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Synthesis, molecular docking and evaluation of thiazolyl-pyrazoline derivatives containing benzodioxole as potential anticancer agents

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ABSTRACT

A series of novel thiazolyl-pyrazoline derivatives containing benzodioxole (**C1–C20**) have been designed and synthesized. Among of the synthesized compounds, 2-(5-(benzo[*d*][1,3]dioxol-5-yl)-3-(4-bromophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)-4-(4-bromophenyl)thiazole (**C6**) displayed the most potent inhibitory activity for HER-2 (IC_{50} = 0.18 μM for HER-2). Antiproliferative assay results indicated that compound **C6** owned high antiproliferative activity against MCF-7 and B16-F10 in vitro, with IC_{50} value of 0.09 and 0.12 μM, respectively, being comparable with the positive control Erlotinib. Docking simulation was further performed to determine the probable binding model. Based on the preliminary results, compound **C6** with potent inhibitory activity in tumor growth would be a potential anticancer agent.

1. Introduction

Cancer is a multifactor disease with superfluous and robust biological networks.[1] It may require treatment with compounds that could target multiple intracellular components.[2] Recently, these novel anticancer drugs are those targeting mutant or aberrantly expressed oncogenic growth factor receptor and non-receptor tyrosine kinases involved in mitogenic or proliferative signal transduction pathways in cancer cells.[3]

Receptor tyrosine kinases (RTKs) and epidermal growth factor receptor (EGFR) play very important roles in regulating tumor cell proliferation, differentiation, survival and apoptosis.[4–6] EGFR family comprises four members, including: EGFR (HER-1/ErbB-1), ErbB-2 (HER-2/neu), ErbB-3 (HER-3) and ErbB-4 (HER-4).[7] Compounds that inhibit the kinase activity of EGFR and/or HER-2 after binding to its ATP binding site are of potential interest as new therapeutic antitumor agents.[8] For example, Erlotinib (Fig. 1) inhibits EGFR/HER-2 that is overexpressed in tumors and is approved antitumor agent.

Small and simple hetero-aromatics often have surprisingly complex biological properties and belong to one of the most important classes of compounds in medicinal chemistry.[9] Benzodioxole, thiazoles and pyrazoles are widely found in both natural products and drugs.[10] Benzodioxole has received significant attention in medicinal and pharmaceutical research as this structural scaffold is found in a variety of drugs, and we could suppose the benzo[*d*][1,3]dioxole-5-carbaldehyde and the 2,3-dihydrobenzo[*b*][1,4]dioxine-6-carbaldehyde as 3,4-dimethoxy derivative and 3,4-diethoxy derivative, respectively, which was reported in our previous research.[11]

On the other hand, thiazoles and their derivatives have been frequently discovered as a vital component of novel that recently found application in drug development for treatment of allergies,[12] schizophrenia[13] and more recently for the treatment of cancer.[14] For example, Micheal, D.G. et al. reported that 6-thiazolylquinazoline derivatives (Fig. 1) showed modest to HER-2/EGFR TK inhibitory activity.[15] Meanwhile, pyrazole derivatives have attracted continuing attention over the years because of their broad spectrum biological activities and strong efficacy. Thus, some representatives of this heterocycle exhibit antitumor,[16] anti-hyperglycemic activity[17] and sedative-hypnotic activity.[18] For example, Claudio, N.C. et al. have discovered N1 (Fig. 1) showed modest EGFR/HER-2 inhibitory activity.[19] And as previous reported in our laboratory, N2 (Fig. 1) displayed the most potent EGFR TK inhibitory activity (IC_{50} = 0.06 μM for EGFR).[20]

In addition, benzodioxole is found in a variety of anticancer drugs with excellent bioavailability and low cytotoxicity,[21,22] which will enhance the anticancer activity of thiazole and pyrazole motif, thus might exhibit synergistic anticancer effect and the modification in this paper also creates new possibility to reinforce the combination between our compounds and the receptor.[23] In an effort to extend our research on anticancer compounds with HER-2

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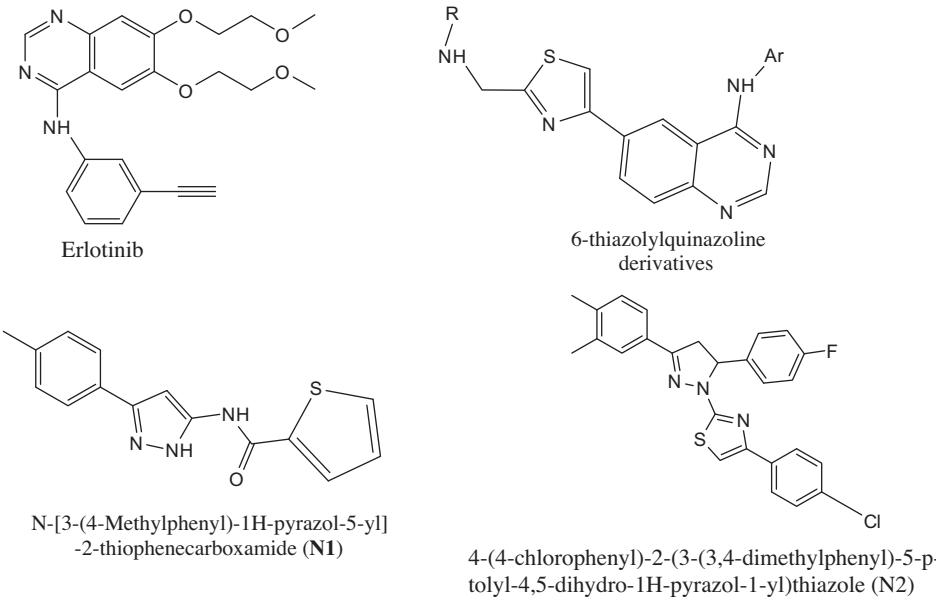


