Design and Synthesis of Novel Bispecific Molecules for Inducing BRD4 Protein Degradation

WANG Shihui[1], SONG Yuming[2], WANG Yue[1], GAO Yang[1], YU Shanshan[1], ZHAO Qianqian[1], JIN Xiangqun[1*] and LU Haibin[1*]

1. College of Pharmacy, Jilin University, Changchun 130021, P. R. China;

2. China-Japan Union Hospital of Jilin University, Changchun 130033, P. R. China

Abstract Proteolysis targeting chimeras(PROTACs) are bispecific molecules containing a target protein binder and a ubiquitin ligase binder connected by a linker. Recently, some heterobifunctional small molecule bromodomain-containing protein 4(BRD4) degraders based on the concept of PROTACs were designed to induce the degradation of BRD4 protein. Herein, we synthesized a new class of PROTAC BRD4 degraders. One of the most promising compound **22**f exhibited robust potency of BRD4 inhibition with IC_{50} value of (9.4±0.6) nmol/L. Furthermore, compound **22**f potently inhibited cell proliferation in BRD4-sensitive cell lines RS4;11 with IC_{50} value of (27.6±1.6) nmol/L and capable of inducing degradation of BRD4 protein at 0.5—1.0 μmol/L in the RS4;11 cells. These data establish that compound **22**f is a potent and efficacious BRD4 degrader.

Keywords Proteolysis targeting chimera(PROTAC); Bromodomain-containing protein 4(BRD4) degrader; Bromodomain-containing protein 4(BRD4) inhibitor

1 Introduction

Bromodomain-containing protein 4(BRD4) belongs to the bromodomain and extraterminal domain(BET) family of proteins, which is a transcriptional co-activator involved in dynamic transcriptional activation and elongation[1]. BRD4 binds to enhancer and promoter regions adjacent to target genes, *via* recognition of side-chain acetylated lysine on histone proteins and transcription factors(TFs) by twin acetyl-lysine binding modules or bromodomains[2]. It has emerged as an exciting new therapeutic target for cancer and other human diseases[3—5]. Several BRD4 inhibitors, such as **1**[(+)-JQ-1][6] and **2**(OTX015)[7](Fig.1) have been advanced into clinical development.

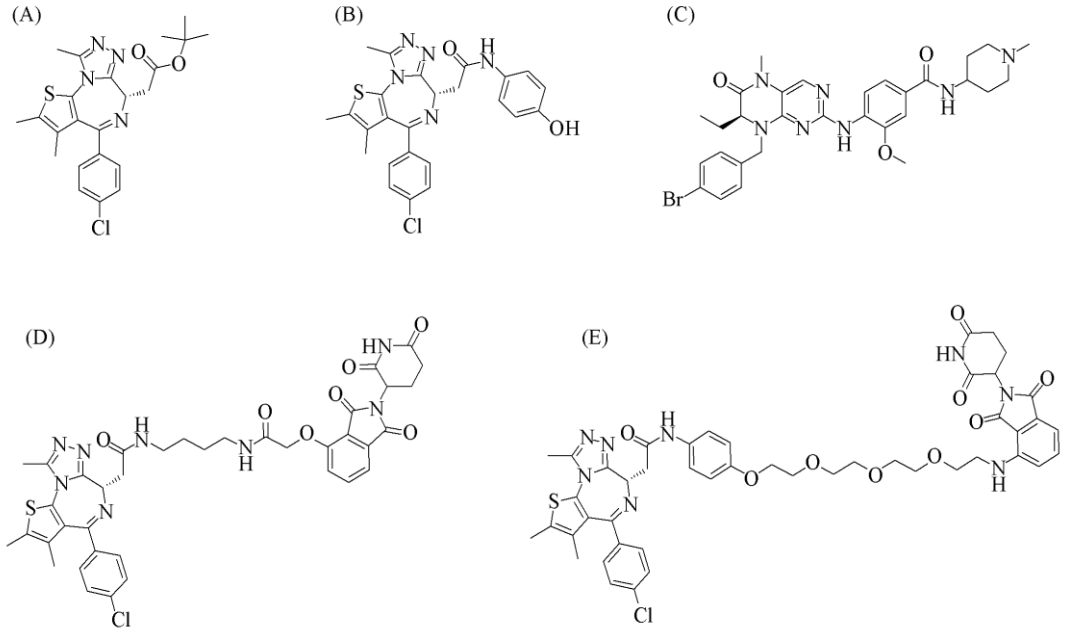

Fig.1 Chemical structures of (+)-JQ-1(A), OTX015(B), BI2536 derivative(C), dBET1(D) and ARV-825(E)

*Corresponding authors. E-mail: jingxq@jlu.edu.cn; haibin1025@163.com

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In addition to small-molecule BRD4 inhibitors, a new approach has recently been developed to target BRD4 protein for degradation based upon the proteolysis targeting chimeras(PROTACs) concept[8]. PROTACs are bispecific molecules containing a target protein binder and an ubiquitin ligase binder connected by a linker. By recruiting an ubiquitin ligase to a target protein, PROTACs promote ubiquitination and proteasomal degradation of the target protein. In this approach, PROTAC molecule is designed to contain a BRD4 inhibitor, which binds to BRD4 protein, another small-molecule ligand, which binds to an E3 ubiquitin ligase complex, and a linker to tether these two ligands together[9,10]. A number of BRD4 degraders have been reported, including compound **4**(dBET1)[11] and compound **5**(ARV-825)[12]. These BRD4 degraders have shown the ability of efficiently inducing BRD4 protein degradation and to be more potent in inhibition of cancer cell growth and in induction of apoptosis than their corresponding BRD4 inhibitors. Compound **3**[13] is a BI2536 derivative, which is a potent BRD4 inhibitor. In this study, we reported the discovery of a new class of small-molecule BRD4 degraders designed based upon compound **3** and thalidomide as ligand for cereblon/Cullin4A. Their biological activities were also evaluated.

2 Experimental

2.1 Materials and Instruments

All necessary chemical materials in the experiments were of analytical grade. ^{1}H NMR spectra of the products were recorded on a Bruker 400 MHz spectrometer with tetramethylsilane(TMS) as internal standard. HRMS were determined on an Agilent Q-TOF-6250 spectrometer.

