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Discovery of 3,5-dimethylisoxazole derivatives as novel, potent inhibitors for bromodomain and extraterminal domain (BET) family

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ABSTRACT

Bromodomain and extra-terminal (BET) is a promising therapeutic target for various hematologic cancers. We used the BRD4 inhibitor compound **13** as a lead compound to develop a variety of compounds, and we introduced diverse groups into the position of the compound **13** orienting toward the ZA channel. A series of compounds (**14–23**, **38–41**, **43**, **47–49**) bearing triazolopyridazine motif exhibited remarkable BRD4 protein inhibitory activities. Among them, compound **39** inhibited BRD4(BD1) protein with an IC_{50} of 0.003 μM was superior to lead compound **13**. Meanwhile, compound **39** possess activity, IC_{50} = 2.1 μM, in antiproliferation activity against U266 cancer cells. On the other hand, compound **39** could arrest tumor cells into the G0/G1 phase and induce apoptosis, which was consistent with its results in inhibiting cell proliferation. Biological and biochemical data suggest that BRD4 protein might be a therapeutic target and that compound **39** is an excellent lead compound for further development.

1. Introduction

MM is the second most hematological malignancy characterized by the uncontrolled proliferation of malignant plasma cells,[1–4] which accounts for ~10% of hematological malignancies.[5] Despite the continuous development of cancer treatment drugs, MM is still an incurable disease because of relapse and drug resistance, and the optimal therapy for advanced-stage multiple myeloma remains poorly defined.[6] Thus, the potent molecules with novel mechanisms are needed to build up the theoretical basis for MM therapies.

BET proteins, such as BRD2/3/4 and BRDT, play a major part in chromatin readers for acetylated lysine residues.[7] BRD4 contains two bromodomains (BD1 and BD2) and an extraterminal (ET) domain, which is similar to all BET family members, and it also contains four α-helices (αZ, αA, αB, and αC) that are linked by two divergent loop regions (ZA and BC loops).[8–10] BD1 and BD2 have high homology in amino acid sequence, achieving >40%. Especially, in the position of the binding pocket, they only have three crucial residues different from each other, showing 95% sequence similarity.[11] Although the two have high homology in the binding pocket, they can specifically recognize different acetylation substrates in H3 and H4 histone tails.[12]

Some studies have proposed that BRD4 interacts with the *N*-terminal regions of histones H3 and H4, and it also plays a crucial role in recruiting the positive transcription elongation factor b (P-TEFb) to transcription initiation area promoting transcriptional elongation in a highly context-dependent manner.[13,14] On the other hand, BRD4 can regulate nuclear factor-κB dependent genes as a response to immune abnormality.[15–17] Furthermore, BRD4 proteins are attracting increasing attention as a therapeutic target for a variety of human diseases, including acute myeloid leukemia (AML), MM, NUT midline carcinoma (NMC), etc.[18–20]

So far, BRD4 bromodomain inhibitors have been designed to modulate the function of BRD4 protein, including JQ1, OTX015, ABBV075 (Fig. 1), some of which have promising therapeutic potential for disease treatment.[18,21] In fact, Several BET inhibitors such as **2** (OTX015) and **3** (I-BET762) have recently entered clinical trials for

Abbreviations: BET, Bromodomain and Extraterminal Domain; MM, Multiple myeloma; WB, Western Blot; HEXIM1, Hexamethylene bis-acetamide inducible protein; MYC, Myelocyto matosis oncogene.

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cancer therapy. As previously mentioned, we reported that compound **13** containing quinazolinone motif as a BRD4 inhibitor exhibit excellent biological activities in vitro.[22] Here, we set up to introduce different functional moieties into the position of compound **13** orienting toward the ZA channel to obtain novel and potent BRD4 inhibitors for the treatment of MM by exploring the structure–activity relationship (SAR) more extensively and comprehensively. Additionally, we also have described the antiproliferation mechanism of compound **39** against U266 cells.

2. Results and discussion

2.1. Chemistry

The synthesis of compounds (**13**–**23**) as sketched in Scheme 1 and their structure were confirmed by ^{1}H NMR, ^{13}C NMR spectrum and mass spectra. Representative spectrometry was displayed in the Supporting Information. First, 2-Aminobenzophenone as the starting material reacted with *N*-Bromosuccinimide, followed by amine reduction with *tert*-butyl sulfinate to produce intermediate **9**, which was reduced by sodium borohydride according to reported methods[22] and then reacted with triphosgene to obtain key intermediate **11**. Intermediate **12** was prepared by methylation of compound **11** with iodomethane. The commercially available 3,5-Dimethylisoxazole pinacol boronate was used to afford the target compound **13** via Suzuki − Miyaura coupling with intermediate **12**. Subsequently, lead compound **13** undergoes nucleophilic substitution with various heterocyclic groups to obtain target compounds **14**–**23**.

Compounds (**39**–**41**, **43**, **47**–**49**) are synthesized as shown in Scheme 2. The commercially available starting material 3,6-dichloropyridazine reacted with Semicarbazide, followed by chlorination with $POCl_3$ to obtain compound **36** and then substituted with piperazine to produce key intermediate **37**. Lead compound **13** was substituted with brominated alkanes of different lengths to afford intermediates **38a–c**. Target compounds **39**–**41** were prepared by using intermediates **38a–c** and key intermediate **37** via nucleophilic substitution. Meanwhile, compound **13** also treated with diethylene glycol ditoluenesulfonate, followed by substitution with compound **37** to give target compound **43**. Besides, Compound **13** could be converted to intermediates **44**–**46** by reacting with brominated fatty acids of different lengths. Target products **47**–**49** were attained through the above methods.

2.2. Design strategy and SAR study

Protein binding pattern analysis of quinazolinone compound **13** with BRD4(BD1) protein revealed that the 3,5-Dimethylisoxazole motif as a Kac mimic extended into the Kac pocket of the BRD4 protein and formed a hydrogen bond with the key amino acid Asn140, allowing compound **13** to be firmly anchored in the Kac pocket. Simultaneously, the 4-position benzene ring of compound **13** extended into the WPF hydrophobic pocket, which formed by hydrophobic amino acids W81, P82 and F83, immobilizing the protein by hydrophobic interactions (Figure 2**A**). Delightfully, we found that the 3-position amide of compound **13** oriented toward the ZA channel of BRD4 protein, which was exposed to the solvent region. It meant that diverse active moieties can be introduced into this position to enhance the physicochemical properties of the lead compound **13**.

Beginning with the parent molecule compound **13** and SAR study, we first designed and synthesized compounds **14**–**23** by introducing different heterocycles into the 3-position of the lead compound **13** to explore the effects of the ZA region on the inhibitory activity. Then, most of these compounds exhibited favorable BRD4 inhibitory activities, especially the inhibition rates of compounds **14**, **15**, **17** and **23** were 100% against BRD4(BD1) protein at 1 μM. Compounds **14** and **23** had outstanding activities against BRD4(BD1) with an IC_{50} of 0.055 and 0.058 μM, respectively, that were similar to the activity of the lead compound **13** (IC_{50} = 0.053 μM). Besides, the introduced heterocyclic group extended into the solvent region outside the ZA region by the docking model of compound **14** to the BRD4 molecule (Figure 2**B**). We confirm that the ZA channel has a strong capacity to accommodate various larger moieties, which can provide a basis for the design of subsequent BRD4 inhibitors (see Table 1).

Considered with the above experimental results, we next designed and synthesized compounds **38**–**41**, **43**, **47**–**49** by introducing a triazolopyridazine structure into this position of the lead compound **13**. We observed that the triazolopyridazine warhead could be able to extend into the solvent region through the ZA channel, thereby enhancing the binding ability of small molecules to the protein (Figure 3**A**). Meanwhile, most of the compounds exhibited significant BRD4 (BD1) protein inhibitory activities with an inhibition rate of 100% against BRD4(BD1) at 0.5 μM in the protein inhibitory activities study, except for compound **49**. Encouragingly, compounds (**39**–**41**) with carbon chains as linkers performed better inhibitory activities than other

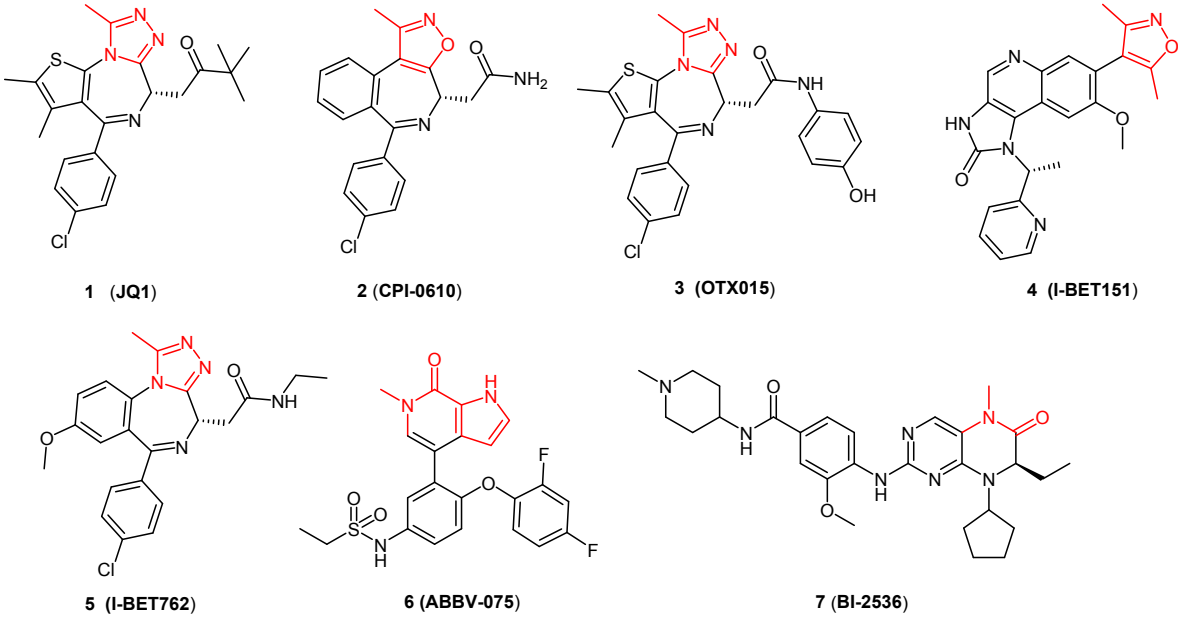
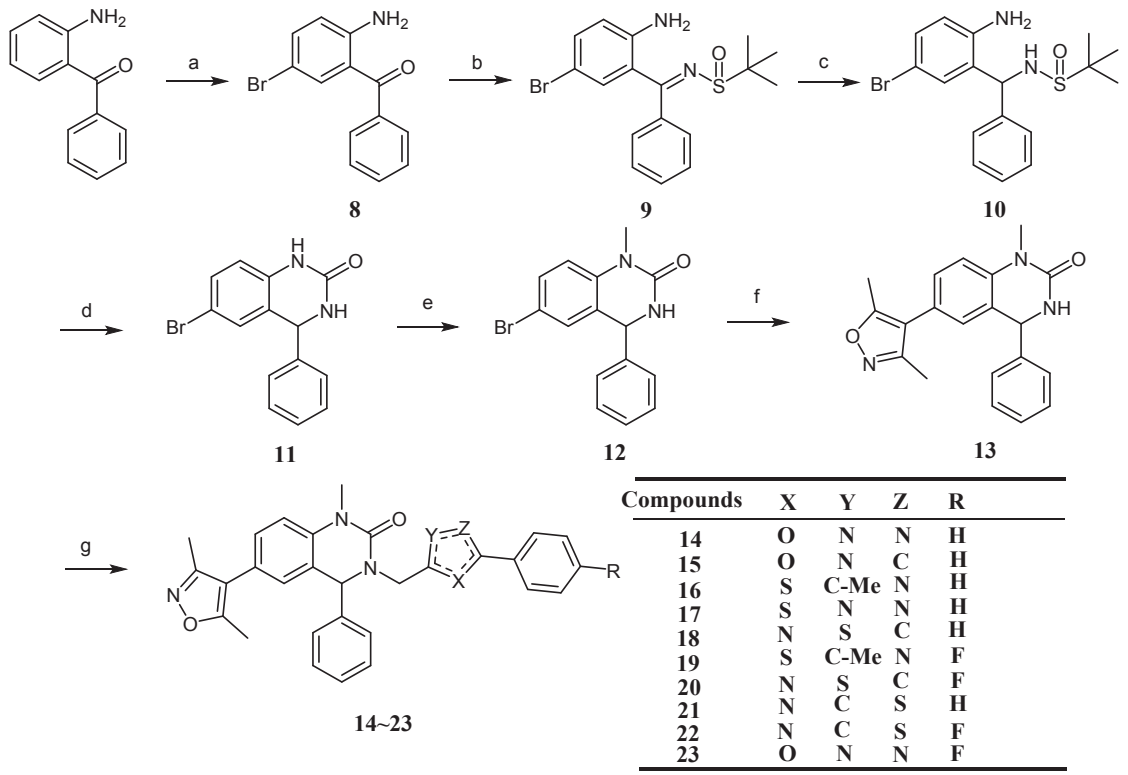


