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ARTICLE TYPE

Design, synthesis and biological evaluation of novel 1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolins as potential EZH2 inhibitors

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The histone lysine methyltransferase EZH2 has been implicated as a key component in cancer aggressiveness, metastasis and poor prognosis. This study discovered a new class of hexahydroisoquinolin derivatives as EZH2 inhibitors. Structure activity relationship study showed that the steric hindrace was important to the activity for EZH2. Preliminary optimization study led to the discovery of several potent compounds with low nanomolar to sub-nanomolar potency for EZH2. Biological evaluation indicated that SKLB1049 was a highly potent with improved solubility than EPZ6438, SAM-competitive, and cell-active EZH2 inhibitor that decreased global H3K27me3 in SU-DHL-6 and Pfeiffer lymphoma cells in a concentration- and time-dependent manner. Further study indicated that SKLB1049 caused cell arrest in G_0/G_1 phase. These compounds would be useful as chemical tools to further explore the biology of EZH2 and provided us a start point to develop new EZH2 inhibitors.

Introduction

Cancer is a major public health problem in the world.[1] Targeting epigenetic regulators has provided us a new strategy for cancer therapy.[2] Enhancer of Zeste Homolog 2 (EZH2) is a member of histone lysine methyltransferase family and the catalytic component of the polycomb repressive complex 2 (PRC2) as well.[3] PRC2 catalyzes the methylation of histone 3 lysine 27 (H3K27), leading to trimethylation of H3K27 (H3K27me3), which is a epigenetic mark of transcriptionally repressive and silences target genes.[4] The over expression of EZH2 has been observed in numerous blood and solid tumors, such as Hodgkin's disease,[5] Non Hodgkin's lymphoma,[6] prostate,[7] breast,[8] renal,[9] melanoma[10] and tongue[11] cancers, and is widely implicated in cancer progression, metastasis and poor prognosis.[8, 12] Recently, somatic mutations of tyrosine Y641, Alanine A677 and A687 of EZH2 were reported to be associated with germinal center B-cell like diffuse large B-cell lymphoma (GCB-DLBCL) and Follicular Lymphoma (FL).[13-15] Thus, inhibition of EZH2 is considered as an attractive therapeutic target for the treatment of cancer.

Over the recent years, multiple groups, including GlaxoSmithKline,[16] Epizyme,[17-19] Novartis,[20] UNC Eshelman[21] and Constellation,[22, 23] have reported various structures of small molecule inhibitors of EZH2 (Fig. 1). The most potent compound EPZ6438 was reported to inhibit EZH2 with an IC_{50} of 2.5 nM, and exhibited significant tumor growth inhibition in several human DLBCL[17] and malignant rhabdoid[18] xenograft models. In 2013, a phase I/II study of EPZ6438 for the treatment of advanced solid tumors and B cell lymphomas was initiated. However, the effective dose of EPZ6438, 600 mg/kg once a day, was very high in the WSU-DLCL2 xenograft model, which could attribute to its inferior physicochemical properties, such as solubility. Thus highly potent EZH2 inhibitor with improved physicochemical properties is emergently needed.

Our research group designed and synthesized a series of novel EZH2 inhibitors bearing hexahydroisoquinolins, and the potency of these compounds for EZH2 was characterized. Biological evaluation indicated that SKLB1049 was highly potent for EZH2 methyltransferase with improved solubility than EPZ6438. In cell-based assays, SKLB1049 remarkably decreased global H3K27me3 levels, and selectively killed two DLBCL cell lines harboring the EZH2 mutant, SU-DHL-6 and Pfeiffer cells, but no apparent impact on normal cell lines. Further study indicated that SKLB1049 caused cell cycle arrest in G_0/G_1 phase. This study may contribute to the further design of novel scaffolds against EZH2.

Results and discussion

Chemistry

The compounds described in this study were synthesized according to Scheme 1. Briefly, treatment of ketone **1** with ethyl

acetate under sodium hydride[24] or acetyl chloride under LDA[25] afforded 1,3-diones **2**. Then the key building blocks pyridine-2-(*1H*)-ones **3** were prepared by treating 1,3-diones with cyanoacetamide in the presence of triethylenediamine (DABCO) in refluxing ethanol.[26] Subsequently, reduction of **3** by sodium borohydride and iodine afforded amines **4**,[27] which were subjected to coupling reaction with various acids **8** afforded **9**[28]. Next, the title compounds **10** were gained by Suzuki coupling reaction with various boronic acids or boronic esters. Compound **7** used for preparation of acid **8** was synthesized under a two-step reductive amination reaction, starting from methyl 3-amino-5-bromo-2-methylbenzoate **5**. The structures of all the tested compounds were fully characterized by ^{1}H NMR, ^{13}C NMR and ESI-MS analysis.