2.2 Synthesis of the Target Compounds

*2.2.1 Synthesis of (R)-Methyl 2-Aminobutanoate(**6**)*

D-2-Aminobutyric acid(10.0 g, 97 mmol) was suspended in 10 mL of methanol and slowly combined with thionyl chloride(15 mL, 204 mmol) at 0 ºC. The mixture was refluxed for 1.5 h and then the solvent was evaporated. The remaining oil was combined with 100 mL of ether, stirred for 0.5 h and filtered to obtain compound **6**(11 g, 97%). ^{1}H NMR(400 MHz, $CDCl_3$), δ: 8.72(s, 2H, NH_2), 4.25—4.02(m, 1H, CH), 3.68(s, 3H, CH_3), 2.26—2.01(m, 2H, CH_2), 1.09(t, *J*=7.0 Hz, 3H, CH_3).

2.2.2 Synthesis of (R)-Methyl 2-[(4-Bromobenzyl)-amino]butanoate(7)

Compound **6**(10 g, 85 mmol) and 4-bromobenzaldehyde (12 g, 66 mmol) were dissolved in 100 mL of dichloromethane. Sodium acetate(5.5 g, 66 mmol) and sodium triacetoxyborohydride(20 g, 95 mmol) were then added at 0 ºC. The reaction mixture was stirred for 16 h at room temperature and then 200 mL of 20% $NaHCO_3$ solution was added. The aqueous phase was extracted with dichloromethane. The combined organic phase was washed with water, dried over $MgSO_4$ and the solvent was removed under reduced pressure affording compound **7**(18 g, 96%). ^{1}H NMR(400 MHz, $CDCl_3$), δ: 7.48(s, 1H, Ph-H), 7.37(d, *J*=7.6 Hz, 1H, Ph-H), 7.27—7.20(m, 2H, Ph-H), 3.85(d, *J*=14.0 Hz, 1H, CH), 3.74(s, 3H, CH_3), 3.68(d, *J*=14.0 Hz, 1H, CH), 3.24(m, 1H, CH), 1.64—1.56(m, 2H, CH_2), 0.96(t, *J*=4.0 Hz, 3H, CH_3).

*2.2.3 Synthesis of (R)-Methyl 2-[(4-Bromobenzyl)(2-chloro-5-nitropyrimidin-4-yl)amino]butanoate(**8**)*

Compound **7**(8.8 g, 31 mmol) and potassium carbonate(4.3 g, 31 mmol) were suspended in 100 mL of acetone and combined with 2,4-dichloro-5-nitropyrimidin(6.5 g, 34 mmol) in 30 mL of acetone at 0 °C. The reaction mixture was stirred overnight at room temperature and the volatiles were removed under reduced pressure. Ethyl acetate was added to the residue and the mixture was washed with water. The aqueous phase was extracted with ethyl acetate. The combined organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude was purified by chromatography on silica gel[*V*(petroleum ether):*V*(ethyl acetate)=20:1] to give compound **8**(6.8 g, 50%) as a yellow powder. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 8.64(s, 1H, pyrimidine-H), 7.43—7.40(m, 2H, Ph-H), 7.26—7.21(m, 1H, Ph-H), 7.13(t, *J*=7.2 Hz, 1H, Ph-H), 4.78—4.66(m, 2H, CH_2), 4.56(d, *J*=15.6 Hz, 1H, CH), 3.83(s, 3H, CH_3), 2.28—2.21(m, 1H, CH), 2.08—2.02(m, 1H, CH), 1.06(t, *J*=6.8 Hz, 3H, CH_3).

*2.2.4 Synthesis of (R)-8-(4-Bromobenzyl)-2-chloro-7-ethyl-7,8-dihydropteridin-6(5H)-one(**9**)*

Compound **8**(5.0 g, 11 mmol) was dissolved in 50 mL of glacial acetic acid, then 1.5 g of iron powder was added at 70 °C. The mixture was stirred for 1 h at 70 °C, then for 1.5 h at 100 °C. The solution was filtered, evaporated under reduced pressure and purified by chromatography on silica gel [*V*(petroleum ether):*V*(ethyl acetate)=20:1] to give compound **9**(2.1 g, 50%) as a yellow powder. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 8.84(brs, 1H, NH), 7.69(s, 1H, pyrimidine-H), 7.25—7.18(m, 4H, Ph-H), 5.18—5.13(m, 1H, CH), 4.45(d, *J*=14.8 Hz, 1H, CH), 4.18—4.14(m, 1H, CH), 1.84—1.78(m, 2H, CH_2), 0.73(t, *J*=7.2 Hz, 3H, CH_3).

*2.2.5 Synthesis of (R)-8-(4-Bromobenzyl)-2-chloro-7-ethyl-5-methyl-7,8-dihydropteridin-6(5H)-one(**10**)*

Compound **9**(2.0 g, 5.3 mmol) was dissolved in 20 mL of *N*,*N*-dimethylformamide before methyl iodide(0.6 mL, 9.5 mmol) was added. The reaction was cooled down to –10 °C and NaH(60%, 0.4 g, 9.5 mmol) was added. The reaction mixture was stirred for 0.5 h at 0 °C, then for 1 h at room temperature. The reaction was then quenched by adding crushed ice. Solvent was evaporated under reduced pressure. Ethyl acetate was added to the residue and washed twice with water. The organic layer was dried using $MgSO_4$ and the volatiles were removed under reduced pressure affording compound **10**(2.0 g, 96%) as a light yellow solid . ^{1}H NMR(400 MHz, DMSO-d_6), δ: 7.92(s, 1H, pyrimidine-H), 7.63(s, 1H, Ph-H), 7.48(d, *J*=8.0 Hz, 1H, Ph-H), 7.38(d, *J*=7.6 Hz, 1H, Ph-H), 7.31(t, *J*=8.0 Hz, 1H, Ph-H), 5.18(d, *J*=16.0 Hz, 1H, CH), 4.45(d, *J*=14.8 Hz, 1H, CH), 4.28(t, *J*=4.4 Hz, 1H, CH), 3.28(s, 3H, CH_3), 1.84—1.78(m, 2H, CH_2), 0.72(t, *J*=6.8 Hz, 3H, CH_3).

*2.2.6 Synthesis of (R)-4-{[8-(4-Bromobenzyl)-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl]amino}-3-methoxybenzoic Acid(**11**)*

Compound **10**(2.0 g, 5.1 mmol) and 4-amino-3-methoxybenzoic acid(1.0 g, 6.0 mmol) were suspended in 3 mL of ethanol, 15 mL of water and 15 μL of concentrated hydrochloric acid, then refluxed for 48 h. Solvent was evaporated under reduced pressure and purified by chromatography on silica gel [*V*(methanol):*V*(dichloromethane)=1:10] to give compound **11**(1.9 g, 70%) as a yellow powder. ^{1}H NMR(400 MHz, $CDCl_3$), *δ*: 7.94(d, *J*=7.6 Hz, 1H, pyrimidine-H), 7.64(d, *J*=14.8 Hz, 2H, Ph-H), 7.56—7.49(m, 4H, Ph-H), 7.31(t, *J*=8.0 Hz, 1H, Ph-H), 5.16(d, *J*=15.6 Hz, 1H, CH), 4.43(d, *J*=16.0 Hz, 1H, CH), 4.29(t, *J*=4.0 Hz, 1H, CH), 3.92(s, 3H, CH_3), 3.26(s, 3H, CH_3), 1.82—1.78(m, 2H, CH_2), 0.72(t, *J*=7.2 Hz, 3H, CH_3).