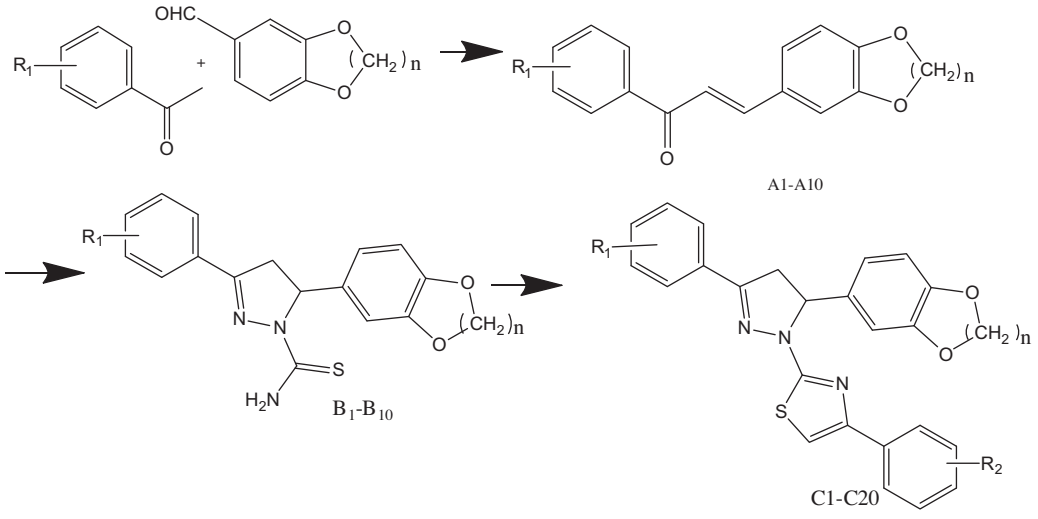
Figure 1. Chemical structures of some reported compounds.

inhibitory activity, we synthesized a series of compounds building of benzodioxole combined with thiazolyl-pyrazoline derivatives as potential anticancer agents. But as we know, as a member of the EGFR family, although in recent years it has evolved to become an important biomarker and target of therapy for the disease, there is a little literature reported about HER-2[24–26] and we want to investigate the effect of HER-2, so we choose HER-2 to test the inhibitory activity and do docking simulation. And the HER-2 is the homologous protein of human EGFR, it can heterodimerise with any of the other three receptors and is considered to be the preferred dimerisation partner of the other ErbB receptors, and the activity of HER-2 is often stronger than the other heterodimer. And the drugs we study eventually will be acting on the human body, so it will has more practical significance of the study on HER-2. According to the activity data and biological evaluation result, the compounds have potent HER-2 inhibitory activity, which eventually would be proved to be potential anticancer agents.

2. Results and discussion

2.1. Chemistry

Synthesis of compounds **C1–C20** is followed the general pathway outlined in Scheme 1. Firstly, the benzodioxole compounds were obtained by protocatechuic aldehydes reacting with dibromomethane or 1,2-dibromoethane in acetonitrile. Secondly, the chalcone derivatives were obtained by direct condensation by the benzodioxole compounds and the substituted acetophenone, using 40% potassium hydroxide as catalyst in ethanol. Thirdly, cyclization of different chalcone derivatives with thiosemicarbazide under basic condition leads to the formation of pyrazole derivatives containing thiourea skeleton. Finally, thiazolyl-pyrazoline derivatives **C1–C20** were obtained by reacting compound **B** with two different kinds of substituted 2-bromoacetophenone. All of these compounds are reported for the first time. All of the synthesized



Scheme 1. General synthesis of thiazolyl-pyrazoline derivatives containing benzodioxole (**C1–C20**). Reagents and conditions: (i) 40% aqueous potassium hydroxide solution, ethanol, 4 h rt; (ii) thiosemicarbazide, KOH, ethanol, reflux, 12 h; (iii) substituted 2-bromoacetophenone, ethanol, reflux, 8 h.

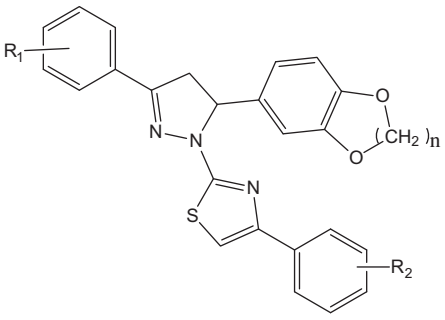
compounds gave satisfactory analytical and spectroscopic data, which were in full consistent with their depicted structures.

2.2. Anticancer activity and structure–activity relationship of the thiazolyl-pyrazoline derivatives containing benzodioxole C1–C20

To test the anticancer activities of the synthesized compounds, we evaluated antiproliferative activities of compounds **C1–C20** against MCF-7 (human breast cancer cell lines) and B16-F10 cells (mouse melanoma cell lines). The results were summarized in Table 2. It was observed that thiazolyl-pyrazoline derivatives which containing benzodioxole showed fairly good inhibiting MCF-7 and B16-F10 activities displaying IC_{50} values between 0.09 and 4.53 μM, 0.12 and 4.43 μM, respectively. Among them, compound **C6** displayed the most potent inhibitory activity (IC_{50} = 0.09 μM for MCF-7, IC_{50} = 0.12 μM for B16-F10). Subsequently SAR studies were performed by modification of the parent compound to determine how the substituents of the subunits affect the HER-2 inhibitory activities. Inspection of the chemical structures of the compounds **C1–C20** suggested that they could be divided into three subunits: A-, B- and C-rings (Scheme 1 and Table 1).

As shown in Table 1, structure–activity relationships in compounds **C1–C20** demonstrated that compounds bearing one bromine atom at *para*- at A-ring (compounds **C5**, **C6**, **C15**, **C16** and the value of IC_{50}, respectively: 0.85, 0.18, 1.32 and 0.46 μM) showed better HER-2 inhibitory activity than those with fluorin (**C1, C2, C11, C12**), chlorine (**C3, C4, C13, C14**), methyl (**C7, C8, C17, C18**) and methoxyl (**C9, C10, C19, C20**) substituents at the same position, basically in the order of Br > Cl > F, Br > Me > OMe. This meant that a weaker electron-withdrawing substituent at *para*-position of A-ring (Br) had better HER-2 inhibitory activity than that with a relatively stronger electron-withdrawing substituents (Cl, F), and that compound with a faintish electron-donating substituents at *para*-position of A-ring (Me) had better HER-2 inhibitory activity than that with a relatively stronger electron-donating substituents (OMe).