Fig. 1. Structures of small-molecule BET Bromodomain inhibitors.



Compounds	X	Y	Z	R
14	O	N	N	H
15	O	N	C	H
16	S	C-Me	N	H
17	S	N	N	H
18	N	S	C	H
19	S	C-Me	N	F
20	N	S	C	F
21	N	C	S	H
22	N	C	S	F
23	O	N	N	F

Scheme 1. Reagents and conditions: (a) NBS, DCM, 0 ℃; (b) *tert*-butanesulfinamide, tetraethyl titanate, THF, reflux; (c) $NaBH_4$, THF, H_2O, r.t; (d) bis(trichloromethyl) carbonate, THF, r.t; (e) dimethylsulfate; Cs_2CO_3, acetonitrile, r.t; (f) phenylboronic acids, Pd $(PPh_3)_4$, Na_2CO_3, N_2, H_2O/EtOH/toluene, 80 ℃; (g) **25a, b** or **26** or **28** or **30a, b** or **32a, b** or **34a,b**, Cs_2CO_3, DMF, r.t.

types of compounds. Among them, compounds **39** and **41** showed the best activity with an IC_{50} of 0.003 and 0.0031 μM, respectively (Table 2). Especially, compound **39** increased an 8-fold inhibitory of BRD4(BD1) protein comparing with the lead compound **13**. These results implied that the introduction of the triazolopyridazine group remarkably enhanced the BRD4 protein inhibitory activity of the lead compound **13**. Therefore, we evaluated the antiproliferative activities of these compounds in diverse cells.

2.3. Biological evaluation

2.3.1. Anti-proliferation in cancer cell lines

We next evaluated the antiproliferative potency of compounds **14–23**, **39–41**, **43**, and **47–49** by in vitro human tumor cell line screening, including MCF10A, CT26, MCF7, HepG2, HCT116 and U266 cells. Together, a majority of compounds possessed excellent antiproliferative activity against U266 and nearly no toxicity to normal breast cells. Most important was that the inhibition rates of compounds **39–41** were around 50% at 0.1 μM (Table 3), which were consistent with their potent BRD4 protein activities.

In terms of inhibition of cell proliferation to CT26, HepG2 and U266 cells, compounds **13, 14, 39–41** and **43** exhibited higher antiproliferative potency. Therefore, we further sought to the IC_{50} of these compounds on CT26, HepG2 and U266 cells (Table 4). It was worth noting that U266 cells were more sensitive to those compounds comparing to CT26 and HepG2 cells. The inhibitory activity of compounds **39** and **40** on U266 cells (IC_{50} = 2.11 and 1.99 μM, respectively) were similar to that of ABBV-075(IC_{50} = 2.03 μM) but the inhibitory activity of compounds **39** and **40** (IC_{50} = 15.77 and 11.77 μM, respectively) on CT26 was much better than that of ABBV-075 (IC_{50} = 48.26 μM). Surprisingly, the inhibitory ability of compound **39** on U266 cells was about 17-fold that of compound **13** (IC_{50} = 35.35 μM).

2.3.2. Compound 39 induced cell cycle arrest in U266 cells

To validate the effect of compound **39** on the cell cycle distribution of U266 cells, we used flow cytometry to analyze the cycle distribution of U266 cells after 48 h treatment with compound **39** at 1 and 2 μM, respectively. We found that U266 cells could be arrested into the G0/G1 phase after treatment with compound **39** comparing with the control (Fig. 4), indicating that compound **39** induced U266 cells cycle arresting into G0/G1 phase.

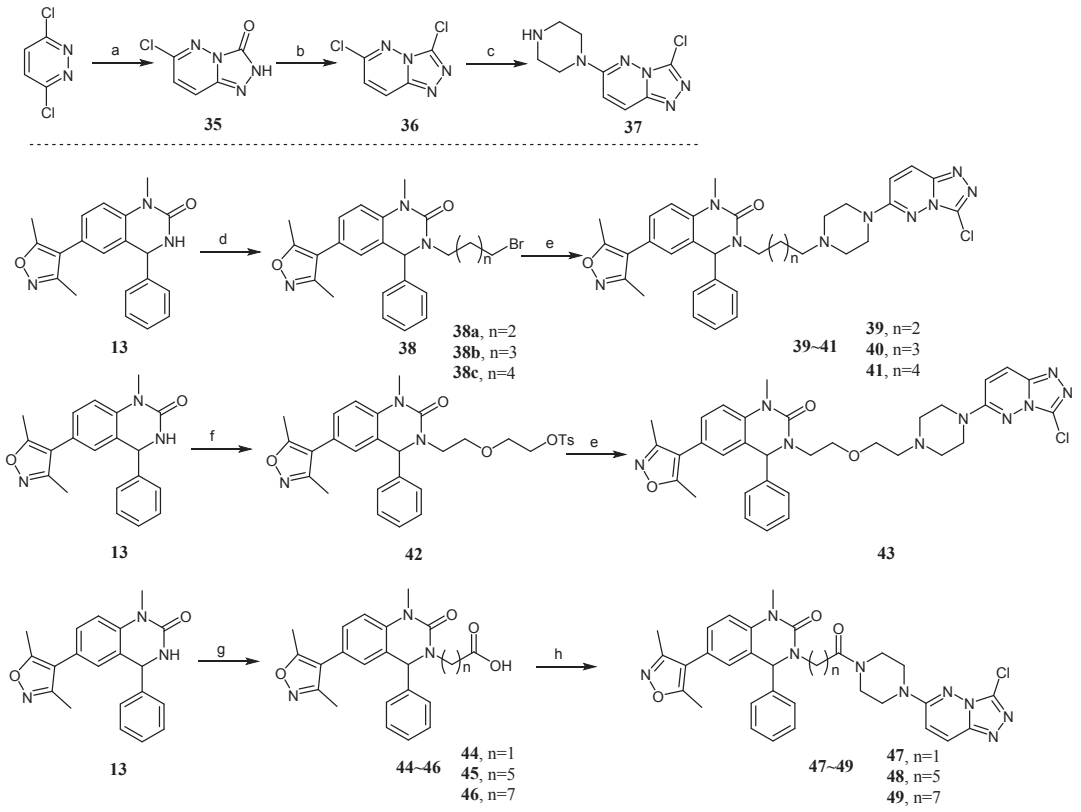
2.3.3. Compound *39* induced apoptosis in U266 cells

Followed by, to investigate whether compound **39** could cause U266 cell apoptosis, DAPI staining was used to analyze the morphology of U266 cell nucleus after 48 h treatment with compound **39**. The result showed that U266 nucleus underwent dramatic condensation comparing with the negative control, indicating that compound **39** induced apoptosis in U266 cells through U266 cell nucleus condensation (Figure 5**A**).

We used flow cytometry to further verified U266 cells apoptosis after 48 h treatment with compound **39**. Compound **39** led to significant U266 cells apoptosis in a concentration-dependent manner comparing with control (Figure 5**B, C**). Thus, compound **39** had a significant ability to induce U266 cell apoptosis which might be the reason for its antiproliferative effect.

3. Conclusion

In this study, we performed further structural optimization and activity evaluation of quinazolinone compound **13**, which is previously reported in our group. The inhibitory activities of compounds **14–23** against BRD4(BD1) protein indicate that compound **13** can accommodate large heterocycles at the position orienting toward the ZA channel. Herein, we introduced a triazolopyridazine structure in this position to enhance inhibitory potency. The BRD4(BD1) protein assay results



Scheme 2. Reagents and conditions: (a) semicarbazide hydrochloride, conc. HCl, EtOH, H_2O, reflux; (b) $POCl_3$ reflux; (c) piperazine, triethylamine, acetonitrile, r.t; (d) 1,4-dibromobutane or 1,5-dibromopentane or 1,6-dibromohexane, NaH, DMF, 0 ℃ to r.t; (e) **37**, NaH, DMF, 0 ℃ to r.t; (f) 1,1′-bis(4-methylbenzenesulfonate), NaH, DMF, 0 ℃ to r.t; (g) (1) ethyl bromoacetate or ethyl 6-bromohexanoate or ethyl 8-bromooctanoate, NaH, DMF, 0 ℃ to r.t; (2) LiOH, MeOH, H_2O, r.t; (h) **37**, EDCI, HOBT, triethylamine, DMF, 0 ℃ to r.t.