*2.2.7 Synthesis of 2-(2,6-Dioxopiperidin-3-yl)-4-hydroxyisoindoline-1,3-dione(**14**)*

3-Hydroxyphthalic anhydride **12**(5.0 g, 61 mmol), 3-aminoperidine-2,6-dione hydrochloride **13**(10 g, 61 mmol) and triethylamine(10 mL, 67 mmol) were added in toluene(500 mL). The resulting mixture was heated to reflux for 12 h with Dean-Stark trap equipment. After cooling to room temperature, evaporation of most of the solvent afforded a crude product, which was purified by flash column chromatography [*V*(methanol):*V*(dichloromethane)=1:10] to obtain compound **14** as a slightly white solid(15 g, 90%). ^{1}H NMR(400 MHz, DMSO-d_6), *δ*: 11.16(s, 1H, NH), 11.08(s, 1H, OH), 7.65(t, *J*=7.6 Hz, 1H, Ph-H), 7.32(d, *J*=7.2 Hz, 1H, Ph-H), 7.25(d, *J*=8.4 Hz, 1H, Ph-H), 5.07(dd, J_1=12.8 Hz, J_2=5.2 Hz, 1H, CH), 2.93—2.84(m, 1H, CH), 2.61—2.46(m, 1H, CH), 2.05—2.01(m, 1H, CH).

*2.2.8 Synthesis of tert-Butyl 2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]oxy}acetate(**15**)*

Compound **14**(15 g, 55 mmol) was dissolved in 100 mL of *N*,*N*-dimethylformamide. KI(0.91 g, 5.5 mmol) and $KHCO_3$(8.3 g, 82.5 mmol) were added to the stirred solution. Then tert-butyl bromoacetate(9.8 mL, 66 mmol) was added dropwise. The resulting mixture was stirred at 60 °C for 12 h. After cooling to room temperature, 500 mL of saturated brine was added, then extracted by ethyl acetate, the combined organic phase was dried over Na_2SO_4. After filtration and evaporation, the residue was purified by flash column chromatography[*V*(methanol):*V*(dichloromethane)=1:20] to get compound **15** as a white solid(17 g, 80%). ^{1}H NMR(400 MHz, DMSO-d_6), *δ*: 11.13(s, 1H, NH), 7.80(t, *J*=8.0 Hz, 1H, Ph-H), 7.48(d, *J*=7.2 Hz, 1H, Ph-H), 7.38(d, *J*=8.4 Hz, 1H, Ph-H), 5.13(dd, J_1=12.8 Hz, J_2=5.2 Hz, 1H, CH), 4.97(s, 2H, CH_2), 2.97—2.85(m, 1H, CH), 2.65—2.52(m, 2H, CH_2), 2.14—2.03(m, 1H, CH), 1.43(s, 9H, CH_3).

*2.2.9 Synthesis of 2-{[2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]oxy}acetic Acid(**16**)*

Compound **15**(10 g, 26 mmol) was dissolved in 50 mL of trifluoroacetic acid. The reaction mixture was stirred at room temperature for 2 h. After the evaporation of the solvent, the residue was washed by ether to afford compound **16**(8.2 g, 95%) as a white solid. ^{1}H NMR(400 MHz, DMSO-d_6), *δ*: 13.16(s, 1H, COOH), 11.11(s, 1H, NH), 7.80(t, *J*=8.0 Hz, 1H, Ph-H), 7.48(d, *J*=7.2 Hz, 1H, Ph-H), 7.40(d, *J*=8.8 Hz, 1H, Ph-H), 5.11(dd, J_1=12.8 Hz, J_2=5.2 Hz, 1H, CH), 4.99(s, 2H, CH_2), 2.95—2.86(m, 1H, CH), 2.63—2.48(m, 2H, CH_2), 2.08—2.03(m, 1H, CH).

*2.2.10 Preparation of Compounds **18**a and **18**b*

Compound **16**(0.5 g, 1.5 mmol) was dissolved in 5 mL of anhydrous *N*,*N*-dimethylformamide. Mono-Boc protected alkyl diamine(2.0 mmol), HATU(0.76 g, 2.0 mmol) and *N*,*N*-diisopropylethylamine(1 mL, 0.9 mmol) were added sequentially. The reaction mixture was stirred at room temperature for 2 h. After added 100 mL of saturated brine and extracted by ethyl acetate, the combined organic phase was dried over Na_2SO_4. After filtration and evaporation, the residue was purified by flash column chromatography [*V*(methanol):*V*(dichloromethane)=1:10], which was then dissolved in the mixture of 20 mL of dichloromethane and 10 mL of trifluoroacetic acid. After stirring for 1 h, the solvent was evaporated and the residue **17** was used as a stored solution(0.1 mol/L in DMF) in the next step without further purification.

HATU(0.6 g, 1.5 mmol), *N*,*N*-diisopropylethylamine(0.54 mL, 3.0 mmol), compound **11**(0.8 g, 1.5 mmol) and the amine intermediate **17**(0.1 mol/L in 15 mL of DMF, 1.5 mmol) were added sequentially to *N*,*N*-dimethylformamide(15 mL). The reaction mixture was stirred at room temperature for 2 h. After added saturated brine(100 mL) and extracted by ethyl acetate, the combined organic phase was dried over Na_2SO_4. After filtration and evaporation, the residue was purified by flash column chromatography[*V*(methanol):*V*(dichloromethane)= 1:20] to get compound **18**.