Table 1
Chemical structures of thiazolyl-pyrazoline derivatives containing benzodioxole



Compounds	R1	*n*	R2	Compounds	R1	*n*	R2
C1	F	1	OMe	**C11**	F	2	OMe
C2	F	1	Br	**C12**	F	2	Br
C3	Cl	1	OMe	**C13**	Cl	2	OMe
C4	Cl	1	Br	**C14**	Cl	2	Br
C5	Br	1	OMe	**C15**	Br	2	OMe
C6	Br	1	Br	**C16**	Br	2	Br
C7	Me	1	OMe	**C17**	Me	2	OMe
C8	Me	1	Br	**C18**	Me	2	Br
C9	OMe	1	OMe	**C19**	OMe	2	OMe
C10	OMe	1	Br	C20	OMe	2	Br

Table 2
Antiproliferative activities of compounds **C1–C20** against MCF-7 and B16-F10 (IC_{50}, μM)

Compounds	MCF-7	B16-F10	Compounds	MCF-7	B16-F10
C1	3.65 ± 0.36	3.98 ± 0.41	**C11**	4.53 ± 0.46	4.43 ± 0.48
C2	3.43 ± 0.38	3.81 ± 0.35	**C12**	4.20 ± 0.41	4.11 ± 0.43
C3	2.41 ± 0.25	2.32 ± 0.25	**C13**	3.03 ± 0.32	3.12 ± 0.33
C4	1.72 ± 0.18	1.51 ± 0.16	**C14**	2.23 ± 0.20	2.15 ± 0.22
C5	0.27 ± 0.02	0.32 ± 0.04	**C15**	0.87 ± 0.07	1.12 ± 0.14
C6	0.09 ± 0.008	0.12 ± 0.01	**C16**	0.32 ± 0.05	0.43 ± 0.06
C7	1.43 ± 0.14	1.52 ± 0.14	**C17**	2.68 ± 0.24	2.87 ± 0.26
C8	1.41 ± 0.12	1.39 ± 0.12	**C18**	1.59 ± 0.14	1.68 ± 0.18
C9	2.73 ± 0.27	2.67 ± 0.28	**C19**	2.73 ± 0.30	3.01 ± 0.28
C10	2.47 ± 0.25	2.54 ± 0.24	**C20**	2.54 ± 0.24	2.52 ± 0.26
Erlotinib	0.02 ± 0.004	0.05 ± 0.008	Erlotinib	0.02 ± 0.004	0.05 ± 0.008

In the case of constant A ring substituents, changes of substituents on B ring can also affect the activities of these compounds. Compared to the six-membered ring compounds (the value of IC_{50} range from 0.32 to 4.53 μM, 0.43 to 4.43 μM for MCF-7 and B16-F10, respectively), the five-membered ring compounds showed more inhibitory activity (the value of IC_{50} range from 0.09 to 3.65 μM, 0.12 to 3.98 μM for MCF-7 and B16-F10, respectively). We can arrive at the conclusion that five-membered ring structure is more similar to the receptor (the five-membered ring is smaller in volume and is more hydrophobic, which would be easier to insert into receptor's hydrophobic pocket), so that they showed better affinity properties than the six-membered ring compounds.

At the same time, changes of substituents on C ring can also affect the activities of these compounds. According to the results of activities data, we know that electron-withdrawing substituents at 4-position derivatives had more potent HER-2 inhibitory activities than the electron-donating substituents ones.

The above results indicated that benzodioxole, thiazole and pyrazole rings in the thiazolyl-pyrazoline derivatives containing benzodioxole might play an important role in the process of apoptosis. And the changes of substituents on the A-, B-, C-rings had effects on the activity of the compound, which might be explained that substituents on the three different rings had certain influence on the molecular bonding between the receptor and the compounds. Molecular docking of all the compounds of this series had demonstrated the point and this was conformed to our estimate.

2.3. HER-2 inhibitory activity and molecular docking study

The series of thiazolyl-pyrazoline derivatives containing benzodioxole (**C1–C20**) were evaluated for their ability to inhibit the autophosphorylation of HER-2 kinases using a solid-phase ELISA assay. The inhibition constant (IC_{50}) of the compounds were summarized in Table 3. As was shown in Table 3, out of the top six compounds, compounds **C6** and **C16**, showed potent inhibitory activity of HER-2 (IC_{50} = 0.18 and 0.46 μM, respectively), indicating

Table 3
Inhibition activities of compounds **C4**, **C5**, **C6**, **C8**, **C15**, **C16** (IC_{50}, μM)

Compounds	HER-2
C4	2.38 ± 0.22
C5	0.85 ± 0.09
C6	0.18 ± 0.02
C8	2.23 ± 0.25
C15	1.32 ± 0.16
C16	0.46 ± 0.06
Erlotinib	0.16 ± 0.007

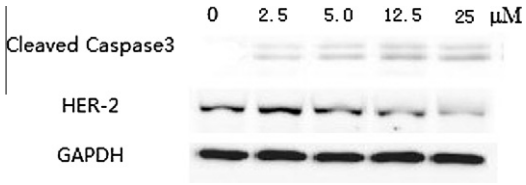


Figure 2. Western blotting was performed to detect the effect of compound **C6** on HER-2.

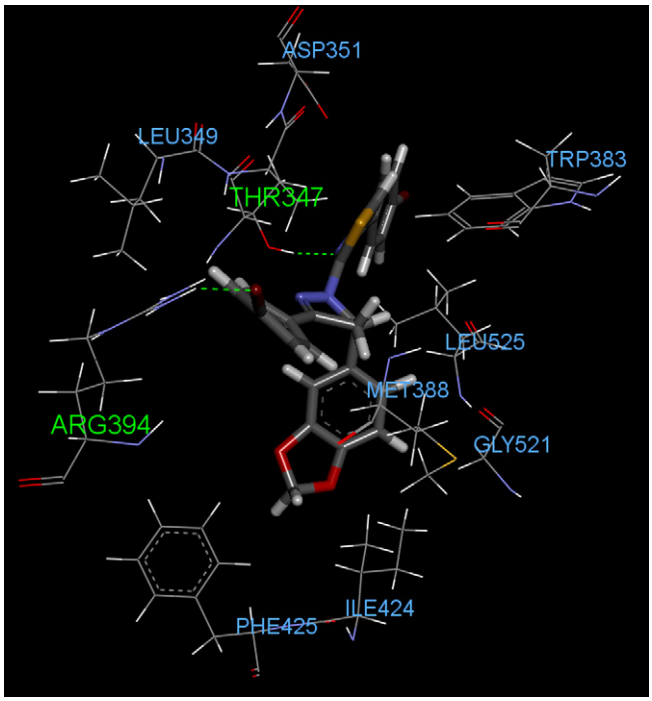


Figure 3A. Molecular docking 3D modeling of compound **C6** with HER-2: for clarity, only interacting residues are displayed. The H-bond (green lines) is displayed as dotted lines.