SAR analysis

Modifications of 4- and 5-Positions of pyridone moiety

Table 1 and 2 provided the biological data of these compounds. All synthesize derivatives were evaluated for its EZH2 inhibitory activity in AlphaLISA[29] assay and the antiproliferation activities of SU-DHL-6 and Pfeiffer cells in MTT assay. To investigate the structure–activity relationships of C4 and C5 position of pyridone moiety, we first synthesized compounds **10a–c** wherein pyridone C4–C5 position together formed an alkyl ring. Evaluation of the ring size of the fused system demonstrated that the seven- and eight-membered rings (**10b** and **10c**) were equipotent and inferior to the six-membered ring (**10a**, EZH2^{WT} IC_{50} = 14 nM; EZH2^{Y641} IC_{50} = 27.2 nM; EZH2^{A677G} IC_{50} = 7.0 nM), which was chosen for further SAR study. Simple methyl substitution at the 7-position of the terminal fused ring (**10d**) improved the potency on all three EZH2 enzymes: EZH2^{WT}, EZH2^{Y641F} and EZH2^{A677G}, while the ethyl (**10e**), tert-butyl (**10f**) and dimethyl analogs (**10g**) were less active. This revealed that a relative small substituent in this position was more potent, and the volume of the substitutents was a crucial factor for the activity. Introduction of a methyl to C6 position of 1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin resulted in a less potent compound (**10h**). Comparison of **10h** and **10d** suggested that methyl substituted at the 7-position of the hexahydroisoquinoline was preferred relative to the 6-position. Interestingly, introduction of a phenyl ring fused to cyclohexane moiety led to an inactive compound **10i**, which suggested that a bulky substituent in C5 and C6 of hexahydroisoquinoline might change its conformation and was harmful to the anti-EZH2 activity. In this series of pyridone analogs (**10a**-**10i**), **10d** displayed the most active for EZH2 inhibition. However, it did not show remarkably enhanced antiproliferation activities for SU-DHL-6 and Pfeiffer cells compared with **10a** in this 4-days assay.

Substitutions of R_2 Position of central phenyl

Subsequent SAR study of R_2 position of central phenyl was further explored with a series of aryl and heteroaryl (Table 2). For this purpose, the fused cyclohexane group was held constant. The different R_2 substituents had considerable influence on the activity for EZH2. Among aryl substitutuents, compound **10q** was the most potent compound with an IC_{50} of 7.3 nM for EZH2^{WT}, which was 2-fold improvement compared with **10a**. On the other hand, replacement of the 4-(morpholinomethyl) phenyl moiety (**10a**) with 1-methyl-1H-pyrazole-4-yl (**10r**), 2-fluoropyridin-5-yl (**10s**) or 2-chloropyridin-5-yl (**10t**) retained excellent potency for EZH2, and introduction of 2-piperazinyl pyridin-5-yl (**10u**, EZH2^{WT} IC_{50} = 4.0 nM) in this position led to a 4-fold improvement of potency. In this series (**10a**, **10j**-**10u**), most compounds did not show obvious effect on tumor cells viability. However, **10q** and **10u** displayed slightly improved antiproliferative activities for SU-DHL-6 and Pfeiffer cells compared with **10a**.

The solubility assay of hexahydroisoquinolin analogs

Structure activity relationship study led to the discovery of several potent compounds with low nanomolar to sub-nanomolar potency for EZH2. The 1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin which replaced pyridone moiety also led to improved solubility in some cases. The solubility of **10a**, **10q** (SKLB1049) and **10u** was significantly improved over that of EPZ6438 (Table 3). In particular， SKLB1049 exhibited an apparent 60-fold increase of solubility in water (185.3 μg/mL vs <3.0 μg/mL) compared with EPZ6438.