4-{[(*S*)-8-(4-Bromobenzyl)-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl]amino}-*N*-[4-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]oxy}acetamido)butyl]-3-methoxybenzamide(**18**a): yield 65%, purity 98%(HPLC), ^{1}H NMR(400 MHz, CD_3OD), *δ*: 8.01—7.96(m, 2H, Ph-H), 7.88(s, 1H, pyrimidine-H), 7.81(t, *J*=7.6 Hz, 1H, Ph-H), 7.62—7.56(m, 2H, Ph-H), 7.48(s, 1H, Ph-H), 7.39—7.34(m, 2H, Ph-H), 7.27—7.17(m, 2H, Ph-H), 5.14(d, *J*=15.6 Hz, 1H, CH), 5.11(dd, J_1=12.8 Hz, J_2=5.2 Hz, 1H, CH), 4.78(s, 2H, CH_2), 4.44(d, *J*=16.0 Hz, 1H, CH), 4.29(t, *J*=4.0 Hz, 1H, CH), 3.92(s, 3H, CH_3), 3.30—3.25(m, 5H, CH_2, CH_3), 3.22—3.16(m, 2H, CH_2), 2.89—2.85(m, 1H, CH), 2.60—2.45(m, 2H, CH_2), 2.05—2.01(m, 1H, CH), 1.51—1.49(m, 4H, CH_2), 1.82—1.78(m, 2H, CH_2), 0.72(t, *J*=7.6 Hz, 3H, CH_3). HRMS, *m/z*, calcd. for $C_{43}H_{44}BrN_9O_9$([M+H]$^+$) 910.2445; found 910.2442.

4-{[(*S*)-8-(4-Bromobenzyl)-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl]amino}-*N*-{2-[2-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]oxy}acetamido)-ethoxy]ethyl}-3-methoxybenzamide(**18**b): yield 55%, purity 96%(HPLC), ^{1}H NMR(400 MHz, CD_3OD), *δ*: 8.04—7.98(m, 2H, Ph-H), 7.89(s, 1H, pyrimidine-H), 7.82(dd, J_1=8.6 Hz, J_2=7.2 Hz, 1H, Ph-H), 7.62—7.54(m, 2H, Ph-H), 7.46(s, 1H, Ph-H), 7.41(d, *J*=8.6 Hz, 1H, Ph-H), 7.39—7.34(m, 1H, Ph-H), 7.27—7.16(m, 2H, Ph-H), 5.16(d, *J*=15.6 Hz, 1H, CH),

5.12(dd, J_1=12.8 Hz, J_2=5.6 Hz, 1H, CH), 4.81(s, 2H, CH_2), 4.44(d, J=16.0 Hz, 1H, CH), 4.30(t, J=4.0 Hz, 1H, CH), 3.92(s, 3H, CH_3), 3.59(t, J=5.2 Hz, 2H, CH_2), 3.51(t, J=5.7 Hz, 2H, CH_2), 3.26(s, 3H, CH_3), 3.36(q, J=5.7 Hz, 2H, CH_2), 2.99(t, J=5.7 Hz, 2H, CH_2), 2.94—2.84(m, 1H, CH), 2.64—2.47(m, 2H, CH_2), 2.11—1.99(m, 1H, CH), 1.82—1.76(m, 2H, CH_2), 0.72(t, J=7.6 Hz, 3H, CH_3). HRMS, *m/z*, calcd. for $C_{43}H_{44}BrN_9O_{10}$([M+H]$^+$): 926.2395, found: 926.2392.

*2.2.11 Synthesis of 2-(2,6-Dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione(**20**)*

3-Fluorophthalic anhydride **19**(5.0 g, 30 mmol), 3-aminopiperidine-2,6-dione hydrochloride **13**(5.0 g, 30 mmol) and sodium acetate(3.7 g, 45 mmol) were added to acetic acid(100 mL). The resulting mixture was heated to reflux for 12 h. After cooling to room temperature, acetic acid was evaporated and the residue was purified by flash column chromatography[*V*(methanol):*V*(dichloromethane)=1:10] to obtain compound **20** as a slightly yellow solid(7.5 g, 90%). ^{1}H NMR (400 MHz, DMSO-d_6), δ: 11.15(s, 1H, NH), 7.98—7.93(m, 1H, Ph-H), 7.80—7.72(m, 2H, Ph-H), 5.17(dd, J_1=13.2 Hz, J_2=5.2 Hz, 1H, CH), 2.95—2.86(m, 1H, CH), 2.64—2.47(m, 2H, CH_2), 2.10—2.06(m, 1H, CH).

*2.2.12 Preparation of Compounds **22a—22f***

Mono-Boc protected alkyl diamine(2.0 mmol) of different length was added to a stirred solution of compound **20**(0.5 g, 1.8 mmol) in 10 mL of *N*,*N*-dimethylformamide and 0.7 mL of *N*,*N*-diisopropylethylamine(4.0 mmol). The reaction mixture was stirred at 90 °C for 12 h. Then the mixture was cooled to room temperature, poured into water and extracted twice with ethyl acetate. The combined organic phase was washed with brine and dried over anhydrous Na_2SO_4. After filtration and evaporation, the crude residue was purified by flash column chromatography[*V*(methanol)/*V*(dichloromethane)=1:10] to give the intermediate, which was dissolved in dichloromethane(20 mL) and trifluoroacetic acid(10 mL). After stirring for 1 h, the solvent was evaporated to give the crude product **21**, which was used in the next step without further purification.

HATU(0.4 g, 1.0 mmol), *N*,*N*-diisopropylethylamine(0.36 mL, 0.15 mmol) and the amine intermediate **21**(0.5 mmol) were added sequentially to a stirred solution of compound **11**(0.26 g, 0.5 mmol) in *N*,*N*-dimethylformamide(20 mL). The solution was stirred at room temperature for 2 h, then poured into water and extracted twice with ethyl acetate. The combined organic phase was washed with brine and dried over anhydrous Na_2SO_4. After filtration and evaporation, the crude residue was purified by flash column chromatography[*V*(methanol):*V*(dichloromethane)=1:10] to give the pure product **22**.

4-{[(*S*)-8-(4-Bromobenzyl)-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl]amino}-*N*-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]amino}ethyl)-3-methoxybenzamide(**22**a): yield 60%, purity 98%(HPLC), ^{1}H NMR(400 MHz, CD_3OD), δ: 7.96(d, J=7.6 Hz, 1H, Ph-H), 7.88(s, 1H, pyrimidine-H), 7.63—7.58(m, 2H, Ph-H), 7.55—7.48(m, 2H, Ph-H), 7.39—7.34(m, 1H, Ph-H), 7.26—7.12(m, 3H, Ph-H), 7.01(d, J=7.1 Hz, 1H, Ph-H), 5.14(d, J=15.6 Hz, 1H, CH), 5.05—4.95(m, 1H, CH), 4.44(d, J=16.0 Hz, 1H, CH), 4.29(t, J=4.0 Hz, 1H, CH), 3.92(s, 3H, CH_3), 3.72—3.70(m, 2H, CH_2), 3.66—3.62(m, 2H, CH_2), 3.26(s, 3H, CH_3), 2.83—2.73(m, 2H, CH_2), 2.71—2.58(m, 2H, CH_2), 1.99—1.97(m, 1H, CH), 1.82—1.78(m, 2H, CH_2), 0.72(t, J=7.6 Hz, 3H, CH_3). HRMS, *m/z*, calcd. for $C_{39}H_{38}BrN_9O_7$([M+H]$^+$): 824.2078, found: 824.2075.