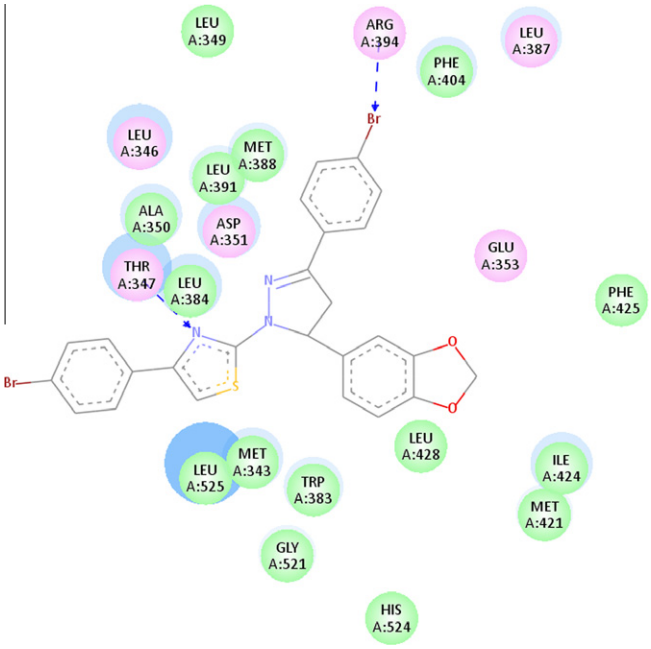


Figure 3B. Molecular docking 2D modeling of **C6** with HER-2: for clarity, only interacting residues are displayed. The H-bond (blue lines) is displayed as arrow dots.

that these thiazolyl-pyrazoline derivatives containing benzodioxole were potent antitumor agents. In particular, compound **C6** had demonstrated significant inhibitory activity in tumor growth inhibition and displayed potential HER-2 inhibitory activity.

To determine whether target compounds suppress activation of HER-2, selected compound **C6** were tested for its suppression on HER-2 and Cleaved Caspase 3 (an important enzyme in apoptosis). As shown in Figure 2, HER-2 was dose-dependent lower regulated upon **C6** treated for 24 h. The activity of Cleaved Caspase 3 was dose-dependent up-regulated upon **C6** treated. It can be conferred from the result that the apoptosis induced by **C6** may be mediated by the inhibition of PI3K/AKT pathway.[27,28]

In order to gain better understanding on the potency of the compound **C6** and guide further SAR studies, we proceeded to examine the interaction of it with HER-2 (PDB code: 1UOM) by molecular docking, which was performed by simulation of the compound into the ATP binding site in HER-2. The binding model of compound **C6** and HER-2 was depicted in Figures 3A and 3B. The amino acid residues which had interaction with HER-2 were labeled. In the binding mode, compound **C6** was nicely bound to the ATP binding site of HER-2 through hydrophobic interaction, and the two H-bonds played an important effect in strengthening the interaction: N···H–O/Thr347 (distance: 2.17612 Å, angle: 162.968°) and Br···H–N/Arg394 (distance: 2.15122 Å, angle: 145.903°). Based on the favorable HER-2 inhibitory activity of thiazolyl-pyrazoline derivatives, it could be concluded that the hydrophobic pockets of ATP binding site were all nicely occupied by these compounds. This molecular docking result, along with the enzyme assay data, suggesting that compound **C6** is a potential inhibitor of HER-2.

3. Conclusions

In this paper, a series of thiazolyl-pyrazoline derivatives containing benzodioxole that may function as inhibitors of HER-2 kinases have been synthesized and their biological activities were evaluated. And some of them displayed potent HER-2 inhibitory activities and antiproliferative activities against MCF-7 cell lines and B16-F10 cell lines. Among them, compound **C6** showed the most potent HER-2 inhibition activities (IC_{50} = 0.18 μM) and anticancer activities (IC_{50} = 0.09 μM for MCF-7 and IC_{50} = 0.12 μM for B16-F10). Molecular docking was further performed to study the inhibitor-HER-2 protein interactions. After analysis of the binding model of compound **C6** with HER-2, it was found that two hydrogen bonds interaction with the protein residues in the ATP binding site might play a crucial role in its HER-2 inhibition and antiproliferative activities. Among these compounds, it could be concluded that compound **C6** had been demonstrated to show significant HER-2 and tumor growth inhibitory activity as a potential anticancer agent. The result of this work might be helpful for the design and synthesis of HER-2 inhibitors with stronger activities.

4. Experiments

4.1. Materials and measurements

All chemicals and reagents used in current study were of analytical grade. Separation of the compounds by column chromatography was carried out with silica gel 60 (200–300 mesh ASTM, E. Merck). The quantity of silica gel used was 50–100 times the weight charged on the column. Then, the eluates were monitored using TLC. Melting points (uncorrected) were determined on a XT4 MP apparatus (Taike Corp., Beijing, China). ESI mass spectra were obtained on a Mariner System 5304 mass spectrometer, and ^{1}H NMR spectra were recorded on a Bruker PX500 or DPX300 spectrometer at 25 °C with TMS and solvent signals

allotted as internal standards. Chemical shifts were reported in ppm (δ). Elemental analyses were performed on a CHN-O-Rapid instrument and were within ±0.4% of the theoretical values.

4.2. Synthesis

4.2.1. General synthetic procedure of benzodioxole compounds (benzo[*d*][1,3]dioxole-5-carbaldehyde and 2,3-dihydrobenzo[*b*][1,4]dioxine-6-carbaldehyde)

Equimolar portions of the appropriately protocatechuic aldehydes (50 mmol, 1 equiv) and dibromomethane or 1, 2-dibromoethane (50 mmol, 1 equiv) were dissolved in acetonitrile. The mixture was allowed to stir for several minutes at 90 °C to let dissolve. Then potassium carbonate (20.7 g, 3 equiv) was slowly added to the reaction as acid binding agent. The reaction solution was refluxed approximately 24 h. Most commonly, filtered and removed the precipitation, spin dry the solvent, extraction and recrystallization, then you will get benzodioxole compounds as reaction raw materials.

4.2.2. General synthetic procedure of chalcones derivatives (A1–A10)

Equimolar portions of the appropriately benzodioxole compounds (5 mmol, 1 equiv) and substituted acetophenone (5 mmol, 1 equiv) were dissolved in approximately 20 mL of ethanol. The mixture was allowed to stir for several minutes at 10 °C to let dissolve. Than a 1 mL aliquot of a 40% aqueous potassium hydroxide solution was then slowly added dropwise to the reaction flask via a selfequalizing addition funnel. The reaction solution was allowed to stir at room temperature for approximately 4–6 h. Most commonly, a precipitate formed and was then collected by suction filtration.

4.2.3. General synthetic procedure of pyrazole derivatives (B1–B10)

A mixture of chalcone derivatives (3 mmol), thiosemicarbazide (4.5 mmol), and KOH (3 mmol) was refluxed in ethanol (30 mL) for 12 h. After cooling, the solution was poured into mass of ice-water and stirred for a few minutes. The precipitate was filtered and crystallized from ethanol.

