 3353, 3278 (2NH), 1706 (COPh), 1660 (CONH), 1600 (C=C). ^{1}H NMR (DMSO-d_6) δ_{ppm} = 7.15 (bs, 2H, NH_2), 7.42–8.05 (m, 15H, Ar–H), 9.68 (s, 1H, NH), 12.15 (s, 1H, NH, amidic). ^{13}C NMR (DMSO-d_6): δ_{ppm} = 93.6, 121.8, 125.9, 127.5, 128.8, 129.0, 129.2, 130.3, 131.0, 132.8, 133.5, 140.3, 141.7, 155.2, 156.3, 157.2, 159.2, 165.2, 185.3. MS (EI, 70 ev) m/z (%) = 481 (M^+, 4.0), 377 (1.7), 335 (1.5), 319 (4.3), 215 (0.9), 160 (1.4), 105 (100), 77 (95.9). Anal. Calcd. for $C_{26}H_{19}N_5O_3S$ (481.53): C, 64.85; H, 3.98; N, 14.54%; Found: C, 64.92; H, 4.03; N, 14.51%.

5.7. Synthesis of (N-(5-1-cyano-2-(methylthio)-2-(phenylamino) vinyl)-1,3,4-oxadiazol-2-yl)benzamide (**10**)

To a stirred solution of potassium hydroxide (60 mg, 1 mmol) in dimethylformamide (15 mL), compound **2** (0.23 g, 1 mmol) was added. After stirring for 30 min, phenyl isothiocyanate (0.135 g, 1 mmol) was added to the resulting mixture. The stirring was continued for a further 6 h, then methyl iodide (0.141 g, 1 mmol) was added and the reaction mixture was further stirred 4 h then diluted with cold water (5 mL). The solid product was filtered off, washed with water, dried, and finally recrystallized from ethanol to afford compound **10**.

Yellow crystals; yield 90%; m.p. 200–201 °C. IR (KBr): ν/cm^{-1} = 3430 (NH), 3218 (NH–Ph), 2206 (CN), 1704 (CO), 1630 (C=C), 1580 (C=N). ^{1}H NMR (DMSO-d_6): δ_{ppm} = 2.31 (s, 3H, SCH_3), 7.23–7.99 (m, 10H, Ar–H), 10.46 (s, 1H, NH), 11.94 (s, 1H, NH, amidic). MS (EI, 70 ev) m/z (%) = 377 (M^+, 56.7), 377 (3.5), 364 (30.0), 340 (48.4), 326 (18.0), 305 (8.0), 283 (25.0), 265 (4.9), 256 (10.5), 242 (31.6), 231 (16.0), 227 (25.8), 197 (5.0), 195 (30.0), 170 (14.9), 160 (3.6), 153 (30.5), 126 (8.6), 99 (100.0), 92 (3.9), 85 (17.9), 73 (38.0), 63 (2.5). Anal. Calcd. for $C_{19}H_{15}N_5O_2S$ (377.42): C, 60.46; H, 4.01; N, 18.56%; Found: C, 60.41; H, 4.07; N, 18.53%.

5.8. Reaction of N-(5-(1-cyano-2-(methylthio)-2-(phenylamino) vinyl)-1,3,4-oxadiazol-2-yl)benzamide (**10**) with hydrazines

A mixture of an equimolar amounts of **10** (0.38 g, 0.001 mol) and hydrazine derivatives namely; hydrazine hydrate (0.05 g, 0.001 mol) or phenyl hydrazine (0.11 g, 0.001 mol) in ethanol (20 mL) was refluxed for 4 h. The reaction mixture was left to cool then poured onto ice-cold water (10 mL), filtered off, washed with water, dried, and recrystallized from ethanol to afford compounds **11a** and **11b**, respectively.