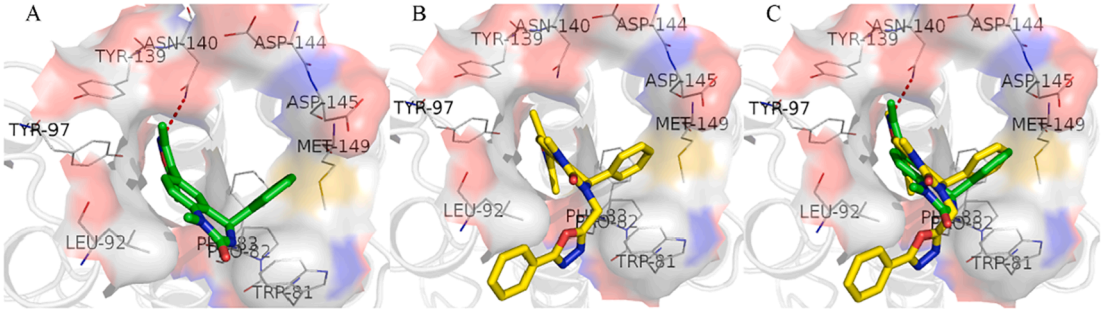


Fig. 2. Molecular docking conformation. (A) Conformation of compound **13** (green) in BRD4(BD1) protein (2YEL). (B) Conformation of compound **14** (yellow) in BRD4(BD1) protein (2YEL). (C) The superposition of compound **13** (green) and **14** (yellow) in BRD4(BD1) protein (2YEL).

exhibit that compound **39** possesses excellent inhibitory activity with an IC_{50} of 0.003 μM. Biological test results show that compound **39** effectively inhibits the proliferation of U266 myeloma cells with an IC_{50} of 2.1 μM, because it can not only arrest the U266 cells cycle into the G0/G1 phase, but also has a strong ability to induce cell apoptosis. Due to the significant in vitro biological activities of compound **39** against malignant tumors, further molecular binding model and in vivo activity studies are in progress.

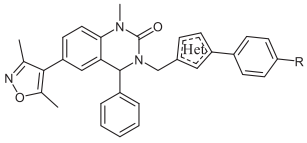
4. Experiment section

4.1. Chemistry

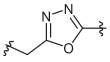
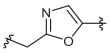
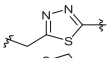
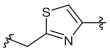
The solvents or reagents used in the chemical experiments were commercially available analytical or chemically pure products, which were not treated, unless otherwise specified. The melting point was determined using an RY-1 melting point meter from Tianjin Analytical Instrument Factory. Infrared spectroscopy (IR) was determined using potassium bromide for slicing and Nicolet Impact 410 IR tester. Nuclear magnetic resonance hydrogen (^{1}H NMR) and carbon (^{13}C NMR) spectra were determined using Bruker AV-300 or Bruker AV-400 magnetic resonance instruments with tetramethylsilane (TMS) as the internal standard compound. Mass spectrometry (MS) was determined using an Agilent 110 liquid combination system. Column chromatography was performed using 200–300 mesh silica gel from Qingdao Ocean Chemical Co. All reactions were monitored using high-efficiency thin-layer plates under 254 nm and 365 nm wavelength UV lamp with either petroleum ether/ethyl acetate or dichloromethane/methanol systems.

*(2-Amino-5-bromophenyl)(phenyl) methanone (**8**).* To the solution of 2-Amino benzophenone (2 g, 10.14 mmol) in dichloromethane (30 mL), *N*-bromosuccinimide (1.9 g, 10.65 mmol) was added slowly at 0 °C and stirred in the ice bath for two hours. After completion of the reaction, the

Table 1
BRD4(BD1) inhibitory activities of compounds **13–23**.



14~23

Compound No.	Het	R	BRD4(BD1) 1 μM (%)	BRD4(BD1) IC_{50} μM[a]
13			100	0.053 ± 0.013
14		H	100	0.055 ± 0.011
15		H	100	0.105 ± 0.012
16		H	82	NT [b]
17		H	100	0.074 ± 0.014
18		H	89	NT
19		F	98	NT
20		F	74	NT
21		H	76	NT
22		F	69	NT
23		F	100	0.058 ± 0.015
JQ1			100	0.042 ± 0.011

[a] The values are presented as means ± SD from three separate determinations.
[b] NT: no test.

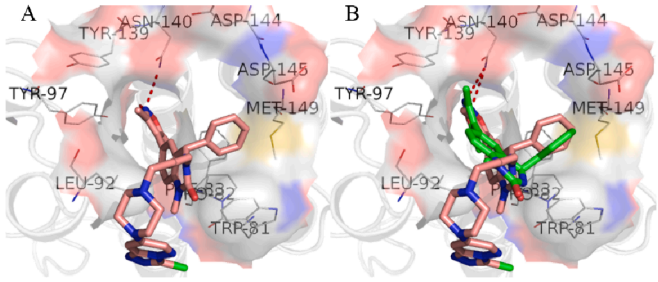
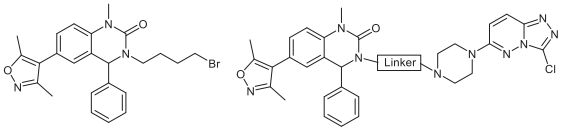


Fig. 3. Molecular docking conformation. (A) Conformation of compound **39** (orange) in BRD4(BD1) protein (2YEL). (B) The superposition of compound **13** (green) and **39** (orange) in BRD4(BD1) protein (2YEL).

reaction was quenched by water (40 mL), followed by dichloromethane (20 mL) solution. The organic phase was separated and dried over anhydrous sodium sulfate. The crude was purified by column chromatography (petroleum ether/ethyl acetate = 10/1–8/1) to obtain compound **8a** (2.4 g, 86% yield) as a light yellow solid.^{1}H NMR (300 MHz, DMSO-d_6) δ 7.61–7.45 (m, 5H), 7.38 (dd, J = 8.9, 2.4 Hz, 1H), 7.28 (d, J = 2.4 Hz, 1H), 7.14 (s, 2H), 6.76 (d, J = 8.9 Hz, 1H).

*(E)-N-((2-Amino-5-bromophenyl)(phenyl) methylene)-2-methylpropane-2-sulfinamide (**9**).* **8** (5 g, 18.11 mmol) and *tert*-butyl sulfinateamine (6.58 g, 54.32 mmol) were added to the tetrahydrofuran (60 mL) solution and stirred for ten minutes at room temperature. Then tetraethyltitanate (11.47 mL, 54.32 mmol) was added to the above reaction solution, the temperature was slowly increased to 80 °C and the mixture was stirred for 8 h. After cooling the reaction media down to ambient temperature, abundant tetrahydrofuran solution was evaporated under reduced pressure distillation. The resulting crude product was purified by column chromatography (petroleum ether/ethyl acetate = 15/1–1/1) to give

Table 2
BRD4(BD1) inhibitory activities of compounds **38–41**, **43**, and **47–49**.



38 39~41, 43, 47~49

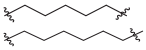
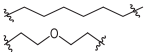
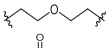
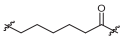
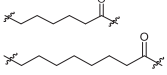
Compound No.	Linker	BRD4(BD1) 1 μM(%)	BRD4(BD1) IC_{50} μM
38		100	0.013
39		100	0.003
40		100	0.0088
41		100	0.0031
43		100	0.012
47		100	0.0056
48		100	0.0092
49		98	0.037
13		100	0.053
JQ1		100	0.011

Table 3
Anti-proliferation activities of **39–41**, **43**, and **47–49** in cancer cell lines.

Compound No.	Inhibitory% (2 μM) MCF10A	CT26	MCF7	HepG2	HCT116	(0.1 μM) U266
13	6.8	44.0	33.5	32.5	27.8	13.1
14	12.4	30.1	34.1	34.7	31.4	26.7
15	3.7	21.2	34.0	50.6	30.0	15.1
16	4.9	20.1	35.1	29.2	28.3	7.19
17	3.3	28.1	35.7	35.2	26.9	20.3
18	2.7	15.5	29.1	44.5	26.7	12.7
19	6.5	28.8	33.4	26.4	26.0	10.0
20	8.5	16.4	27.4	41.1	33.3	12.6
21	4.4	23.5	17.5	34.0	28.4	10.4
22	6.8	17.0	37.9	31.3	31.8	8.4
23	0.7	24.0	26.7	52.8	36.6	26.8
39	11.6	41.0	35.5	46.7	61.8	52.3
40	9.3	33.8	33.4	39.8	39.0	48.4
41	5.3	20.3	33.1	51.9	44.7	45.5
43	6.0	25.1	34.7	36.7	18.7	29.5
47	10.9	27.5	33.4	28.5	48.9	19.8
48	14.7	25.1	33.6	30.7	38.4	31.3
49	0.5	8.8	28.7	13.6	14.1	8.5
JQ1	6.5	37.9	35.4	22.2	30.3	35.7

Table 4
The IC_{50} of **14**, **39–41**, and **43** in different cancer cell lines.

Compound No.	IC_{50} (μM) ± SD [a] CT26	HepG2	U266
14	21.18 ± 6.31	19.49 ± 3.28	3.45 ± 0.27
39	15.77 ± 4.00	16.73 ± 3.45	2.11 ± 0.02
40	11.77 ± 0.84	14.04 ± 1.01	1.99 ± 0.01
41	7.95 ± 1.64	9.45 ± 0.33	2.12 ± 0.21
43	22.89 ± 4.01	20.98 ± 3.44	3.05 ± 0.37
13	35.98 ± 18.78	18.77 ± 3.28	35.35 ± 0.22
JQ1	28.67 ± 27.84	18.39 ± 1.88	3.45 ± 0.48
ABBV-075	48.26 ± 9.82	NT [b]	2.03 ± 0.18

[a] Three titrations were performed and data were used to calculate average IC_{50} and SD values.
[b] NT: no test.

compound **9** (6.1 g, 89% yield) as a light yellow solid.