4.2.4. General synthetic procedure of benzodioxole thiazolyl-pyrazoline derivatives (C1–C20)

A mixture of compound B (1 mmol) and substituted 2-bromoacetophenone (1 mmol) was dissolved in ethanol (20 mL) and kept stirring in 80 °C for 8 h. After cooling, the solution was poured into mass of ice-water and stirred for a few minutes. The precipitate was filtered and crystallized from methylene dichloride/ethanol = 1:1.

4.2.4.1. 2-(5-(Benzo[d][1,3]dioxol-5-yl)-3-(4-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-methoxyphenyl)thiazole (C1).

Yield: 78%. Mp 189–191 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.32 (dd, J_1 = 5.35 Hz, J_2 = 17.40 Hz, 1H), 3.83 (s, 3H), 3.90 (dd, J_1 = 11.90 Hz, J_2 = 17.35 Hz, 1H), 5.93 (d, J = 2.15 Hz, 3H), 6.65 (s, 1H), 6.79 (d, J = 8.10 Hz, 2H), 6.87–6.91 (m, 2H), 7.06 (s, 1H), 7.12–7.16 (m, 2H), 7.69 (d, J = 8.65 Hz, 2H), 7.63–7.69 (m, 2H). ESI-MS: 474.52 ($C_{26}H_{21}FN_3O_3S$, $[M+H]^+$). Anal. Calcd for $C_{26}H_{20}FN_3O_3S$: C, 65.95; H, 4.26; N, 8.87. Found: C, 66.07; H, 4.28; N, 8.81.

4.2.4.2. 2-(5-(Benzo[d][1,3]dioxol-5-yl)-3-(4-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-bromophenyl)thiazole (C2).

Yield: 82%. Mp 218–220 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.32 (dd, J_1 = 5.43 Hz, J_2 = 17.69 Hz, 1H), 3.92 (dd, J_1 = 8.78 Hz, J_2 = 18.04 Hz, 1H), 5.92 (d, J = 5.35 Hz, 1H), 5.98 (s, 2H), 6.78–6.84 (m, 3H), 7.28 (s, 1H), 7.32 (d, J = 9.34 Hz, 2H), 7.69 (d, J = 6.74 Hz, 2H), 7.68 (d, J = 6.32 Hz, 2H), 7.83 (d, J = 7.81 Hz, 2H). ESI-MS: 523.39 ($C_{25}H_{18}BrFN_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{25}H_{17}BrFN_3O_2S$: C, 57.48; H, 3.28; N, 8.04. Found: C, 57.56; H, 3.24; N, 8.01.

4.2.4.3. 2-(5-(Benzo[d][1,3]dioxol-5-yl)-3-(4-chlorophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-methoxyphenyl)thiazole (C3).

Yield: 75%. Mp 198–200 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.29 (dd, J_1 = 5.95 Hz, J_2 = 17.55 Hz, 1H), 3.86 (s, 3H), 3.88 (d, J = 12.05 Hz, 1H), 5.82 (s, 1H), 5.93 (d, J = 4.25 Hz, 2H), 6.68 (s, 1H), 6.79 (d, J = 7.90 Hz, 1H), 6.87–6.89 (m, 3H), 6.97 (d, J = 7.90 Hz, 1H), 7.41 (d, J = 8.40 Hz, 2H), 7.65–7.71 (m, 4H). ESI-MS: 490.97 ($C_{26}H_{21}ClN_3O_3S$, $[M+H]^+$). Anal. Calcd for $C_{26}H_{20}ClN_3O_3S$: C, 63.73; H, 4.11; N, 8.58. Found: C, 63.85; H, 4.03; N, 8.62.

4.2.4.4. 2-(5-(Benzo[d][1,3]dioxol-5-yl)-3-(4-chlorophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-bromophenyl)thiazole (C4).

Yield: 78%. Mp 220–222 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.34 (d, J = 17.55 Hz, 1H), 3.90 (d, J = 16.83 Hz, 1H), 5.94 (s, 2H), 6.14 (s, 1H), 6.80 (s, 2H), 6.86 (s, 1H), 7.04 (d, J = 7.50 Hz, 1H), 7.43 (d, J = 8.35 Hz, 2H), 7.49 (d, J = 8.40 Hz, 2H), 7.62 (d, J = 8.25 Hz, 2H), 7.71 (d, J = 8.40 Hz, 2H). ESI-MS: 538.84 ($C_{25}H_{18}BrClN_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{25}H_{17}BrClN_3O_2S$: C, 55.72; H, 3.18; N, 7.80. Found: C, 55.84; H, 3.11; N, 7.72.

4.2.4.5. 2-(5-(Benzo[d][1,3]dioxol-5-yl)-3-(4-bromophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-methoxyphenyl)thiazole (C5).

Yield: 81%. Mp 226–228 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.28 (dd, J_1 = 6.25 Hz, J_2 = 17.40 Hz, 1H), 3.82 (s, 3H), 3.87 (d, J = 11.90 Hz, 1H), 5.77 (s, 1H), 5.93 (d, J = 4.10 Hz, 2H), 6.68 (s, 1H), 6.79 (d, J = 7.95 Hz, 1H), 6.86–6.89 (m, 3H), 6.96 (d, J = 7.45 Hz, 1H), 7.56 (d, J = 8.55 Hz, 2H), 7.62–7.66 (m, 4H). ESI-MS: 535.42($C_{26}H_{21}BrN_3O_3S$, $[M+H]^+$). Anal. Calcd for $C_{26}H_{20}BrN_3O_3S$: C, 58.43; H, 3.77; N, 7.86. Found: C, 58.59; H, 3.70; N, 7.81.

4.2.4.6. 2-(5-(Benzo[d][1,3]dioxol-5-yl)-3-(4-bromophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-bromophenyl)thiazole (C6).

Yield: 84%. Mp 241–243 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.29 (dd, J_1 = 6.25 Hz, J_2 = 17.40 Hz, 1H), 3.88 (d, J = 12.05 Hz, 1H), 5.77 (s, 1H), 5.93 (d, J = 4.90 Hz, 2H), 6.79 (d, J = 7.95 Hz, 1H), 6.83 (d, J = 14.50 Hz, 2H), 6.95 (d, J = 7.95 Hz, 1H), 7.46 (d, J = 8.05 Hz, 2H), 7.56–7.68 (m, 6H). ESI-MS: 584.29 ($C_{25}H_{18}Br_2N_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{25}H_{17}Br_2N_3O_2S$: C, 51.48; H, 2.94; N, 7.20. Found: C, 51.32; H, 2.98; N, 7.14.

4.2.4.7. 2-(5-(Benzo[d][1,3]dioxol-5-yl)-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-methoxyphenyl)thiazole (C7).