5.8.1. N-(5-(3-amino-5-(phenylamino)-1H-pyrazol-4-yl)-1,3,4-oxadiazol-2-yl)benzamide (**11a**)

Colorless powder; yield 76%; m.p. 184–185 °C. IR (KBr): ν/cm^{-1} = 3448–3375 (NH_2), 3343, 3220 (2NH), 1675 (CO), 1600 (C=N). ^{1}H NMR (DMSO-d_6): δ_{ppm} = 6.93 (bs, 2H, NH_2), 7.32–7.83 (m, 10H, Ar–H), 8.96 (s, 1H, NH–pyrazole), 10.32 (s, 1H, NH), 11.53 (s, 1H, NH, amidic). ^{13}C NMR (DMSO-d_6): δ_{ppm} = 88.9, 115.9, 116.3, 118.9, 121.9, 125.9, 129.2, 130.6, 142.8, 144.4, 150.5, 151.2, 166.2, 170.6. MS (EI, 70 ev) m/z (%) = 361 (M^+, 10.5), 303 (1.68), 258 (2.13), 243 (45.7), 174 (100.0), 160 (80.7), 104 (82.4), 77 (74.9). Anal. Calcd. for $C_{18}H_{15}N_7O_2$ (361.36): C, 59.83; H, 4.18; N, 27.13%; Found: C, 59.86; H, 4.22; N, 27.18%.

5.8.2. N-(5-(3-amino-1-phenyl-5-(phenylamino)-1H-pyrazol-4-yl)-1,3,4-oxadiazol-2-yl)benzamide (**11b**)

Colorless powder; yield 69%; m.p. 287–288 °C. IR (KBr): ν/cm^{-1} = 3444–3385 (NH_2), 3203 (NH), 1704 (CO), 1600 (C=N). ^{1}H NMR ($CDCl_3$): δ_{ppm} = 6.25 (bs, 2H, NH_2), 7.27–7.45 (m, 15H, Ar–H), 9.07 (s, 1H, NH). MS (EI, 70 ev) m/z (%) = 437 (M^+, 4.2), 347 (1.6), 333 (34.7), 319 (5.9), 294 (0.7), 286 (11.7), 263 (5.4), 255 (39.0), 229 (39.1), 224 (10.4), 221 (2.3), 197 (9.9), 188 (15.6), 169 (7.6), 129 (1.5), 103 (80.0), 100 (20.5), 95 (100.0), 87 (4.7), 76 (66.2), 65 (4.4). Anal. Calcd. for $C_{24}H_{19}N_7O_2$ (437.45): C, 65.89; H, 4.38; N, 22.41%; Found: C, 65.92; H, 4.42; N, 22.49%.

5.9. Reaction of compound **2** with aromatic aldehydes

A few drops of triethylamine were added to an ethanolic solution (25 mL) of compound **2** (0.23 g, 1 mmol) and each of *p*-anisaldehyde (0.14 g, 1 mmol) and 1,3-diphenyl-1*H*-pyrazole-4-carbaldehyde (0.25 g, 1 mmol), and the reaction mixture was refluxed for 6 h. The solvent was evaporated under reduced pressure, and the residue was treated with ethanol. The solid product was filtered off, washed with ethanol, dried, and purified by recrystallization from ethanol to afford compounds **12a**,**b**.

*5.9.1. N-(5-(1-cyano-2-(4-methoxyphenyl)vinyl)-1,3,4-oxadiazol-2-yl)benzamide (**12a**)*

Yellow crystals; yield 74%; m.p. 270–271 °C. IR (KBr): ν/cm^{-1} = 3405 (NH), 2206 (CN), 1695 (CO), 1622 (C=C), 1582 (C=N). ^{1}H NMR ($CDCl_3$) δ_{ppm} = 3.92 (s, 3H, OCH_3), 7.29–7.82 (m, 5H, Ar–H), 8.23 (s, 1H, CH=), 8.42 (d, J = 6.8 Hz, 2H, Ar–H), 8.53 (d, J = 6.8 Hz, 2H, Ar–H), 11.13 (s, 1H, exchangeable with D_2O, NH, amidic). MS (EI, 70 ev) m/z (%) = 346 (M^+, 2.7), 345 ($M^+ - 1$, 22.0), 341 (10.9), 340 (4.4), 310 (2.6), 309 (10.9), 283 (1.0), 269 (4.3), 231 (2.4), 228 (18.2), 203 (15.2), 198 (1.2), 186 (22.9), 170 (3.4), 146 (2.6), 122 (2.3), 103 (8.0), 95 (100.0), 82 (5.9), 76 (36.9), 64 (2.3). Anal. Calcd. for $C_{19}H_{14}N_4O_3$ (346.34): C, 65.89; H, 4.07; N, 16.18%; Found: C, 65.82; H, 4.04; N, 16.18%.