*N-((2-Amino-5-bromophenyl)(phenyl) methyl)-2-methylpropane-2-sulfinamide (**10**).* To a stirred solution of compound **9** (1 g, 2.64 mmol) in tetrahydrofuran (20 mL) was then slowly added sodium borohydride (0.4 g, 10.55 mmol) and the reaction mixture was stirred for six hours at room temperature. After completion of the reaction,

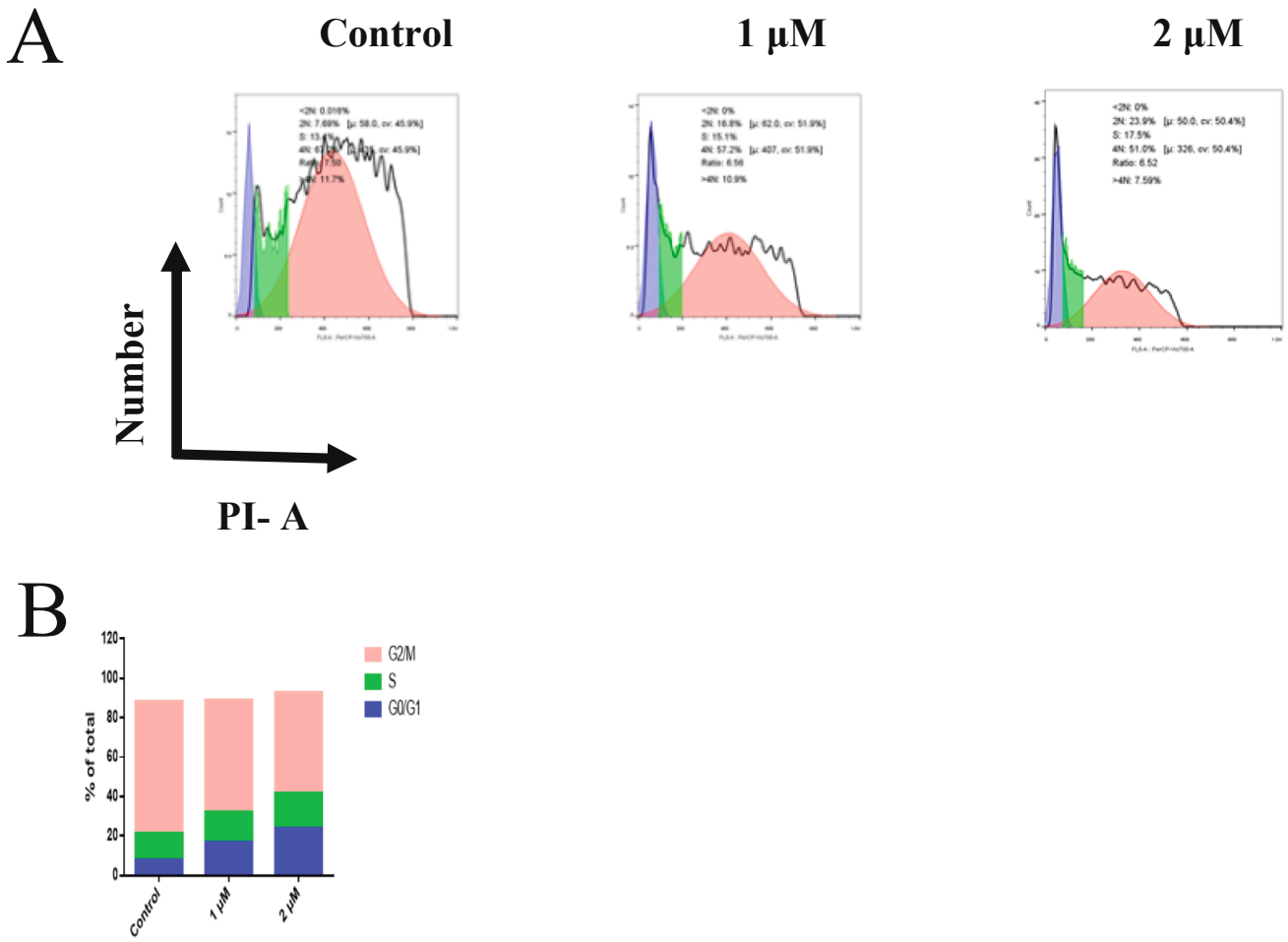


Fig. 4. Compound **39** induced cell cycle arrest in U266 cells. (A, B) U266 cells were stimulated with 1 and 2 μM of compound **39** for 48 h, and the cell cycle ratio of U266 cells was detected by cell cycle staining kit and flow cytometry.

tetrahydrofuran was removed from the reaction solution by reduced pressure distillation. The residue was purified by column chromatography (petroleum ether/ethyl acetate = 11/4–2/1) to afford compound **10** (0.9 g, 90% yield) as a white solid.^{1}H NMR (300 MHz, DMSO) δ 7.36 (d, J = 6.9 Hz, 4H), 7.26 (s, 1H), 7.11 (dd, J = 20.2, 11.6 Hz, 2H), 6.65–6.56 (m, 1H), 5.99 (dd, J = 26.1, 6.2 Hz, 1H), 5.54 (dd, J = 30.2, 6.2 Hz, 1H), 5.25 (s, 2H), 1.13 (d, J = 7.0 Hz, 9H).

6-Bromo-4-phenyl-3,4-dihydroquinazolin-2(1H)-one ***(11)***. Bis (trichloromethyl) carbonate (1.17 g, 3.93 mmol) was slowly added to a solution of compound **10** (1 g, 2.62 mmol) in tetrahydrofuran (15 mL) and the reaction mixture was stirred for eight hours at ambient temperature. After the reaction was completed, the pH of the reaction solution was adjusted to pH 7.6–10.4 with saturated sodium carbonate aqueous solution. Then water (20 mL) and ethyl acetate (20 mL) were added to the above mixture, and the organic phase was separate, the aqueous phase was extracted twice with ethyl acetate (20 mL). The organic phase was evaporated and purified by column chromatography (petroleum ether/ethyl acetate = 5/1–2/1) to obtain compound **11** (0.65 g, 82% yield) as a white solid.

6-Bromo-1-methyl-4-phenyl-3,4-dihydroquinazolin-2(1H)-one ***(12)***. To a solution of **11** (1 g, 3.30 mmol) and cesium carbonate (3.22 g, 9.90 mmol) in acetonitrile (15 mL), dimethyl sulfate (0.5 g, 3.96 mmol) was slowly dripped and the above reaction was stirred for 6 h at room temperature. Upon completion, the solvent was evaporated and the residue was isolated by column chromatography (petroleum ether/ethyl acetate = 5/1–2/1) to give compound **12** (0.8 g, 76% yield) as a white solid. ^{1}H NMR (300 MHz, DMSO) δ 7.80 (s, 1H), 7.54–7.15 (m, 7H), 6.94 (d, J = 8.5 Hz, 1H), 5.53 (s, 1H), 3.19 (s, 3H).

6-(3,5-Dimethylisoxazol-4-yl)-1-methyl-4-phenyl-3,4-dihydroquinazolin-2(1H)-one ***(13)***. To a solution of **12** (0.3 g, 0.95 mmol) in a mixture of toluene (3 mL) and ethanol (1 mL) was added, 3,5-dimethylisoxazole-4-boronic acid pinacol ester (0.23 g, 1.0 mmol), 1 mol/L aqueous sodium carbonate solution (3 mL) and tetraphenylphosphine palladium (0.109 g, 0.096 mmol) were added in batches. The mixture was heated to 80℃ and stirred for 12 h under nitrogen. After completion, the reaction solution was concentrated and purified by column chromatography (petroleum ether/ethyl acetate = 5/1–1/1) to obtain compound **13** (0.21 g, 67% yield) as a white solid in 97% purity. IR (KBr, cm^{-1}) ν: 3415.94, 1679.16, 1638.13, 1617.58, 1522.13, 1476.05, 1437.20, 1393.99, 1334.82, 1275.37, 1123.55, 721.72, 699.70, 599.58, 568.80, 541.92, 524.80, 489.63; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37 (s, 5H), 7.26 (s, 1H), 7.14 (s, 1H), 7.01 (s, 1H), 6.65 (s, 1H), 5.62 (s, 1H), 3.41 (s, 3H), 2.27 (s, 3H), 2.12 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 165.22, 158.58, 154.52, 144.71, 138.14, 132.51, 132.03, 131.92, 129.29, 129.17, 129.06, 127.86, 127.60, 126.43, 124.92, 123.39, 115.77, 114.04, 55.92, 40.62, 40.42, 40.21, 40.00, 39.79, 39.58, 39.37, 29.61, 11.73, 10.90.MS (ESI, m/z): 334.0$[M+H]^+$.

6-(3,5-Dimethylisoxazol-4-yl)-1-methyl-4-phenyl-3-((5-phenyl-1,3,4-oxadiazol-2-yl) methyl)-3,4-dihydroquinazolin-2(1H)-one ***(14)***. To a solution of compound **13** (0.2 g, 0.6 mmol) in N, *N*-dimethylformamide (6 mL), cesium carbonate (0.59 g, 1.80 mmol) was slowly added and stirred for 10 min at room temperature. Compound **25a** (0.14 g, 0.72 mmol) was added to the above reaction solution, and the reaction was stirred for further two hours at room temperature. After the reaction was