Yield: 74%. Mp 207–209 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 2.34 (s, 3H), 3.32 (dd, J_1 = 9.75 Hz, J_2 = 18.95 Hz, 1H), 3.40 (s, 3H), 3.90 (d, J = 19.85 Hz, 1H), 5.91 (d, J = 7.55 Hz, 1H), 6.07 (s, 2H), 6.66 (s, 1H), 6.80 (d, J = 12.65 Hz, 2H), 6.91 (s, 1H), 6.98 (d, J = 13.70 Hz, 2H),7.14 (d, J = 14.60 Hz, 2H), 7.75 (d, J = 8.85 Hz, 2H), 7.78 (d, J = 8.80 Hz, 2H). ESI-MS: 470.55 ($C_{27}H_{24}N_3O_3S$, $[M+H]^+$). Anal. Calcd for $C_{27}H_{23}N_3O_3S$: C, 69.06; H, 4.94; N, 8.95. Found: C, 68.95; H, 4.90; N, 9.01.

4.2.4.8. 2-(5-(Benzo[d][1,3]dioxol-5-yl)-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-bromophenyl)thiazole (C8).

Yield: 82%. Mp 219–221 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 2.42 (s, 3H), 3.33 (dd, J_1 = 5.80 Hz, J_2 = 17.55 Hz, 1H), 3.90 (dd, J_1 = 11.70 Hz, J_2 = 17.50 Hz, 1H), 5.93 (d, J = 4.45 Hz, 1H), 5.98 (s, 2H), 6.78–6.81 (m, 2H), 6.87 (s, 1H), 7.00 (d, J = 7.95 Hz, 1H), 7.24–7.27 (m, 2H), 7.47 (d, J = 8.40 Hz, 2H), 7.61 (d, J = 8.55 Hz, 2H), 7.67 (d,

J = 8.10 Hz, 2H). ESI-MS: 519.42 ($C_{26}H_{21}BrN_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{26}H_{20}BrN_3O_2S$: C, 60.24; H, 3.89; N, 8.11. Found: C, 60.39; H, 3.82; N, 8.03.

4.2.4.9. 2-(5-(Benzo[d][1,3]dioxol-5-yl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-methoxyphenyl)thiazole (C9). Yield: 74%. Mp 190–192 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.33 (d, J = 17.55 Hz, 1H), 3.83 (s, 6H), 3.88 (d, J = 14.25 Hz, 1H), 5.91 (s, 1H), 5.94 (s, 2H), 6.63 (s, 1H), 6.79 (d, J = 7.95 Hz, 1H), 6.89 (d, J = 10.70 Hz, 3H), 6.96 (d, J = 8.85 Hz, 2H), 7.06 (s, 1H), 7.69–7.78 (m, 4H). ESI-MS: 486.55 ($C_{27}H_{24}N_3O_4S$, $[M+H]^+$). Anal. Calcd for $C_{27}H_{23}N_3O_4S$: C, 66.79; H, 4.77; N, 8.65. Found: C, 66.64; H, 4.70; N, 8.71.

4.2.4.10. 2-(5-(Benzo[d][1,3]dioxol-5-yl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-bromophenyl)thiazole (C10). Yield: 76%. Mp 207–209 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.34 (d, J = 17.55 Hz, 1H), 3.83 (s, 3H), 3.94 (d, J = 10.25 Hz, 1H), 5.93 (d, J = 9.15 Hz, 1H), 5.98 (s, 2H), 6.79–6.99 (m, 3H), 7.21 (d, J = 7.65 Hz, 1H), 7.47 (d, J = 5.60 Hz, 2H), 7.53 (d, J = 8.10 Hz, 2H), 7.68 (d, J = 7.30 Hz, 2H), 7.75 (d, J = 8.40 Hz, 2H). ESI-MS: 535.42 ($C_{26}H_{21}BrN_3O_3S$, $[M+H]^+$). Anal. Calcd for $C_{26}H_{20}BrN_3O_3S$: C, 58.43; H, 3.77; N, 7.86. Found: C, 58.31; H, 3.71; N, 7.92.

4.2.4.11. 2-(5-(2,3-Dihydrobenzo[b][1,4]dioxin-6-yl)-3-(4-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-methoxyphenyl)thiazole (C11). Yield: 70%. Mp 218–220 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.28 (dd, J_1 = 6.15 Hz, J_2 = 14.25 Hz, 1H), 3.83 (s, 3H), 3.94(dd, J_1 = 7.55 Hz, J_2 = 15.20 Hz, 1H), 4.12 (s, 2H), 4.15 (s, 2H), 5.95 (s, 1H), 6.70 (s, 1H), 6.79 (d, J = 7.05 Hz, 2H), 6.88 (s, 1H), 6.93 (d, J = 5.80 Hz, 2H), 7.03–7.08 (m, 2H), 7.22 (d, J = 9.35 Hz, 2H), 7.68 (d, J = 7.95 Hz, 2H). ESI-MS: 488.55 ($C_{27}H_{23}FN_3O_3S$, $[M+H]^+$). Anal. Calcd for $C_{27}H_{22}FN_3O_3S$: C, 66.51; H, 4.55; N, 8.62. Found: C, 66.69; H, 4.50; N, 8.55.

4.2.4.12. 4-(4-Bromophenyl)-2-(5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(4-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl)thiazole (C12). Yield: 72%. Mp 230–232 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.29 (dd, J_1 = 6.75 Hz, J_2 = 17.25 Hz, 1H), 3.87 (d, J = 12.55 Hz, 1H), 4.16 (s, 2H), 4.22 (s, 2H), 5.88 (s, 1H), 6.76–6.80 (m, 2H), 6.86 (s, 1H), 6.98 (d, J = 8.05 Hz, 1H), 7.18 (d, J=7.35 Hz, 2H), 7.40 (d, J = 7.30 Hz, 2H), 7.64 (d, J = 6.95 Hz, 2H), 7.70 (d, J = 8.60 Hz, 2H). ESI-MS: 537.42 ($C_{26}H_{20}BrFN_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{26}H_{19}BrFN_3O_2S$: C, 58.22; H, 3.57; N, 7.83. Found: C, 58.38; H, 3.51; N, 7.74.