*5.9.2. N-(5-(1-cyano-2-(1,3-diphenyl-1H-pyrazol-4-yl)vinyl)-1,3,4-oxadiazol-2-yl)benzamide (**12b**)*

Yellow powder; yield 81%; m.p. 307–308 °C. IR (KBr): ν/cm^{-1} = 3422 (NH), 2205 (CN), 1697 (CO), 1626 (C=C), 1589 (C=N). ^{1}H NMR (DMSO-d_6) δ_{ppm} = 7.29–8.02 (m, 15H, Ar–H), 8.41 (s, 1H, CH=), 9.25 (s, 1H, pyrazole–H_5), 12.31 (s, br., 1H, NH). MS (EI, 70 ev) m/z (%) = 458 (M^+, 5.5), 425 (1.3), 382 (1.6), 353 (1.0), 340 (12.2), 295 (1.8), 268 (1.1), 248 (8.4), 227 (24.4), 188 (1.5), 158 (1.5), 122 (1.8), 115 (5.5), 95 (100.0), 88 (6.8), 76 (36.5), 65 (1.9). Anal. Calcd. for $C_{27}H_{18}N_6O_2$ (458.47): C, 70.73; H, 3.96; N, 18.33%; Found: C, 70.78; H, 4.02; N, 18.39%.

*5.10. Synthesis of pyrazolyl–oxadiazoles **13a**,**b***

Hydrazine hydrate (80%, 0.1 mL, 1 mmol) was added to an equimolar amounts of solution of arylidene derivatives **12a**–**b** (1 mmol) in 20 mL of ethanol. The reaction mixture was heated under reflux for 4 h, then left to cool. The solid product obtained was filtered off, washed with ethanol, dried, and recrystallized from ethanol to afford compounds **13a**,**b**.

*5.10.1. N-(5-(3-amino-5-(4-methoxyphenyl)-1H-pyrazol-4-yl)-1,3,4-oxadiazol-2-yl)benzamide (**13a**)*

Colorless powder; yield 76%; m.p. 212–213 °C. IR (KBr): ν/cm^{-1} = 3407 (NH), 3276–3196 (NH_2), 3141 (NH), 1686 (CO), 1598 (C=N). ^{1}H NMR (DMSO-d_6) δ_{ppm} = 3.91 (s, 3H, OCH_3), 6.65 (s, 2H, NH_2), 7.26–8.17 (m, 9H, Ar–H), 8.47 (s, 1H, pyrazole–NH), 11.12 (s, 1H, amidic NH). Anal. Calcd. for $C_{19}H_{16}N_6O_3$ (376.37): C, 60.63; H, 4.28; N, 22.33%; Found: C, 60.58; H, 4.18; N, 22.28%.

*5.10.2. N-(5-(5-amino-1′,3′-diphenyl-1′H,2H-3,4′-bipyrazol-4-yl)-1,3,4-oxadiazol-2-yl)benzamide (**13b**)*

Colorless powder; yield 71%; m.p. 220–221 °C. IR (KBr): ν/cm^{-1} = 3423–3395 (NH_2), 3345 (NH), 3141 (NH), 1674 (CO), 1598 (C=N). ^{1}H NMR (DMSO-d_6) δ_{ppm} = 6.15 (s, 2H, NH_2), 7.28–8.35 (m, 15H, Ar–H), 8.68 (s, 1H, pyrazole–H_5), 10.89 (s, 1H, pyrazole–NH), 11.19 (s, 1H, amidic NH). MS (EI, 70 ev) m/z (%) = 488 (M^+, 44.2), 388 (5.6), 360 (2.1), 322 (5.8), 246 (82.5), 216 (4.7), 116 (18.6), 77 (100.0). Anal. Calcd. for $C_{27}H_{20}N_8O_2$ (488.5): C, 66.38; H, 4.13; N, 22.94%; Found: C, 66.32; H, 4.08; N, 22.87%.

*5.11. Synthesis of N-(6-amino-7H-pyrazolo[5,1-c][1,2,4]triazol-3-yl)benzamide (**15**)*

A mixture of compound **2** (0.23 g, 1 mmol) and hydrazine hydrate (80%, 0.2 mL, 2 mmol) was heated in an oil bath at 160 °C for 1 h, then left to cool. The obtained solid product was triturated with ethanol (10 mL), filtered off, washed with ethanol, dried, and recrystallized from DMF to afford compound **15**.