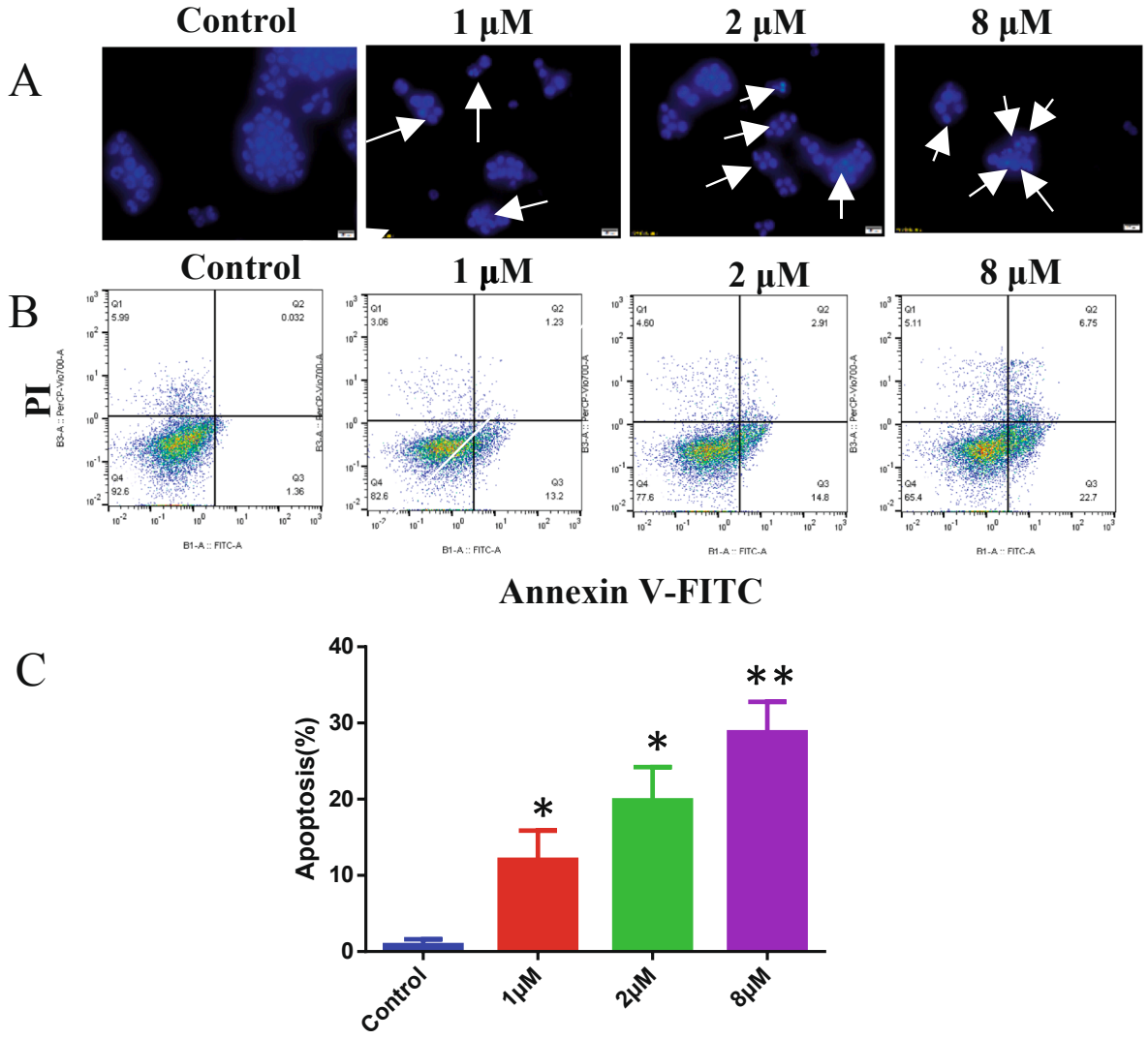


Fig. 5. Compound **39** induced apoptosis in U266 cells. (A) U266 cells were stimulated with 1, 2 and 8 μM of compound **39** for 48 h, and the nuclear condensation was detected by DAPI staining, and representative photographs were shown. (B, C) U266 cells were treated with compound **39** at 1, 2 and 8 μM for 48 h, and apoptotic cells were determined by flow cytometry (*p < 0.05, **p < 0.001).

completed, the above reaction solution was added dropwise to water (20 mL), then ethyl acetate (20 mL) was added and stirred for 10 min at room temperature to separate the organic phase. The organic phase was collected and purified by column chromatography (petroleum ether/ethyl acetate = 5/1–1/1) to obtain compound **14** (0.18 g, 61% yield) as a light yellow solid in 97% purity. m.p.: 136–138 °C. IR (KBr, cm^{-1}) *ν*: 3415.62, 1660.20, 1644.05, 1612.81, 1551.70, 1518.37, 1477.56, 1443.76, 1422.74, 1402.98, 1330.44, 1272.67, 1240.61, 1207.66, 1151.59, 1106.89, 1011.42, 830.04, 793.52, 772.73, 736.57, 709.30, 699.81, 689.52, 601.80, 565.95, 526.19, 506.75; ^{1}H NMR (500 MHz, DMSO) *δ* 7.88 (d, *J* = 7.4 Hz, 2H), 7.62 (dt, *J* = 23.5, 7.4 Hz, 3H), 7.40 (d, *J* = 7.6 Hz, 2H), 7.36–7.30 (m, 4H), 7.23 (t, *J* = 7.3 Hz, 1H), 7.16 (d, *J* = 8.3 Hz, 1H), 5.93 (s, 1H), 5.19 (d, *J* = 16.3 Hz, 1H), 4.64 (d, *J* = 16.3 Hz, 1H), 3.40 (s, 3H), 2.35 (s, 3H), 2.18 (s, 3H). ^{13}C NMR (75 MHz, DMSO) *δ* 164.81, 164.18, 163.25, 158.01, 153.30, 141.37, 136.51, 131.91, 129.30, 128.87, 128.76, 127.89, 126.84, 126.43, 125.99, 124.10, 123.63, 123.15, 115.07, 114.00, 61.56, 41.15, 40.35, 40.08, 39.80, 39.52, 39.25, 38.97, 38.70, 30.27, 11.21, 10.33. MS (ESI, *m/z*): 492.2[M+H] $^{+}$.

6-(3,5-Dimethylisoxazol-4-yl)-1-methyl-4-phenyl-3-((5-phenyloxazol-2-yl) methyl)-3,4–dihydroqu inazolin-2(1H)-one ***(15)***. The synthesis method used was similar to that used in the preparation of compound **14** and compound **15** (0.12 g, 74% yield) was given as a grayish-white solid in 99% purity. m.p.: 90–92 °C. IR (KBr, cm^{-1}) *ν*: 3414.82, 1661.77, 1615.17, 1519.30, 1475.23, 1447.42, 1400.72, 1340.83, 1269.33, 1106.88, 763.00, 695.82, 617.81; ^{1}H NMR (300 MHz, DMSO) *δ* 7.67–7.51 (m, 4H), 7.36 (t, *J* = 15.8 Hz, 9H), 7.11 (s, 1H), 5.85 (s, 1H), 5.12 (d, *J* = 15.1 Hz, 1H), 4.32 (d, *J* = 17.4 Hz, 1H), 3.35 (d, *J* = 1.9 Hz, 3H), 2.32 (d, *J* = 3.2 Hz, 3H), 2.15 (d, *J* = 3.2 Hz, 3H). ^{13}C NMR (75 MHz, MeOD) *δ* 163.48, 156.50, 152.48, 139.29, 134.87, 134.83, 126.99, 126.73, 126.54, 126.32, 125.97, 125.40, 125.15, 124.17, 122.29, 121.84, 119.08, 111.79, 60.84, 46.45, 41.18, 27.60, 7.92, 7.17. MS (ESI, *m/z*): 491.3[M+H] $^{+}$.

6-(3,5-Dimethylisoxazol-4-yl)-1-methyl-3-((4-methyl-2-phenylthiazol-5-yl) methyl)-4-phenyl-3,4-dihydroquinazolin-2(1H)-one ***(16)***. The preparation method was similar to that of compound **14** and compound **16** (0.14 g, 79% yield) was synthesized as a gray-white solid in 98% purity. m.p.: 104–106 °C. IR (KBr, cm^{-1}) *ν*: 3414.82, 1639.62, 1615.83, 1517.62, 1441.78, 1400.30, 1338.36, 1267.91, 1233.38, 1192.11, 1154.95, 1102.46, 1003.90, 838.17, 699.88, 618.46, 582.16, 522.64; ^{1}H NMR (500 MHz, DMSO) *δ* 7.87 (dd, *J* = 8.7, 5.4 Hz, 2H), 7.36 (d, *J* = 7.4 Hz, 2H), 7.33–7.23 (m, 7H), 7.07 (d, *J* = 8.5 Hz, 1H), 5.75 (s, 1H), 5.10 (d, *J* = 15.7 Hz, 1H), 4.28 (d, *J* = 15.7 Hz, 1H), 3.28 (s, 3H), 2.31 (s, 3H), 2.24 (s, 3H), 2.14 (s, 3H). ^{13}C NMR (75 MHz, DMSO) *δ* 164.75, 163.52, 161.36, 157.99, 153.38, 150.97, 141.84, 136.43, 129.69, 128.80, 128.61, 128.18, 128.07, 127.96, 127.82, 126.78, 125.74, 124.06,

123.39, 116.22, 115.93, 115.05, 113.77, 60.80, 41.86, 40.35, 40.06, 39.78, 39.51, 39.23, 38.95, 30.13, 14.87, 11.22, 10.34. MS (ESI, *m/z*): 521.3[M+H] $^{+}$.

6-(3,5-Dimethylisoxazol-4-yl)-1-methyl-4-phenyl-3-((5-phenyl-1,3,4-thiadiazol-2-yl) methyl)-3,4-dihydroquinazolin-2(1H)-one ***(17)***. Compound **17** (0.083 g, 89% yield) was obtained as a light yellow solid in 98% purity by similar synthesis procedure used in the preparation of compound **14**. m.p.: 238–240 °C. IR (KBr, cm^{-1}) *ν*: 3414.33, 1655.48, 1612.21, 1517.71, 1475.19, 1466.37, 1455.32, 1442.19, 1419.93, 1404.05, 1331.82, 1301.69, 1271.04, 1239.18, 1109.93, 978.53, 829.83, 770.50, 753.69, 736.41, 693.67, 641.92, 635.13, 617.51, 562.01, 595.52; ^{1}H NMR (300 MHz, DMSO) *δ* 7.89 (d, *J* = 5.5 Hz, 2H), 7.52 (d, *J* = 6.3 Hz, 3H), 7.31 (dd, *J* = 17.2, 7.0 Hz, 6H), 7.23 (d, *J* = 6.2 Hz, 1H), 7.12 (d, *J* = 9.0 Hz, 1H), 5.91 (s, 1H), 5.26 (d, *J* = 15.7 Hz, 1H), 4.61 (d, *J* = 16.0 Hz, 1H), 2.31 (s, 3H), 2.14 (s, 3H). ^{13}C NMR (75 MHz, DMSO) *δ* 166.79, 164.83, 158.29, 153.55, 141.78, 136.54, 132.01, 131.98, 131.51, 131.38, 131.26, 129.38, 128.90, 128.80, 128.76, 128.64, 127.94, 127.53, 126.84, 126.12, 124.07, 123.65, 114.04, 61.79, 45.88, 40.05, 39.77, 39.49, 39.21, 38.94, 30.20, 11.24, 10.36. MS (ESI, *m/z*): 508.4[M+H] $^{+}$.

6-(3,5-Dimethylisoxazol-4-yl)-1-methyl-4-phenyl-3-((4-phenylthiazol-2-yl)methyl)-3,4-dihydroq uinazolin-2(1H)-one ***(18)***. The synthesis method was similar to that of compound **14**. Compound **18** (0.091 g, 87% yield) was obtained as a pale yellow solid in 97% purity. m.p.: 94–96 °C. IR (KBr, cm^{-1}) *ν*: 3415.44, 1655.21, 1615.26, 1519.05, 1475.27, 1442.70, 1493.61, 1339.18, 1399.45, 1306.83, 1269.39, 1240.19, 1105.94, 827.66, 735.92, 696.64, 617.59, 478.80; ^{1}H NMR (300 MHz, DMSO) *δ* 7.99 (s, 1H), 7.88 (d, *J* = 7.1 Hz, 2H), 7.45–7.25 (m, 10*H*), 7.13 (d, *J* = 8.5 Hz, 1H), 5.92 (s, 1H), 5.33 (d, *J* = 16.1 Hz, 1H), 4.40 (d, *J* = 16.2 Hz, 1H), 3.38 (s, 3H), 2.33 (s, 3H), 2.16 (s, 3H). ^{13}C NMR (75 MHz, DMSO) *δ* 167.62, 164.80, 158.03, 153.95, 153.54, 141.47, 136.62, 133.97, 128.89, 128.73, 128.68, 127.95, 127.89, 126.75, 125.99, 125.94, 124.22, 123.60, 115.08, 114.67, 113.93, 61.56, 47.88, 40.05, 39.77, 39.49, 39.21, 38.93, 30.23, 11.23, 10.35. MS (ESI, *m/z*): 507.3[M+H] $^{+}$.