4.2.4.13. 2-(3-(4-Chlorophenyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-methoxyphenyl)thiazole (C13). Yield: 69%. Mp 228–230 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.32(dd, J_1 = 8.75 Hz, J_2 = 15.95 Hz, 1H), 3.83 (s, 3H), 3.92 (d, J = 5.20 Hz, 1H), 4.22 (s, 4H), 5.85 (s, 1H), 6.71–6.73 (m, 1H), 6.77 (d, J = 8.15 Hz, 1H), 6.88 (d, J = 8.25 Hz, 2H), 6.99 (d, J = 8.05 Hz, 2H), 7.17 (d, J = 5.85 Hz, 2H), 7.23 (d, J = 6.75 Hz, 2H), 7.68 (d, J = 9.15 Hz, 2H). ESI-MS: 505.00 ($C_{27}H_{23}ClN_3O_3S$, $[M+H]^+$). Anal. Calcd for $C_{27}H_{22}ClN_3O_3S$: C, 64.34; H, 4.40; N, 8.34. Found: C, 64.47; H, 4.46; N, 8.27.

4.2.4.14. 4-(4-Bromophenyl)-2-(3-(4-chlorophenyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-4,5-dihydro-1H-pyrazol-1-yl)thiazole (C14). Yield: 75%. Mp 245–247 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.33 (d, J = 15.25 Hz, 1H), 3.90 (d, J = 11.75 Hz, 1H), 4.25 (s, 4H), 5.94 (s, 1H), 6.75 (d, J = 8.25 Hz, 2H), 6.87 (d, J = 6.75 Hz, 1H), 7.02 (d, J = 8.35 Hz, 1H), 7.39–7.41 (m, 2H), 7.51 (d, J = 7.30 Hz, 2H), 7.67 (d, J = 8.65 Hz, 2H), 7.78–7.81 (m, 2H). ESI-MS: 553.87 ($C_{26}H_{20}BrClN_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{26}H_{19}BrClN_3O_2S$: C, 56.48; H, 3.46; N, 7.60. Found: C, 56.32; H, 3.51; N, 7.69.

4.2.4.15. 2-(3-(4-Bromophenyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-methoxyphenyl)thiazole (C15). Yield: 75%. Mp 247–249 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.32 (d, J = 15.85 Hz, 1H), 3.83 (s, 3H), 3.91 (dd, J_1 = 7.10 Hz, J_2 = 17.45 Hz, 1H), 4.26 (s, 4H), 5.91 (s, 1H), 6.72 (s, 1H), 6.75 (d, J = 8.25 Hz, 2H), 6.97 (d, J = 8.10 Hz, 1H), 6.91–6.94 (m, 2H), 7.03 (d, J = 8.35 Hz, 2H), 7.38 (d, J = 6.25 Hz, 2H), 7.71 (d, J = 8.75 Hz, 2H). ESI-MS: 549.45 ($C_{27}H_{23}BrN_3O_3S$, $[M+H]^+$). Anal. Calcd for $C_{27}H_{22}BrN_3O_3S$: C, 59.13; H, 4.04; N, 7.66. Found: C, 59.01; H, 4.09; N, 7.72.

4.2.4.16. 4-(4-Bromophenyl)-2-(3-(4-bromophenyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-4,5-dihydro-1H-pyrazol-1-yl)thiazole (C16). Yield: 74%. Mp 261–263 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.31 (dd, J_1 = 5.95 Hz, J_2 = 16.05 Hz, 1H), 3.92 (d, J_1 = 6.35 Hz, J_2 = 14.35 Hz, 1H), 4.28 (s, 4H), 5.94 (s, 1H), 6.79–6.82 (m, 3H), 6.95 (d, J = 7.25 Hz, 1H), 7.06 (d, J = 7.45 Hz, 2H), 7.24 (d, J = 8.05 Hz, 2H), 7.56 (d, J = 7.35 Hz, 2H), 7.71 (d, J = 7.95 Hz, 2H). ESI-MS: 598.32 ($C_{26}H_{20}Br_2N_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{26}H_{19}Br_2N_3O_2S$: C, 52.28; H, 3.21; N, 7.03. Found: C, 52.14; H, 3.28; N, 7.09.

4.2.4.17. 2-(5-(2,3-Dihydrobenzo[b][1,4]dioxin-6-yl)-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-methoxyphenyl)thiazole (C17). Yield: 68%. Mp 217–219 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 2.33 (s, 3H), 3.29 (dd, J_1 = 7.05 Hz, J_2 = 17.45 Hz, 1H), 3.83 (s, 3H), 3.90 (d, J_1 = 7.34 Hz, J_2 = 16.85 Hz, 1H), 4.25 (s, 4H), 5.85 (s, 1H), 6.63 (s, 1H), 6.77 (d, J = 7.75 Hz, 2H), 6.82 (d, J = 8.15 Hz, 1H), 6.98 (d, J = 5.70 Hz, 2H), 7.14 (d, J = 6.60 Hz, 2H), 7.35 (d, J = 7.85 Hz, 2H), 7.67 (d, J = 7.80 Hz, 2H). ESI-MS: 484.58 ($C_{28}H_{26}N_3O_3S$, $[M+H]^+$). Anal. Calcd for $C_{28}H_{25}N_3O_3S$: C, 69.54; H, 5.21; N, 8.69. Found: C, 69.42; H, 5.27; N, 8.61.

4.2.4.18. 4-(4-Bromophenyl)-2-(5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)thiazole (C18). Yield: 77%. Mp 231–233 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 2.33 (s, 3H), 3.32 (dd, J_1 = 7.15 Hz, J_2 = 16.75 Hz, 1H), 3.93 (dd, J_1 = 5.75 Hz, J_2 = 17.25 Hz, 1H), 4.24 (s, 4H), 5.81 (s, 1H), 6.67 (s, 1H), 6.74 (d, J = 7.25 Hz, 2H), 6.85 (d, J = 6.75 Hz, 1H), 7.13 (d, J = 7.05 Hz, 2H), 7.31 (d, J = 7.85 Hz, 2H), 7.53 (d, J = 7.55 Hz, 2H), 7.70 (d, J = 8.25 Hz, 2H). ESI-MS: 533.45 ($C_{27}H_{23}BrN_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{27}H_{22}BrN_3O_2S$: C, 60.90; H, 4.16; N, 7.89. Found: C, 60.84; H, 4.10; N, 7.95.

4.2.4.19. 2-(5-(2,3-Dihydrobenzo[b][1,4]dioxin-6-yl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-methoxyphenyl) thiazole (C19). Yield: 72%. Mp 220–222 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.29 (dd, J_1 = 7.45 Hz, J_2 = 18.75 Hz, 1H), 3.82 (s, 6H), 3.90 (d, J = 15.35 Hz, 1H), 4.24 (s, 4H), 5.91 (d, J = 4.45 Hz, 1H), 6.67 (s, 1H), 6.76 (d, J = 8.05 Hz, 2H), 6.84 (d, J = 7.30 Hz, 1H), 7.01 (d, J = 5.35 Hz, 2H), 7.04 (d, J = 7.15 Hz, 2H), 7.41 (d, J = 6.75 Hz, 2H), 7.73 (d, J = 7.25 Hz, 2H). ESI-MS: 500.58 ($C_{28}H_{26}N_3O_4S$, $[M+H]^+$). Anal. Calcd for $C_{28}H_{25}N_3O_4S$: C, 67.32; H, 5.04; N, 8.41. Found: C, 67.20; H, 5.09; N, 8.48.