Colorless powder; yield 65%; m.p. 263–264 °C. IR (KBr): ν/cm^{-1} = 3386–3304 (NH_2), 3165 (NH), 2986 (CH, aliphatic), 1675 (CO), 1615 (C=N). ^{1}H NMR (DMSO-d_6) δ_{ppm} = 2.61 (s, 2H, CH_2), 6.86 (s, 2H, NH_2), 7.21–7.87 (m, 5H, Ar–H), 12.13 (s, 1H, amidic NH). Anal. Calcd. for $C_{11}H_{10}N_6O$ (242.24): C, 54.54; H, 4.16; N, 34.69%; Found: C, 54.47; H, 4.08; N, 34.57%.

*5.12. Synthesis of coumarin **17** and naphthoxazines **19** and **21** derivatives*

To a solution of compound **2** (0.23 g, 1 mmol) in ethanol (25 mL) containing a few drops of piperidine, either salicylaldehyde (0.12 g, 1 mmol), 1-nitroso-2-naphthol (0.173 g, 1 mmol), or 2-nitroso-1-naphthol (0.173 g, 1 mmol) was added. The reaction mixture, in each case, was heated under reflux for 4 h. The solid products formed upon pouring onto an ice-water mixture containing few drops of hydrochloric acid was collected by filtration, dried, and recrystallized from ethanol to afford compounds **16**–**18**.

*5.12.1. N-(5-(2-oxo-2H-chromen-3-yl)-1,3,4-oxadiazol-2-yl) benzamide (**17**)*

Orange Crystals; yield 72%; m.p. 185–186 °C. IR (KBr): ν/cm^{-1} = 3345 (NH), 1735 (CO), 1671 (CO), 1605 (C=C), 1543 (C=N). ^{1}H NMR (DMSO-d_6) δ_{ppm} = 7.13–7.76 (m, 9H, Ar–H), 8.43 (s, 1H, coumarin-H_4), 10.68 (s, 1H, NH, amidic). MS (EI, 70 ev) m/z (%) = 332 ($M^+ - 1$, 3.9), 186 (10.8), 130 (7.7), 105 (38.8), 51 (100). Anal. Calcd. for $C_{18}H_{11}N_3O_4$ (333.3): C, 64.86; H, 3.33; N, 12.61%; Found: C, 64.95; H, 3.35; N, 12.68%.

*5.12.2. N-(5-(3-oxo-3H-naphtho[2,1-b][1,4]oxazin-2-yl)-1,3,4-oxadiazol-2-yl)benzamide (**19**)*

Violet crystals; yield 85%; m.p. 215–216 °C. IR (KBr): ν/cm^{-1} = 3336 (NH), 1730 (CO), 1668 (CO), 1602 (C=C), 1583 (C=N). ^{1}H NMR (DMSO-d_6) δ_{ppm} = 7.27–8.36 (m, 11H, Ar–H), 11.22 (s, 1H, NH, amidic). ^{13}C NMR (DMSO-d_6): δ_{ppm} = 117.5, 118.2, 120.8, 121.6, 123.3, 125.5, 126.2, 127.6, 128.3, 128.7, 129.3, 131.0, 131.2, 133.9, 150.4, 155.0, 158.4, 164.2, 165.4. MS (EI, 70 ev) m/z (%) = 384 (M^+, 20.0), 322 (40.1), 305 (20.3), 277 (20.2), 196 (20.0), 170 (100.0), 154 (40), 133 (40.3), 105 (12.7), 83 (20.1), 69 (40.1). Anal. Calcd. for $C_{21}H_{12}N_4O_4$ (384.34): C, 65.62; H, 3.15; N, 14.58%; Found: C, 65.67; H, 3.21; N, 14.66%.