6-(3,5-Dimethylisoxazol-4-yl)-3-((2-(4-fluorophenyl)-4-methylthiazol-5-yl) methyl)-1-methyl-4-ph enyl-3,4-dihydroquinazolin-2(1H)-one ***(19)***. The synthesis method used was similar to that used in the preparation of compound **14** and compound **19** (0.101 g, 83% yield) was given as a light yellow solid in 99% purity. m.p.: 98–100 °C. IR (KBr, cm^{-1}) *ν*: 3416.13, 2924.92, 1654.99, 1613.46, 1519.11, 1473.93, 1460.42, 1441.76, 1399.91, 1339.14, 1305.31, 1268.24, 1239.47, 1192.13, 1145.85, 1103.16, 1003.60, 826.86, 763.11, 692.01, 524.63; ^{1}H NMR (300 MHz, DMSO) *δ* 7.82 (d, *J* = 3.9 Hz, 2H), 7.47–7.41 (m, 3H), 7.40–7.28 (m, 5H), 7.24 (s, 2H), 7.07 (d, *J* = 8.4 Hz, 1H), 5.76 (s, 1H), 5.12 (d, *J* = 15.7 Hz, 1H), 4.28 (d, *J* = 15.6 Hz, 1H), 3.37 (s, 3H), 2.31 (s, 3H), 2.25 (s, 3H), 2.14 (s, 3H). ^{13}C NMR (75 MHz, DMSO) *δ* 164.75, 158.07, 153.44, 150.97, 141.84, 136.45, 133.04, 129.96, 129.08, 128.81, 128.62, 128.05, 127.83, 126.78, 125.75, 124.06, 123.38, 115.15, 113.77, 60.78, 41.85, 40.35, 40.07, 39.79, 39.52, 39.24, 38.96, 38.67, 30.14, 14.90, 11.22, 10.34. MS (ESI, *m/z*): 521.3[M+H] $^{+}$.

6-(3,5-Dimethylisoxazol-4-yl)-3-((4-(4-fluorophenyl)thiazol-2-yl) methyl)-1-methyl-4-phenyl-3,4-dihydroquinazolin-2(1H)-one ***(20)***. The synthesis method used was similar to that used in the preparation of compound **14** and compound **20** (0.77 g, 82% yield) was given as a light yellow solid in 98% purity. m.p.: 98–100 °C. IR (KBr, cm^{-1}) *ν*: 3416.42, 1655.58, 1613.36, 1519.30, 1495.81, 1474.18, 1442.93, 1399.71, 1339.11, 1308.76, 1269.76, 1223.25, 1155.48, 1105.89, 840.50, 749.99, 700.67; ^{1}H NMR (300 MHz, DMSO) *δ* 8.00–7.88 (m, 3H), 7.40–7.21 (m, 9H), 7.13 (d, *J* = 8.5 Hz, 1H), 5.91 (s, 1H), 5.30 (d, *J* = 16.1 Hz, 1H), 4.41 (d, *J* = 16.0 Hz, 1H), 3.38 (s, 3H), 2.33 (s, 3H), 2.16 (s, 3H). ^{13}C NMR (75 MHz, DMSO) *δ* 167.77, 164.80, 163.47, 160.22, 158.02, 153.52, 152.87, 141.49, 136.60, 130.57, 128.88, 128.73, 128.04, 127.93, 127.88, 126.74, 125.98, 124.21, 123.59, 115.68, 115.39, 115.07, 114.51, 113.94, 61.56, 47.89, 40.32, 40.05, 39.77, 39.49, 39.21, 38.93, 38.66, 30.22, 11.23, 10.35. MS (ESI, *m/z*): 525.3 [M+H] $^{+}$.

6-(3,5-Dimethylisoxazol-4-yl)-1-methyl-4-phenyl-3-((2-phenylthiazol-4-yl)methyl)-3,4-dihydroqu inazolin-2(1H)-one ***(21)***. The synthesis method used was similar to that used in the preparation of compound **14** and compound **21** (0.79 g, 85% yield) was given as a light yellow solid in 97% purity. m.p.: 170–172 °C. IR (KBr, cm^{-1}) *ν*: 3415.09, 3079.91, 1654.81, 1614.12, 1519.00, 1475.40, 1462.70, 1421.84, 1400.07, 1334.20, 1270.06, 1205.32, 1105.49, 1002.44, 764.46, 733.63, 692.70, 619.11, 518.74; ^{1}H NMR (300 MHz, DMSO) *δ* 7.87 (d, *J* = 3.4 Hz, 2H), 7.46 (dd, *J* = 6.0, 2.9 Hz, 4H), 7.36 (dd, *J* = 14.2, 6.8 Hz, 5H), 7.26 (d, *J* = 7.3 Hz, 2H), 7.07 (d, *J* = 8.4 Hz, 1H), 5.91 (s, 1H), 5.24 (d, *J* = 15.4 Hz, 1H), 4.11 (d, *J* = 15.5 Hz, 1H), 2.32 (s, 3H), 2.16 (s, 3H). ^{13}C NMR (75 MHz, DMSO) *δ* 167.20, 164.73, 158.04, 153.56, 153.49, 141.76, 136.89, 132.91, 130.18, 129.11, 128.86, 128.59, 127.73, 126.72, 126.03, 125.90, 124.25, 123.33, 117.00, 115.15, 113.67, 60.88, 45.58, 40.36, 40.07, 39.79, 39.51, 39.23, 38.95, 38.67, 30.17, 11.22, 10.34. MS (ESI, *m/z*): 507.2[M+H] $^{+}$.

6-(3,5-Dimethylisoxazol-4-yl)-3-((2-(4-fluorophenyl)thiazol-4-yl) methyl)-1-methyl-4-phenyl-3,4-dihydroquinazolin-2(1H)-one ***(22)***. The synthesis method used was similar to that used in the preparation of compound **14** and compound **22** (0.66 g, 91% yield) was given as a light yellow solid in 96% purity. m.p.: 166–168 °C. IR (KBr, cm^{-1}) *ν*: 3415.70, 3103.02, 2923.64, 1664.50, 1614.13, 1470.96, 1520.90, 1448.72, 1419.85, 1307.80, 1400.61, 1338.31, 1237.51, 1229.08, 1205.29, 1297.86, 1267.51, 1154.15, 1103.56, 1026.74, 1001.17, 837.18, 734.42, 749.03, 694.74, 613.31, 586.04, 566.12, 524.06, 497.79; ^{1}H NMR (500 MHz, DMSO) *δ* 7.94 (dd, *J* = 8.7, 5.4 Hz, 2H), 7.50 (s, 1H), 7.43–7.27 (m, 9H), 7.10 (d, *J* = 8.4 Hz, 1H), 5.90 (s, 1H), 5.23 (d, *J* = 15.5 Hz, 1H), 4.14 (d, *J* = 15.4 Hz, 1H), 3.37 (s, 3H), 2.34 (s, 3H), 2.18 (s, 3H). ^{13}C NMR (75 MHz, DMSO) *δ* 165.99, 164.73, 161.48, 158.03, 153.55, 153.46, 141.77, 136.87, 129.60, 128.85, 128.60, 128.39, 128.28, 127.72, 126.72, 125.88, 124.24, 123.32, 117.16, 116.27, 115.97, 115.14, 113.68, 60.87, 45.56, 40.33, 40.06, 39.79, 39.51, 39.23, 38.95, 38.68, 30.16, 11.21, 10.33. MS (ESI, *m/z*): 525.6[M+H] $^{+}$.

6-(3,5-Dimethylisoxazol-4-yl)-3-((5-(4-fluorophenyl)-1,3,4-oxadiazol-2-yl) methyl)-1-methyl-4-ph enyl-3,4-dihydroquinazolin-2(1H)-one ***(23)***. The synthesis method was similar to that of compound **14**. The target compound **23** (0.78 g, 83% yield) was obtained as a light yellow solid in 98% purity. m.p.: 90–92 °C. IR (KBr, cm^{-1}) *ν*: 3415.54, 1638.16, 1616.26, 1521.00, 1498.63, 1475.38, 1400.25, 1341.28, 1269.45, 1238.85, 1157.51, 1107.62, 845.01, 735.91, 614.72; ^{1}H NMR (300 MHz, DMSO) *δ* 7.91 (dd, *J* = 8.8, 5.4 Hz, 2H), 7.46–7.26 (m, 9H), 7.13 (d, *J* = 8.4 Hz, 1H), 5.90 (s, 1H), 5.15 (d, *J* = 16.3 Hz, 1H), 4.63 (d, *J* = 16.2 Hz, 1H), 3.37 (s, 3H), 2.33 (s, 3H), 2.16 (s, 3H). ^{13}C NMR (75 MHz, MeOD) *δ* 139.32, 127.27, 127.15, 127.05, 126.78, 126.09, 125.20, 124.34, 124.28, 122.43, 122.17, 114.26, 114.20, 114.15, 113.96, 113.84, 111.90, 61.08, 60.20, 46.44, 39.19, 27.60, 8.00, 7.12. MS (ESI, *m/z*): 510.6[M+H] $^{+}$.

6-Chloro-[1,2,4]triazolo [4,3-b]pyridazin-3(2H)-one ***(35)***. To a solution of 3,6-Dichloropyridazine (5 g, 33.56 mmol) in ethanol (30 mL), aminourea hydrochloride (7.5 g, 67.13 mmol) was added slowly and stirred at room temperature for three days. After the reaction was completed, the reaction mixture was filtered and washed with cold water. Then the crude was dried to give compound **35** (4.3 g, 75% yield) as a light yellow solid. ^{1}H NMR (300 MHz, DMSO) *δ* 7.90 (d, *J* = 9.8 Hz, 1H), 7.21 (d, *J* = 9.8 Hz, 1H).

3,6-Dichloro-[1,2,4]triazolo[4,3-b]pyridazine ***(36)***. Compound **35** (4.3 g, 33.56 mmol) was dissolved in phosphorus trichloride (15 mL) and refluxed for 4 h. After the reaction was completed, the reaction was cooled to room temperature and quenched with ice water (20 mL) and ethyl acetate (20 mL). The organic phase was collected and purified by column chromatography (petroleum ether/ethyl acetate = 30/1–10/1) to obtain compound **36** (3.1 g, 65% yield) as a white solid.