4-{[(*S*)-8-(4-Bromobenzyl)-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl]amino}-*N*-(4-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]amino}butyl)-3-methoxybenzamide(**22**b): yield 55%, purity 97%(HPLC), ^{1}H NMR(400 MHz, CD_3OD), δ: 7.96(d, J=7.6 Hz, 1H, Ph-H), 7.88(s, 1H, pyrimidine-H), 7.62—7.58(m, 2H, Ph-H), 7.56(t, J=7.2 Hz, 1H), 7.48(s, 1H, Ph-H), 7.39—7.34(m, 1H, Ph-H), 7.27—7.16(m, 2H, Ph-H)，7.12(d, J=8.4 Hz, 1H, Ph-H), 7.01(d, J=6.8 Hz, 1H, Ph-H), 5.15(d, J=15.6 Hz, 1H, CH), 5.05(dd, J_1=12.8 Hz, J_2=5.2 Hz, 1H, CH), 4.45(d, J=16.0 Hz, 1H, CH), 4.30(t, J=4.0 Hz, 1H, CH), 3.90(s, 3H, CH_3), 3.40—3.30(m, 4H, CH_2), 3.26(s, 3H, CH_3), 2.92—2.83(m, 1H, CH), 2.60—2.49(m, 2H, CH_2), 2.07—2.01(m, 1H, CH), 1.82—1.78(m, 2H, CH_2), 1.65—1.55(m, 4H, CH_2), 0.72(t, J=7.6 Hz, 3H, CH_3). HRMS, *m/z*, calcd. for $C_{41}H_{42}BrN_9O_7$([M+H]$^+$): 852.2391, found: 852.2394.

4-{[(*S*)-8-(4-Bromobenzyl)-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl]amino}-*N*-(6-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]amino}hexyl)-3-methoxybenzamide(**22**c): yield 70%, purity 97%(HPLC), ^{1}H NMR(400 MHz, CD_3OD), δ: 7.96(d, J=7.6 Hz, 1H, Ph-H), 7.86(s, 1H, pyrimidine-H), 7.62—7.54(m, 3H, Ph-H), 7.48(s, 1H, Ph-H), 7.38—7.32(m, 1H, Ph-H), 7.27—7.16(m, 2H, Ph-H)，7.08(d, J=8.4 Hz, 1H, Ph-H), 7.00(d, J=6.8 Hz, 1H, Ph-H), 5.14(d, J=15.6 Hz, 1H, CH), 5.04(dd, J_1=12.8 Hz, J_2=5.6 Hz, 1H, CH), 4.44(d, J=16.0 Hz, 1H, CH), 4.29(t, J=4.0 Hz, 1H, CH), 3.92(s, 3H, CH_3), 3.37—3.22(m, 7H, CH_3, CH_2), 2.92—2.83(m, 1H, CH), 2.59—2.49(m, 2H, CH_2), 2.05—2.02(m, 1H, CH), 1.82—1.78(m, 2H, CH_2), 1.73—1.68(m, 2H, CH_2), 1.56—1.48(m, J=7.3 Hz, 2H, CH_2), 1.45—1.25(m, 4H, CH_2), 0.72(t, J=7.6 Hz, 3H, CH_3). HRMS, *m/z*, calcd. for $C_{43}H_{46}BrN_9O_7$([M+H]$^+$): 880.2704, found: 880.2700.

4-{[(*S*)-8-(4-Bromobenzyl)-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl]amino}-*N*-(7-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]amino}heptyl)-3-methoxybenzamide(**22**d): yield 65%, purity 98%(HPLC), ^{1}H NMR(400 MHz, CD_3OD), δ: 7.94(d, J=7.6 Hz, 1H, Ph-H), 7.88(s, 1H, pyrimidine-H), 7.64—7.54(m, 3H, Ph-H), 7.48(s, 1H, Ph-H), 7.39—7.34(m, 1H, Ph-H), 7.27—7.16(m, 2H, Ph-H), 7.10(d, J=8.4 Hz, 1H, Ph-H), 7.02(d, J=6.8 Hz, 1H, Ph-H), 5.14(d, J=15.6 Hz, 1H, CH), 5.04(dd, J_1=12.8 Hz, J_2=5.6 Hz, 1H, CH), 4.44(d, J=16.0 Hz, 1H, CH), 4.30(t, J=4.0 Hz, 1H, CH), 3.92(s, 3H, CH_3), 3.31—3.25(m, 7H, CH_3, CH_2), 2.92—2.83(m, 1H, CH), 2.59—2.49(m, 2H, CH_2), 2.05—2.00(m, 1H, CH), 1.82—1.78(m, 2H, CH_2), 1.61—1.56(m, 2H, CH_2), 1.54—1.49(m, 2H, CH_2), 1.45—1.28(m, 6H, CH_2), 0.72(t, J=7.6 Hz, 3H, CH_3). HRMS, *m/z*, calcd. for $C_{44}H_{48}BrN_9O_7$([M+H]$^+$): 894.2860, found: 894.2864.

4-{[(*S*)-8-(4-Bromobenzyl)-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl]amino}-*N*-{2-[2-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]amino}ethoxy)-ethoxy]ethyl}-3-methoxybenzamide(**22**e): yield 68%, purity 98%(HPLC), ^{1}H NMR(400 MHz, CD_3OD), δ: 7.94(d, J=7.6 Hz, 1H, Ph-H), 7.87(s, 1H, pyrimidine-H), 7.64—7.60(m, 1H, Ph-H), 7.58—7.49(m, 5H, Ph-H), 7.34—7.30(m, 1H, Ph-H), 7.08(d, J=8.4 Hz, 1H, Ph-H), 7.02(d, J=6.8 Hz, 1H, Ph-H), 5.14(d, J=15.6 Hz, 1H, CH), 5.05(dd, J_1=12.8 Hz, J_2=5.6 Hz, 1H, CH), 4.43(d, J=16.0 Hz, 1H, Ph-H), 4.29(t, J=4.0 Hz, 1H, CH), 3.94(s, 3H, CH_3), 3.70—3.54(m, 8H, CH_2), 3.34—3.25(m, 5H, CH_3, CH_2), 3.03—2.86(m, 3H, CH, CH_2), 2.64—2.52(m, 2H, CH_2), 2.08—2.02(m, 1H, CH), 1.80—1.76(m, 2H, CH_2), 0.76(t, J=7.6 Hz, 3H, CH_3). HRMS, *m/z*, calcd. for $C_{43}H_{46}BrN_9O_9$([M+H]$^+$): 912.2602, found: 912.2605.