4.2.4.20. 4-(4-Bromophenyl)-2-(5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)thiazole (C20). Yield: 78%. Mp 235–237 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 3.31 (d, J = 17.15 Hz, 1H), 3.83 (s, 3H), 3.92 (d, J = 16.35 Hz, 1H), 4.24 (s, 4H), 5.92 (s, 1H), 6.66 (s, 1H), 6.76 (d, J = 7.05 Hz, 1H), 6.86–6.90 (m, 2H), 7.02 (d, J = 7.15 Hz, 2H), 7.32 (d, J = 7.30 Hz,

2H), 7.58 (d, J = 7.35 Hz, 2H), 7.75 (d, J = 6.65 Hz, 2H). ESI-MS: 549.45 ($C_{27}H_{23}BrN_3O_3S$, $[M+H]^+$). Anal. Calcd for $C_{27}H_{22}BrN_3O_3S$: C, 59.13; H, 4.04; N, 7.66. Found: C, 59.28; H, 3.98; N, 7.60.

4.3. Preparation, purification of HER-2 and inhibitory assay

A 1.7 Kb cDNA encoded for human HER-2 cytoplasmic domain (HER-2-CD, amino acids 676–1245) was cloned into baculoviral expression vector pBlueBacHis2B. A sequence that encodes $(His)_6$ was located at the 5′ upstream to the HER-2 sequence. Sf-9 cells were infected for 3 days for protein expression. Sf-9 cell pellets were solubilized at 0 °C in a buffer at pH 7.4 containing 50 mM HEPES, 10 mM NaCl, 1% Triton, 10 μM ammonium molybdate, 100 μM sodium vanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 16 μg/mL benzamidine HCl for 20 min followed by 20 min centrifugation. Crude extract supernatant was passed through an equilibrated Ni-NTA superflow packed column and washed with 10 mM and then 100 mM imidazole to remove nonspecifically bound material. Histidinetagged proteins were eluted with 250 and 500 mM imidazole and dialyzed against 50 mM NaCl, 20 mM HEPES, 10% glycerol, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin for 2 h. The entire purification procedure was performed at 4 °C or on ice.

The HER-2 kinase assay was set up to assess the level of autophosphorylation based on DELFIA/Time-Resolved Fluorometry. Compounds (**C4, C5, C6, C8, C15, C16**) were dissolved in 100% DMSO and diluted to the appropriate concentrations with 25 mM HEPES at pH 7.4. The final percentage of DMSO in the buffer solution is less than 0.1%. In each well, 10 μL of compound was incubated with 10 μL (12.5 ng for HER-2) of recombinant enzyme (1:80 dilution in 100 mM HEPES) for 10 min at room temperature. Then, 10 μL of 5 mM buffer (containing 20 mM HEPES, 2 mM $MnCl_2$, 100 μM Na_3VO_4, and 1 mM DTT) and 20 μL of 0.1 mM ATP-50 mM $MgCl_2$ was added for 1 h. Positive and negative controls were included in each plate by incubation of enzyme with or without ATP-$MgCl_2$. At the end of incubation, liquid was aspirated, and plates were washed three times with wash buffer. A 75 μL (400 ng) sample of europium labeled anti-phosphotyrosine antibody was added to each well for another 1 h of incubation. After washing, enhancement solution was added and the signal was detected by Victor (Wallac Inc.) with excitation at 340 nm and emission at 615 nm. The percentage of autophosphorylation inhibition by the compounds was calculated using the following equation: 100% − [(negative control)/(positive control − negative control)]. The IC_{50} was obtained from curves of percentage inhibition with eight concentrations of compound. As the contaminants in the enzyme preparation are fairly low, the majority of the signal detected by the anti-phosphotyrosine antibody is from HER-2.

4.4. Antiproliferation assay

The antiproliferative activities of compounds **C1–C20** were determined using a standard (MTT)-based colorimetric assay (Sigma). Briefly, cell lines were seeded at a density of 7×10^3 cells/well in 96-well microtiter plates (Costar). After 24 h, exponentially growing cells were exposed to the indicated compounds at final concentrations ranging from 0.1 to 100 μg/mL. After 48 h, cell survival was determined by the addition of an MTT solution (10 μL of 5 mg/mL MTT in PBS). After 4 h, 100 μL of 10% SDS in 0.01 N HCl was added, and the plates were incubated at 37 °C for a further 18 h; optical absorbance was measured at 570 nm on an LX300 Epson Diagnostic microplate reader. Survival ratios are expressed in percentages with respect to untreated cells. IC_{50} values were determined from replicates of 6 wells from at least three independent experiments.

4.5. Western blotting

After incubation, cells were harvested and washed with PBS, then lysed in lysis buffer (30 mm Tris, pH 7.5, 150 mm NaCl, 1 mm phenylmethylsulfonyl fluoride, 1 mm Na3VO4, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). After centrifugation at 12,000 g for 5 min, the supernatant was collected as total protein. The concentration of the protein was determined by a BCA™ protein assay kit (Pierce, Rockford, IL, USA). The protein samples were separated by 10% SDS–PAGE and subsequently electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% nonfat milk for 2 h at room temperature. The blocked membrane was probed with the indicated primary antibodies overnight at 4 °C, and then incubated with a horse radish peroxidase (HRP)-coupled secondary antibody. Cleaved Caspase 3 and HER-2 were measured as shown in Figure 2.

4.6. Molecular docking study

Molecular docking of compounds into the 3D HER-2 complex structure (PDB code: 1UOM) was carried out using the Discovery Studio (version 3.1) as implemented through the graphical user interface CDocker protocal. The three-dimensional structures of the aforementioned compounds were constructed using Chem 3D ultra 11.0 software [Chemical Structure Drawing Standard; Cambridge Soft corporation, USA (2009)], then they were energetically minimized by using MOPAC with 5000 iterations and minimum RMS gradient of 0.10. The crystal structures of HER-2 complex were retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.-do). All bound water and ligands were eliminated from the protein and the polar hydrogen was added. The whole HER-2 complex was defined as a receptor and the site sphere was selected based on the ligand binding location of ATP, then the ATP molecule was removed and **C6** was placed during the molecular docking procedure. Types of interactions of the docked protein with ligand were analyzed after the end of molecular docking.

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