3-Chloro-6-(piperazin-1-yl)-[1,2,4]triazolo[4,3-b]pyridazine ***(37)***. To a stirred solution of Piperazine (0.41 g, 4.76 mmol) and triethylamine (1.47 mL, 10.58 mmol) in acetonitrile (15 mL), compound **36** (1 g, 5.29

mmol) diluted with acetonitrile (5 mL) was added dropwise, and the reaction was conducted for ten hours at room temperature. After the completion of the reaction, the mixture was evaporated and dried to obtain compound **37** (0.98 g, 77% yield) as a light yellow. ^{1}H NMR (300 MHz, DMSO) δ 9.51 (s, 1H), 8.20 (d, J = 10.2 Hz, 1H), 7.49 (d, J = 10.2 Hz, 1H), 3.85 (s, 4H), 3.23 (s, 4H).

*3-(4-Bromobutyl)-6-(3,5-dimethylisoxazol-4-yl)-1-methyl-4-phenyl-3,4-dihydroquinazolin-2(1H)-one (**38**).* Compound **13** (0.5 g, 1.50 mmol) was dissolved in N, *N*-dimethylformamide (10 mL) and stirred under ice bath for ten minutes. Then, sodium hydroxide (66 mg, 1.65 mmol) dissolved in N, *N*-dimethylformamide (2 mL) was added to the above reaction solution and stirred under ice bath for further ten minutes. Then, 1,4-dibromobutane (0.32 g, 1.50 mmol) in N, *N*-dimethylformamide (1 mL) was slowly added to the above reaction solution and stirred for eight hours at room temperature. After the reaction was completed, water (20 mL) and ethyl acetate (20 mL) were added to the above reaction solution. The organic phase was separated and purified by column chromatography (petroleum ether/ethyl acetate = 10/1–5/1) to get compound **38** (0.4 g, 57% yield) colorless oil in 97% purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.27 (dt, J = 8.6, 6.0 Hz, 5H), 7.10 (dd, J = 8.3, 1.9 Hz, 1H), 7.02–6.88 (m, 2H), 5.46 (s, 1H), 3.93 (dt, J = 13.6, 6.9 Hz, 1H), 3.53–3.31 (m, 5H), 2.93 (dd, J = 13.7, 6.0 Hz, 1H), 2.30 (s, 3H), 2.17 (d, J = 1.6 Hz, 3H), 1.82 (dd, J = 12.8, 5.2 Hz, 2H), 1.78–1.65 (m, 2H). ^{13}C NMR (75 MHz, DMSO) δ 164.72, 158.29, 153.55, 151.75, 145.53, 142.25, 129.19, 128.85, 128.54, 128.18, 127.66, 126.59, 125.69, 113.24, 86.33, 60.76, 40.37, 40.10, 39.82, 39.54, 39.26, 38.98, 38.71, 34.75, 34.68, 30.07, 29.99, 29.58, 28.13, 26.12, 11.27, 11.05, 10.40, 10.23. MS (ESI, m/z): 468.3$[M+H]^+$.

*3-(4-(4-(3-Chloro-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)piperazin-1-yl)butyl)-6-(3,5-dimethylisoxa zol-4-yl)-1-methyl-4-phenyl-3,4-dihydroquinazolin-2(1H)-one (**39**).* To a stirred solution of compound **38** (0.14 g, 0.3 mmol) and **37** (0.085 g, 0.36 mmol) in N, *N*-dimethylformamide (10 mL), sodium hydroxide (13.1 mg, 0.33 mmol) in N, *N*-dimethylformamide (2 mL) was slowly dropped at 0 °C. The reaction mixture was warmed to room temperature and stirred for ten hours. After the reaction was completed, water (20 mL) and ethyl acetate (20 mL) were added to the above reaction solution and the organic phase was separated. The organic phase was evaporated and purified by column chromatography (dichloromethane/methanol = 80/1–20/1) to obtain compound **39** (0.12 g, 64% yield) as a gray-white solid in 98% purity. m.p.: 108–110 °C. IR (KBr, cm^{-1}) ν: 3452.44, 1641.00, 1553.03, 1477.53, 1405.66, 1268.61, 812.61, 736.48, 699.67, 599.29; ^{1}H NMR (300 MHz, DMSO) δ 8.10 (d, J = 10.1 Hz, 1H), 7.45 (d, J = 10.3 Hz, 1H), 7.40–7.30 (m, 5H), 7.26 (d, J = 7.6 Hz, 2H), 7.04 (d, J = 8.3 Hz, 1H), 5.75 (d, J = 5.4 Hz, 1H), 3.88–3.76 (m, 1H), 3.53 (s, 4H), 3.30 (s, 3H), 2.86 (dd, J = 13.3, 6.7 Hz, 1H), 2.42 (s, 3H), 2.36 (s, 3H), 2.28 (s, 2H), 2.19 (s, 3H), 1.63–1.38 (m, 4H), 0.89–0.81 (m, 1H). ^{13}C NMR (75 MHz, DMSO) δ 164.75, 158.00, 155.51, 153.57, 143.58, 142.49, 137.07, 134.46, 128.82, 128.53, 127.61, 126.59, 125.71, 124.36, 123.06, 115.29, 113.43, 60.76, 57.24, 56.40, 51.98, 46.38, 45.07, 40.39, 40.13, 39.84, 39.57, 39.29, 39.02, 38.75, 29.97, 25.32, 11.26, 10.39. MS (ESI, m/z): 626.3$[M+H]^+$.

*3-(5-(4-(3-Chloro-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)piperazin-1-yl)pentyl)-6-(3,5-dimethylisox azol-4-yl)-1-methyl-4-phenyl-3,4-dihydroquinazolin-2(1H)-one (**40**).* The synthesis method was similar to that of compound **39**, and compound **40** was obtained (0.083 g, 85% yield) as a light yellow solid in 97% purity. m.p .: 110–112 °C. IR (KBr, cm^{-1}) ν: 3448.78, 1648.38, 1552.74, 1477.15, 1406.65, 1266.38, 814.20, 736.52, 699.67, 570.38, 512.94; ^{1}H NMR (300 MHz, DMSO) δ 8.08 (d, J = 10.1 Hz, 1H), 7.47–7.17 (m, 8H), 7.02 (d, J = 8.4 Hz, 1H), 5.73 (s, 1H), 3.81 (d, J = 13.1 Hz, 1H), 3.51 (s, 4H), 3.29 (s, 3H), 2.89–2.75 (m, 1H), 2.50 (s, 2H), 2.40 (s, 3H), 2.34 (s, 3H), 2.17 (s, 3H), 1.37 (d, J = 22.8 Hz, 3H), 1.21 (s, 5H). ^{13}C NMR (75 MHz, DMSO) δ 164.67, 158.05, 155.43, 153.67, 143.43, 142.42, 137.06, 128.82, 125.71, 124.35, 115.27, 113.43, 52.03, 45.10, 40.11, 39.84, 39.57, 39.28, 39.01, 29.96, 27.03, 25.72, 24.03, 11.25, 10.38. MS (ESI, m/z): 640.3$[M+H]^+$.

*3-(6-(4-(3-Chloro-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)piperazin-1-yl)hexyl)-6-(3,5-dimethylisox azol-4-yl)-1-methyl-4-phenyl-3,4-dihydroquinazolin-2(1H)-one (**41**).* The synthetic method for compound **41** was similar to that of compound **39** and compound **41** (0.099 g, 90% yield) was got as a pale yellow solid in 96% purity. m.p.: 116–118 °C. IR (KBr, cm^{-1}) ν: 3461.12, 1647.48, 1552.78, 1520.76, 1476.91, 1406.59, 1338.53, 1301.99, 1268.35, 1241.83, 1104.85, 736.75, 511.09; ^{1}H NMR (300 MHz, DMSO) δ 8.10 (d, J = 10.2 Hz, 1H), 7.46 (d, J = 10.3 Hz, 1H), 7.36 (dd, J = 9.8, 3.1 Hz, 5H), 7.29–7.22 (m, 2H), 7.03 (d, J = 8.4 Hz, 1H), 5.73 (s, 1H), 3.85–3.71 (m, 1H), 3.55 (s, 4H), 3.29 (s, 3H), 2.82 (dd, J = 13.7, 5.7 Hz, 1H), 2.43 (s, 4H), 2.36 (s, 3H), 2.25 (t, J = 7.2 Hz, 2H), 2.19 (s, 3H), 1.48 (d, J = 44.2 Hz, 4H), 1.25 (s, 4H). ^{13}C NMR (75 MHz, DMSO) δ 164.75, 158.01, 155.50, 153.65, 143.44, 142.45, 137.13, 128.82, 128.50, 127.61, 126.57, 125.68, 124.37, 123.01, 115.33, 113.42, 60.72, 57.52, 52.07, 46.45, 45.10, 40.36, 40.09, 39.82, 39.54, 39.26, 38.98, 38.71, 29.96, 27.28, 26.52, 26.08, 25.92, 11.27, 10.40. MS (ESI, m/z): 654.1$[M+H]^+$.

*3-(2-(2-(4-(3-Chloro-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)piperazin-1-yl)ethoxy)ethyl)-6-(3,5-dimethylisoxazol-4-yl)-1-methyl-4-phenyl-3,4-dihydroquinazolin-2(1H)-one (**43**).* The synthesis method of compound **43** was the same as that for compound **39** and compound **43** (0.087 g, 78% yield) was obtained as a pale yellow solid in 94% purity. m.p.: 108–110 °C. IR (KBr, cm^{-1}) ν: 3450.55, 1650.44, 1552.85, 1478.25, 1408.67, 1338.17, 1267.09, 1240.42, 1106.90, 813.55, 736.26, 699.76, 560.52, 514.59; ^{1}H NMR (300 MHz, DMSO) δ 8.08 (d, J = 10.2 Hz, 1H), 7.35 (t, J = 6.2 Hz, 6H), 7.25 (dd, J = 12.2, 6.9 Hz, 2H), 7.04 (d, J = 8.4 Hz, 1H), 5.81 (s, 1H), 3.98 (dd, J = 9.2, 5.1 Hz, 1H), 3.58–3.38 (m, 8H), 3.30 (s, 3H), 3.01–2.91 (m, 1H), 2.46 (s, 6H), 2.31 (s, 3H), 2.14 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 165.17, 158.46, 155.92, 154.12, 143.93, 142.72, 137.44, 135.00, 129.39, 129.07, 128.19, 127.07, 126.20, 124.87, 123.64, 115.75, 115.60, 114.05, 68.61, 58.55, 52.61, 46.52, 45.14, 40.61, 40.40, 40.19, 39.98, 39.77, 39.56, 39.36, 30.53, 11.76, 10.90. MS (ESI, m/z): 642.1$[M+H]^+$.