4-{[(*S*)-8-(4-Bromobenzyl)-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl]amino}-*N*-(2-{2-[2-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]amino}ethoxy)-ethoxy]ethoxy}ethyl)-3-methoxybenzamide(**22**f): yield 50%, purity 97%(HPLC), ^{1}H NMR(400 MHz, CD_3OD), δ: 7.98(d, J=7.6 Hz, 1H, Ph-H), 7.88(s, 1H, pyrimidine-H), 7.64—7.60(m, 1H, Ph-H), 7.58—7.49(m, 5H, Ph-H), 7.32—7.28(m, 1H, Ph-H), 7.08(d, J=8.4 Hz, 1H, Ph-H), 7.02(d, J=6.8 Hz, 1H, Ph-H), 5.14(d, J=15.6 Hz, 1H, CH), 5.06(dd, J_1=12.8 Hz, J_2=5.6 Hz, 1H, CH), 4.43(d, J=16.0 Hz, 1H, CH), 4.32(t, J=4.0 Hz, 1H, CH), 3.92(s, 3H, CH_3), 3.70—3.58(m, 12H, CH_2), 3.34—3.25(m, 5H, CH_3, CH_2), 3.02—2.92(m, 3H, CH, CH_2), 2.64—2.52(m, 2H, CH_2), 2.06—2.00(m, 1H, CH), 1.82—1.78(m, 2H, CH_2), 0.76(t, J=7.6 Hz, 3H, CH_3). HRMS, *m/z*, calcd. for $C_{45}H_{50}BrN_9O_{10}$([M+H]$^+$): 956.2864, found: 956.2860.

2.3 Biological Activity

2.3.1 Fluorescence Anisotropy Binding Assay[14]

The binding of compounds to BRD4 was assessed using a Fluorescence Anisotropy Binding Assay. All the components were dissolved in buffer of composition(50 mmol of HEPES, pH=7.4, 150 mmol of NaCl and 0.5 mmol of CHAPS) with final concentrations of BRD4 being 40 nmol/L, fluorescent ligand being 5 nmol/L. To this reaction mixture were added various concentrations of test compound or DMSO vehicle(5‰ final) in Corning 384 well Black low volume plate and equilibrated in dark for 4 h at room temperature. The fluorescence anisotropy was read on Multi-Mode Microplate Reader(λ_{ex}= 485 nm, λ_{em}=530 nm; Dichroic 505 nmol/L).

2.3.2 CCK-8 Assay [11]

The human acute leukemia RS4;11 cells(purchased from the ATCC) were seeded in 96-well cell culture plates at a density of 1×10^4 cells/well in 100 μL of culture medium(RPMI 1640 media supplemented with 10% FBS and 1% penicillin streptomycin). Each compound tested was serially diluted in the appropriate medium, and 100 μL of the diluted solution containing the tested compound was added to the appropriate wells of the cell plate. After the addition of the tested compound, the cells were incubated for 4 d at 37 °C in an atmosphere of 5% CO_2. The CCK-8 reagent was added to the plate, incubated for at least 1 h, and read at 450 nm. The readings were normalized to the DMSO-treated cells, and the IC_{50} was calculated by GraphPad Prism 6 software.

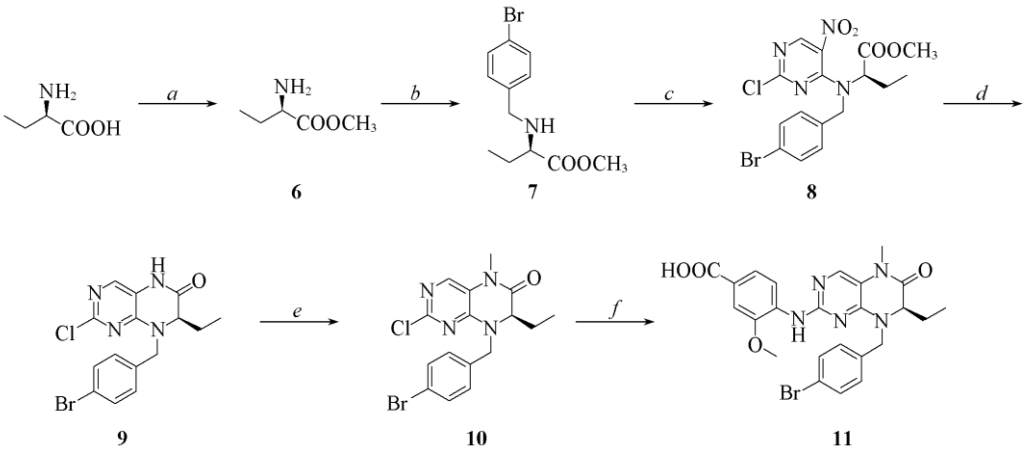
2.3.3 Western Blot Assay[11]

RS4;11 with a density of 2×10^6 cells/well was treated with compounds at the indicated concentrations for various times. The cells were collected and lysed in an RIPA buffer containing protease inhibitors. An amount of 20 μg of lysate was run in each lane of a polyacrylamide gel electrophoresis-sodium dodecyl sulfate(PAGE-SDS) and blotted into polyvinylidene fluoride(PVDF) membranes. The antibodies for immunoblotting BRD4 were purchased from AmyJet Scientific Inc.(BioVision, USA) and GAPDH was purchased from AmyJet Scientific Inc.(Abbkine USA).