*5.12.3. N-(5-(2-oxo-2H-naphtho[1,2-b][1,4]oxazin-3-yl)-1,3,4-oxadiazol-2-yl)benzamide (**21**)*

Violet crystals; yield 78%; m.p. 302–303 °C. IR (KBr): ν/cm^{-1} = 3305 (NH), 1729 (CO), 1674 (CO), 1609 (C=C), 1589 (C=N). ^{1}H NMR (DMSO-d_6) δ_{ppm} = 7.14–8.14 (m, 11H, Ar–H), 11.17 (s, 1H, NH, amidic). Anal. Calcd. for $C_{21}H_{12}N_4O_4$ (384.34): C, 65.62; H, 3.15; N, 14.58%; Found: C, 65.70; H, 3.12; N, 14.51%.

6. Cytotoxicity and antitumor evaluation

6.1. Materials and methods

The reagents RPMI-1640 medium (Sigma Co., St. Louis, USA) Fetal Bovine serum (GIBCO, UK) and the cell lines HepG2, WI38, VERO, MCF-7 which obtained from ATCC were used.

6.1.1. Procedure

The stock samples were diluted with RPMI-1640 medium to desired concentrations ranging from 10 to 1000 μg/mL. The final concentration of dimethylsulfoxide (DMSO) in each sample did not exceed 1% v/v. The cytotoxic activity of the compounds was tested against human hepatocellular liver carcinoma cell line (HepG2), human lung fibroblast cell line (WI 38), human caucasian breast adeno-carcinoma cell line (MCF-7), and normal adult African green

monkey kidney cell line (VERO). The % viability of cell was examined visually. 5-fluorouracil was used as a standard anticancer drug for comparison.

Briefly, cell were batch cultured for 10 d, then seeded in 96-well plates of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 °C for 24 h under 5% CO_2 using a water jacketed carbon dioxide incubator (Shedon.TC2323.Cornelius, OR, USA). The medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of sample to give a final concentrations of (1000, 500, 200, 100, 50, 20, 10 μg/mL). Cells were suspended in RPMI-1640 medium, 1% antibiotic-antimycotic mixture (10^4 ug/mL potassium penicillin, 10^4 μg/mL streptomycin sulfate and 25 μg/mL Amphotericin B) and 1% L-glutamin in 96-well flat bottom microplates at 37 °C under 5% CO_2. After 96 h of incubation, the medium was again aspirated, trays were inverted onto a pad of paper towels, the remaining cells rinsed carefully with medium, and fixed with 3.7% (v/v) formaldehyde in saline for at least 20 min. The fixed cells were rinsed with water, and examined. The cytotoxic activity was identified as confluent, relatively unaltered mono-layers of stained cells treated with compounds.

6.1.2. Calculation of the IC_{50} for each compound

Cytotoxicity was estimated as the concentration that caused approximately 50% loss of monolayer. The assay was used to examine the newly synthesized compounds. 5-Fluorouracil was used as a positive control.

To calculate IC_{50}, you would need a series of dose–response data (e.g., drug concentrations x1, x2, …,xn and growth inhibition y1, y2, …,yn). The values of y are in the range of 0–1.

6.1.3. Linear regression

The simplest estimate of IC_{50} is to plot x–y and fit the data with a straight line (linear regression). IC_{50} value is then estimated using the fitted line, *i.e.*,

$$Y = a^*X + b$$

$$IC_{50} = (0.5 - b)/a$$

6.2. Bleomycin-dependent DNA damage assay [42–44]

To the reaction mixtures in a final volume of 1.0 ml, the following reagents at the final concentrations stated were added: DNA (0.2 mg/ml), bleomycin (0.05 mg/ml), $FeCl_3$ (0.025 mM), magnesium chloride (5 mM), KH_2PO_4–KOH buffer pH 7.0 (30 mM), and ascorbic acid (0.24 mM) or the test fractions diluted in MeOH to give a concentration of (0.1 mg/ml). The reaction mixtures were incubated in a water-bath at 37 °C for 1 h. At the end of the incubation period, 0.1 ml of ethylenediaminetetraacetic acid (EDTA) (0.1 M) was added to stop the reaction (the iron–EDTA complex is unreactive in the bleomycin assay). DNA damage was assessed by adding 1 ml 1% (w/v) thiobarbituric acid (TBA) and 1 ml of 25% (v/v) hydrochloric acid (HCl) followed by heating in a water-bath maintained at 80 °C for 15 min. The chromogen formed was extracted into 1-butanol, and the absorbance was measured at 532 nm.

Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.12.013.

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