*2-(6-(3,5-Dimethylisoxazol-4-yl)-1-methyl-2-oxo-4-phenyl-1,4-dihydroquinazolin-3(2H)-yl)acetic acid (**44**).* Compound **13** (0.15 g, 0.45 mmol) was dissolved in N,*N*-dimethylformamide (10 mL) and stirred under ice bath for ten minutes. Then, sodium hydroxide (21.6 mg, 0.54 mmol) dissolved in N, *N*-dimethylformamide (2 mL) was slowly added to the above reaction solution and stirred under ice bath for ten minutes. Then, ethyl bromide acetate (0.11 g, 0.67 mmol) was added to the above mixture and the reaction was stirred at room temperature for ten hours. After the reaction was completed, water (20 mL) and ethyl acetate (20 mL) were added to the above reaction solution. The organic phase was separated and evaporated under reduced pressure distillation. Methanol (4 mL) and water (2 mL) were added to the residue, and lithium hydroxide (21.5 mg, 0.9 mmol) was added and stirred for two hours at room temperature. After the reaction was completed, the mixture was concentrated and 1 M of dilute hydrochloric acid was added to adjust the pH to pH < 5 and the reaction was filtered to give compound 44 (0.15 g, 85% yield) as a grayish-white solid.

*3-(2-(4-(3-Chloro-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)piperazin-1-yl)-2-oxoethyl)-6-(3,5-dime thylisoxazol-4-yl)-1-methyl-4-phenyl-3,4-dihydroquinazolin-2(1H)-one (**47**).* To a stirred solution of compound **44** (0.3 g, 0.77 mmol), EDCI (0.2 g, 1.07 mmol) and triethylamine (319 mmL, 2.30 mmol) in N, *N*-dimethylformamide (10 mL), HOBT (0.12 g, 0.92 mmol) was added at 0 °C and the reaction mixture was stirred in an ice bath for thirty minutes. Compound **37** (0.2 g, 0.84 mmol) was added to the above mixture at 0 °C and the reaction was stirred at room temperature for ten hours. After the reaction was completed, the reaction was quenched with water (20 mL) and ethyl acetate (20 mL) and the organic phase was collected. The organic phase was evaporated and purified by column chromatography (dichloromethane/methanol = 60/1–20/1) to obtain compound 47 (0.23 g, 49% yield) as a white solid in 98% purity. m.p.: 116–118 °C. IR (KBr, cm^{-1}) ν: 3453.55, 1647.89, 1553.16, 1520.96, 1477.42, 1407.53, 1237.36, 812.37, 736.54, 700.94, 572.56, 527.59; ^{1}H NMR (300 MHz, DMSO) δ 8.13 (d, J = 10.2 Hz, 1H), 7.45 (d, J = 10.2 Hz, 1H), 7.40–7.22 (m, 7H), 7.08 (d, J = 8.9 Hz, 1H), 5.68 (s, 1H), 3.62 (d, J = 16.4 Hz, 8H), 3.30 (s, 3H), 2.94 (s, 2H), 2.32 (s, 3H), 2.15 (s, 3H). ^{13}C NMR

(75 MHz, DMSO) δ 166.59, 164.72, 158.03, 155.34, 153.33, 143.47, 142.03, 136.98, 134.50, 128.85, 128.56, 127.79, 126.81, 126.21, 124.52, 124.13, 123.21, 115.42, 115.18, 113.58, 61.80, 47.38, 44.90, 44.83, 43.21, 40.75, 40.36, 40.08, 39.80, 39.52, 39.25, 38.97, 38.69, 30.10, 11.23, 10.36. MS (ESI, *m/z*): 612.1$[M+H]^+$.

*3-(6-(4-(3-Chloro-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)piperazin-1-yl)-6-oxohexyl)-6-(3,5-dimeth ylisoxazol-4-yl)-1-methyl-4-phenyl-3,4-dihydroquinazolin-2(1H)-one (**48**)*. The synthesis procedure was similar to the method of compound **47** and compound **48** (0.091 g, 88% yield) was given as a pale yellow solid in 97% purity. m.p.: 102–104 °C. IR (KBr, cm^{-1}) ν: 3455.08, 2926.62, 1640.23, 1552.73, 1520.13, 1476.33, 1407.77, 1338.47, 1272.09, 1239.31, 1201.10, 1105.03, 817.45, 736.68; ^{1}H NMR (300 MHz, DMSO) δ 8.14 (d, J = 9.7 Hz, 1H), 7.46 (d, J = 10.7 Hz, 1H), 7.30 (d, J = 23.3 Hz, 7H), 7.02 (d, J = 8.3 Hz, 1H), 5.74 (s, 1H), 3.79 (s, 1H), 3.56 (s, 8H), 3.28 (s, 3H), 2.81 (s, 1H), 2.35 (s, 3H), 2.18 (s, 3H), 1.48 (s, 4H), 1.24 (s, 3H), 0.83 (s, 2H). ^{13}C NMR (75 MHz, DMSO) δ 170.79, 158.05, 155.43, 153.55, 142.45, 137.02, 128.84, 128.50, 127.63, 126.59, 125.69, 124.52, 124.36, 123.01, 115.41, 113.42, 60.69, 46.27, 45.14, 44.91, 43.98, 40.36, 40.28, 40.10, 39.82, 39.54, 39.26, 38.99, 32.01, 29.95, 27.23, 25.93, 24.37, 11.27, 10.41. MS (ESI, *m/z*): 668.3$[M+H]^+$.

*3-(8-(4-(3-Chloro-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)piperazin-1-yl)-8-oxooctyl)-6-(3,5-dimeth ylisoxazol-4-yl)-1-methyl-4-phenyl-3,4-dihydroquinazolin-2(1H)-one (**49**)*. Compound **49** was synthesized using the same methods as described for compound **47** and compound **49** (0.087 g, 83% yield) was obtained as a pale yellow solid in 98% purity. m.p.: 118–120 °C. IR (KBr, cm^{-1}) ν: 3460.99, 2927.88, 2854.78, 1642.31, 1553.15, 1520.82, 1476.74, 1407.57, 1338.89, 1271.97, 1239.22, 1104.91, 736.67, 700.29; ^{1}H NMR (300 MHz, DMSO) δ 8.16 (d, J = 10.2 Hz, 1H), 7.48 (d, J = 10.2 Hz, 1H), 7.41–7.31 (m, 5H), 7.30–7.23 (m, 2H), 7.03 (d, J = 8.4 Hz, 1H), 5.73 (s, 1H), 3.79 (s, 1H), 3.58 (s, 8H), 3.28 (s, 3H), 2.82 (d, J = 5.9 Hz, 1H), 2.36 (s, 3H), 2.31 (d, J = 7.8 Hz, 2H), 2.19 (s, 3H), 1.46 (s, 2H), 1.23 (d, J = 2.4 Hz, 8H). ^{13}C NMR (75 MHz, DMSO) δ 170.87, 164.69, 158.01, 155.38, 153.57, 142.45, 137.04, 128.81, 128.50, 127.60, 126.57, 125.68, 124.50, 124.37, 123.01, 115.41, 113.41, 60.74, 46.47, 45.16, 44.96, 43.98, 40.36, 40.09, 39.81, 39.53, 39.25, 38.98, 38.70, 32.15, 29.94, 28.59, 28.48, 27.30, 26.11, 24.54, 11.26, 10.39. MS (ESI, *m/z*): 696.3$[M+H]^+$.

4.2. Biological methods

4.2.1. BRD4 protein inhibitory activities

A single or gradient concentration of the active compound to be tested was performed in a 384-well plate. The concentration of DMSO in the final solution was ≤0.1%. Then BRD4(BD1) protein solution was prepared with the 1X buffer. 5 μL of protein solution was added dropwise to each well, and 5 μL of buffer was added to the control group as the blank control. After leaving the plate at room temperature for 15 min, 5 μL of substrate solution was added to each well sequentially and incubated for another 1 h. In the dark environment, 15 μL of acceptor and donor solution were added to each well and incubated for another hour at room temperature.[23] Finally, the absorbance value was measured using an EnSpire full-wavelength microplate reader, and the inhibitory activities of compounds were calculated by the formula Inh % = (maximum signal value-compound signal value)/(maximum signal value-minimum signal value) × 100.

4.2.2. Anti-proliferation activities in cancer cell lines

Cryopreserved cells were quickly thawed in a water bath pan (37 °C) and placed in an incubator. After one day, cells were plated at 5000–10,000 cells per well of 96-well plates once sufficient cells were generated. After plating, shake it slowly and incubated overnight in the incubator. After cell attachment, a single concentration of compounds of different gradient concentrations were added to the 96-well plate described above and incubated in the incubator for another 48 h. MTT solution was added to each well of the 96-well plate described above and the absorbance was determined using an enzyme-labeled analyzer.

4.2.3. Cell cycle analysis

U266 cells (1 × 10^5/2 mL per well) were seeded in six-well plates and treated with compound **39** at 1 and 2 μM, respectively. After 48 h, the cells were harvested and fixed in 70% ethanol for 30 min on ice. After washing with phosphate buffer saline (PBS), the cells were labeled with propidium iodide (PI, 0.05 mg/mL) in the presence of RNaseA (0.5 mg/mL) and incubated at room temperature in the dark for 30 min. DNA contents were analyzed using the flow cytometer (MACSQuant® X, Germany).

4.2.4. DAPI staining assay

U266 cells were cultured in 12-well culture plates (20000 cells/well) overnight before treating with compound **39** at 1, 2 and 8 μM for 48 h, and then washed with PBS. After all, the cell incubated with DAPI for 5 min and washed with PBS. Cells were observed and photographed by a fluorescent microscope (Olympus IX53).

4.2.5. Annexin V-FITC apoptosis detection assay

After staining with annexin V-FITC and PI using the Annexin V-FITC apoptosis detection kit (KeyGEN BioTECH, China), cells were detected by flow cytometry to assess the membrane and nuclear events during apoptosis. U266 cells (1 × 10^5/2 mL per well) were seeded in six-well plates and treated with compound **39** at 1, 2 and 8 μM, respectively. After 48 h, cells were collected, washed twice with cold PBS, and resuspended in 500 μL of a binding buffer which was then added to 5 μL of annexin V-FITC and incubated at 37 °C in the dark for 15 min. Subsequently, the buffer was added to 5 μL of PI and incubated at 37 °C in the dark for 5 min. The samples were analyzed by a FACScan flow cytometer (MACSQuant® X, Germany).

4.2.6. Docking study

Molecular docking experiments were used to mimic the way the compounds interact with the BRD4 protein. The structure of the compound was first sketched into Chemdraw and saved in sdf file format. The above small molecules were minimized using the MMFF force field of the Ligand prepare module in the Schrodinger software. Amino acid modifications and force fields were applied for BRD4 protein using the Protein prepare module. Glide files for the BRD4(BD1) protein were generated using the Glide generation module. Finally, small molecule compounds were docked with the BRD4 protein to produce an optimal conformation.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2021.116133.

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