3 Results and Discussion

3.1 Chemistry

The synthesis of compound **11** is shown in Scheme 1[13]. Esterification of *D*-2-aminobutyric acid, then reductive amination with 4-bromobenzaldehyde afforded compound **7**. Compound **7** reacted with 2,4-dichloro-5-nitro pyrimidine to form compound **8**. The reduction of the nitro group was performed using iron and glacial acetic acid leading to an *in situ*



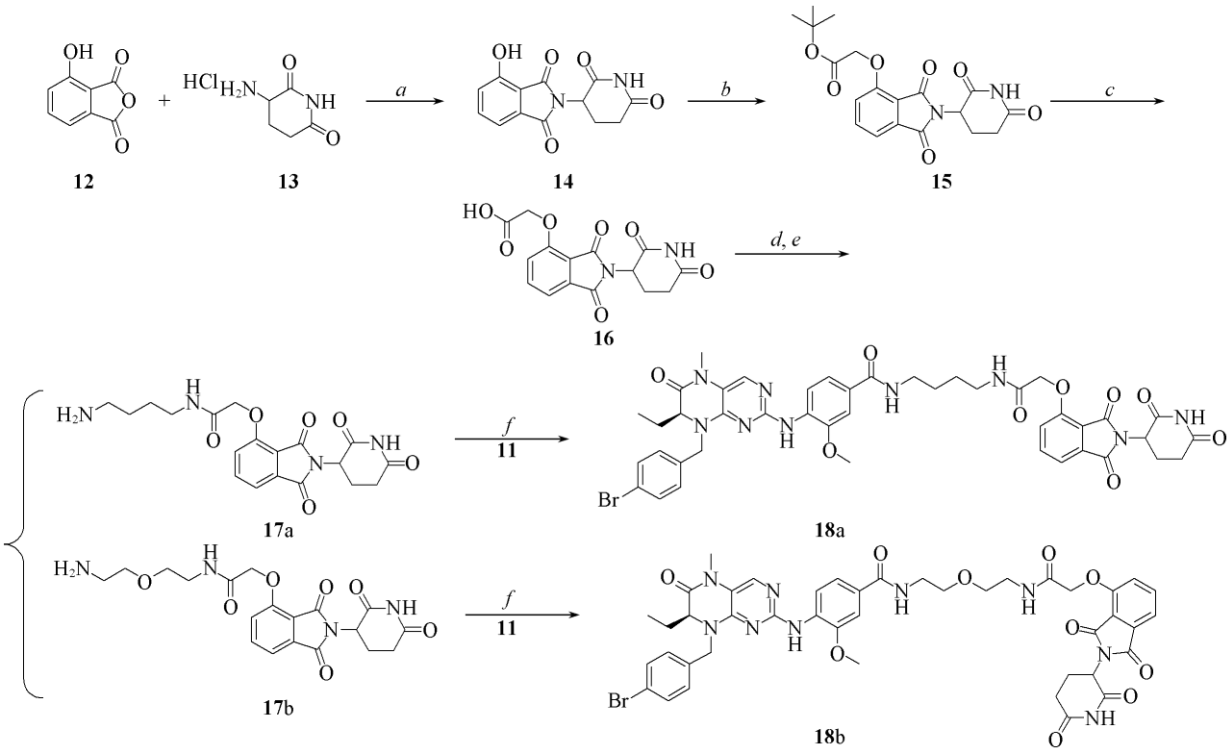
Scheme 1 Synthetic route for compound 11

Reaction conditions: *a*. $SOCl_2$, MeOH; *b*. 4-bromobenzaldehyde, $NaBH(OAc)_3$, NaOAc, DCM; *c*. 2,4-dichloro-5-nitropyrimidin, K_2CO_3, acetone; *d*. Fe, AcOH; *e*. NaH, DMF, CH_3I; *f*. EtOH, HCl, 4-amino-3-methoxybenzoic acid.

intramolecular cyclization affording compound **9**. Then methylation of the secondary amine and amination of the chloropyrimidine by 4-amino-3-methoxybenzoic acid afforded the desired compound **11**.

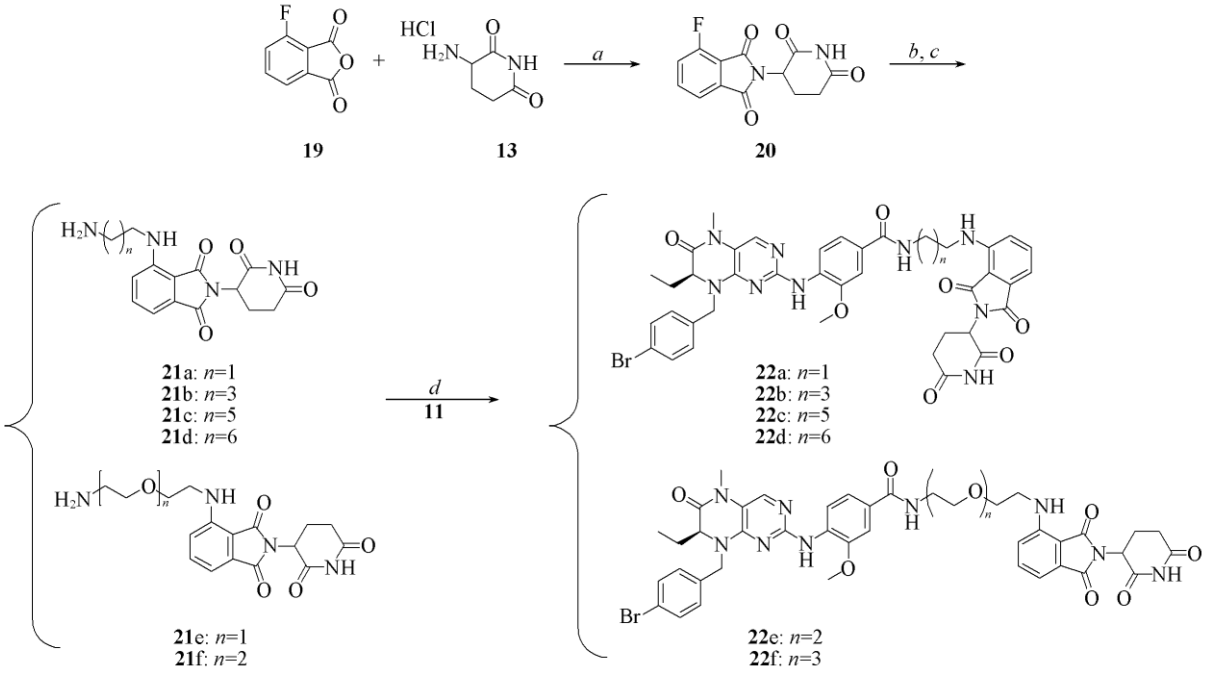
The synthesis of compounds **18**a and **18**b is shown in Scheme 2[15]. Briefly, 3-hydroxyphthalic anhydride(**12**) with 3-aminopiperidine-2,6-dione hydrochloride(**13**) afforded the intermediate compound **14**. Alkylation of compound **14** with *tert*-butyl bromoacetate generated compound **15**, which was subjected to trifluoroacetic acid promoted ester hydrolysis. Different Mono-Boc protected alkyl diamines amidation and Boc-deprotection reactions afforded compounds **17**a and **17**b. Amidation of compound **17** with compound **11** in the presence of HATU and DIPEA gave the target compounds **18**a and **18**b.

Compounds **22**a—**22**f were synthesized according to the route shown in Scheme 3[15]. Briefly, 3-fluorophthalic anhydride(**19**) with 3-aminopiperidine-2,6-dione hydrochloride(**13**) afforded the intermediate **20**. Compounds **21**a—**21**f were



Scheme 2 Synthetic route for compounds 18

Reaction conditions: *a*. TEA, toluene; *b*. *tert*-butyl bromoacetate, KI, $KHCO_3$, DMF; *c*. TFA; *d*. Mono-Boc protected alkyl diamine, HATU, DIPEA, DMF; *e*. TFA, DCM; *f*. HATU, DIPEA, DMF.



Scheme 3 Synthesis of compounds 22

Reaction conditions: *a*. NaOAc, AcOH; *b*. Mono-Boc protected alkyl diamine, DIPEA, DMF; *c*. TFA, DCM; *d*. HATU, DIPEA, DMF.

formed by substitution reaction of compound **20** with different length of Mono-Boc protected alkyl diamines, then Boc-deprotection with trifluoroacetic acid afforded the intermediates **21**a—**21**f, which were subsequently condensed with compound **11** to generate compounds **22**a—**22**f.

3.2 Biological Activity of the Target Compounds

Accordingly, a series of potential BRD4 degraders was synthesized using compound **11** as the BRD4 inhibitor portion, thalidomide as the cereblon ligand and different length and composition linker. At the same time, their BRD4 inhibitory activity, RS4;11 cell growth inhibitory activity and BRD4 degrade activity were evaluated.

BRD4 inhibitory activities of the compounds were evaluated by Fluorescence Anisotropy Binding Assay. All the compounds showed better BRD4 inhibitory activities than compound **3**(synthesized by ourselves, purity: HPLC 97%) (Table 1). First, compound **18**a was synthesized using —$(CH_2)_4$—CO—CH_2—O— as the linker. Replacing the alkyl linker to diethylene glycol linker resulted in compound **18**b, for which the BRD4 inhibitory activity is 2 times better than that of compound **18**a. Next, we chose —$(CH_2)_n$—N— as the linker, and investigated the optimal linker length for BRD4 potencies, which resulted in compounds **22**a—**22**d. Compound **22**a with a linker group(n=2) shorter than that in compound **22**b(n=4) is nearly 2 times less potent than compound **22**b. Similarly, compound **22**b with a linker group(n=4) shorter than that in compound **22**c(n=6) is nearly 2 times less potent than compound **22**c. However, compound **22**d with a linker group(n=7) longer than that in **22**c(n=6) is less potent than compound **22**c. These data demonstrate that for achieving the most potent BRD4 inhibition activity, an optimal linker length should comprise —$(CH_2)_6$—NH— in the compound. Then, replacing the alkyl linker to ethylene glycol resulted in compounds **22**e and **22**f. Compound **22**f with a tetraethylene glycol linker is more potent than compound **22**e with a trithylene glycol linker. In all the compounds, compound **22**f[IC_{50}=(9.4±0.6) nmol/L] showed the best BRD4 inhibitory activity than others.

Table 1 BRD4(BD1+BD2) inhibitory activities of the compounds

Compound	IC_{50}^{*}/(nmol·L^{-1})
3	26.2±2.8
18a	25.6±1.6
18b	12.4±2.3
22a	54.8±3.2
22b	30.4±2.5
22c	12.8±0.8
22d	24.2±1.2
22e	98.4±1.3
22f	9.4±0.6

* Mean values deviation of triplicate experiments.

Then, we investigated the cancer cell growth inhibitory activity of all the compounds on RS4;11 cell lines(Table 2). All other compounds exhibited better anti-proliferative activity than compound **3**, and the structure-activity relationship with the cell growth inhibitory activity is the same to the BRD4 inhibitory activity. Compound **22**f[IC_{50}=(27.6±1.6) nmol/L] showed more potent inhibitory RS4;11 cell growth activity than other compounds.

Table 2 RS4;11 cell growth inhibitory activity of the compounds

Compound	IC_{50}^{*}/(nmol·L^{-1})
3	256.8±12
18a	135.3±5.6
18b	104.4±6.4
22a	152.6±8.9
22b	89.2±5.6
22c	42.7±3.2
22d	76.2±2.8
22e	85.3±2.3
22f	27.6±1.6

* Mean values deviation of triplicate experiments.

We also examined the ability of compounds **3** and **22**f to induce BRD4 degradation in the RS4;11 cell lines, and the data are shown in Fig.2. Western blotting analysis shows that compound **22**f at concentrations of 0.5—1.0 μmol/L is effective in decreasing the level of BRD4 protein in the RS4;11 cells, whereas compound **3** at 10 μmol/L still fails to decrease the level of BRD4 protein. It can be concluded that compound **22**f is a potent BRD4 protein degrader.

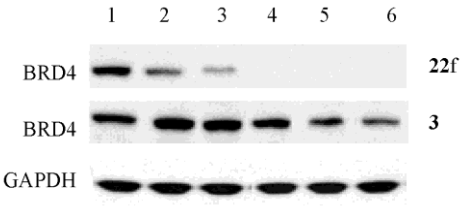


Fig.2 Western blotting analysis of BRD4 proteins in RS4;11 cells treated with compound 3 and 22f

RS4;11 cells were treated for 4 h with each individual compound at indicated concentrations and proteins were probed by specific antibodies. GAPDH was used as the loading control. Lane 1: DMSO; Lane 2—Lane 6: 0.1, 0.5, 1, 5, 10 μmol/L, respectively.

4 Conclusions

In this paper, a new class of PROTAC small-molecule degraders of BRD4 protein were synthesized. Through extensive optimization of the linker length and composition, we have obtained a number of highly potent small-molecule BRD4 protein degraders. One of the most promising compound **22**f exhibited robust potency of BRD4 inhibition with IC_{50} value of (9.4±0.6) nmol/L respectively. Furthermore, compound **22**f potently inhibited cell proliferation in BRD4-sensitive cell lines RS4;11 with IC_{50} value of (27.6±1.6) nmol/L and capable of inducing degradation of BRD4 protein at 0.5—1.0 μmol/L in the RS4;11 leukemia cells. Collectively, our data demonstrate that compound **22**f is a highly potent, efficacious, and promising BRD4 degrader and warrants further evaluation as a potential new therapy for the treatment of human acute leukemia and other types of human cancer.

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