SKLB1049 was a SAM-Competitive, Potent, and Selective Inhibitor of EZH2

On the basis of the high enzyme and cells inhibitory activities and improved solubility, SKLB1049 was selected for subsequent studies for mechanism of action (MOA), selectivity and cellular assays. To elucidate the MOA of EZH2 inhibition by SKLB1049, the IC_{50} values were determined at two different SAM concentrations of 1 μM and 10 μM. As shown in Fig. 2d, the results demonstrated that the increase of SAM concentration could cause an obvious shift of IC_{50} value of SKLB1049 against EZH2^{WT}, which indicated that SKLB1049 was a SAM-competitive EZH2 inhibitor. The MOA for SKLB1049 was consistent with that of structurally similar molecule EPZ6438.

As shown in Fig. 2b, SKLB1049 was highly potent for both Y641F and A677G mutants, with IC_{50} values of 39 nM and 5.9 nM, respectively. The potency of SKLB1049 for WT enzymes was similar with A677G mutant and superior to Y641F mutant. To determine the selectivity profile of SKLB1049, the potency for EZH1 was also evaluated (Fig. 2c). SKLB1049 showed a considerable potency for EZH1, with an IC_{50} value of 84 nM, approximately 12-fold less potent than EZH2.

SKLB1049 Potently Reduced H3K27me3 in Cells

The ability of SKLB1049 to reduce H3K27me3 in intact cells was evaluated in SU-DHL-6 and Pfeiffer cells using Western blotting analysis (Fig. 3). Treatment with SKLB1049 at 5 μM for 4-days and 6-days potently abolished H3K27me3 mark in EZH2 A677G mutant Pfeiffer cells in a concentration- and time-dependent manner. Similarly, SKLB1049 remarkably reduced H3K27me3 and H3K27me2 mark in a concentration-dependent manner in SU-DHL-6 cells, with no affection on EZH2 levels.

SKLB1049 inhibited proliferation of cancer cell line

To further investigate the effect of EZH2 inhibition on cell proliferation, the inhibitory activities of **10a**, SKLB1049 and **10u** against SU-DHL-6 and Pfeiffer cell lines for 6-days were evaluated (Table 4). As shown in Fig. 4, cell lines were effectively killed by EZH2 inhibitors in a time-dependent manner. SKLB1049 and **10u** demonstrated slightly greater potency than **10a** in both cell lines. This was consistent with the better activity

of SKLB1049 and **10u** on EZH2 activity. Pfeiffer was more sensitive to EZH2 inhibition compared with SU-DHL-6, with IC_{50} values of 2.76 μM, 1.19 μM and 1.65 μM for **10a**, SKLB1049 and **10u**, respectively. The difference in sensitivity between Pfeiffer and SU-DHL-6 was not due to different levels of target engagement because the similar significant suppression of global H3K27me3 in both cell lines was observed.

Then, Bright-field microscopy images of SU-DHL-6 cancer cells after incubation with SKLB1049 for 6-days at varying concentrations were performed to assess the activity visually. As shown in Fig. 5, when treated with SKLB1049 for 6-days, the proliferation of SU-DHL-6 cancer cell was remarkably inhibited, and these phenomena were more significant as the concentration increasing.

To further evaluate the safety of our EZH2 inhibitors, the antiproliferation activities of SKLB1049 for 6-days against three normal cell lines, HEK 293, Vero and LO2 were measured by MTT assay (Table 4). All these compounds showed no obvious impact on these normal cells, indicating its good in vitro safety profile.

SKLB1049 could remarkably arrested G_0/G_1 phase

The effect on cell cycle progression of SKLB1049 was also assessed (Fig. 6). Treatment with SKLB1049 in SU-DHL-6 cells at a concentration of 0.625 μM showed no change in cell cycle compared with the DMSO control, while a concentration of 10 μM treatment resulted in an increase of the percentage of cells in G_0/G_1 phase from 44.89% to 66.09%, and a concomitant decrease in S phase from 34.43% to 12.68%. However there was no considerable change in the percentage of cells in the G_2/M phase through 14 days treatment. These results suggested that SKLB1049 could remarkably arrested cell cycle in G_0/G_1 phase in a concentration-dependent manner.

Conclusions

We designed and synthesized a series of novel compounds bearing hexahydroisoquinolin and their biological activities of targeting EZH2 were characterized. Structure activity relationship study showed the steric hindrace was important to the activity for EZH2. Preliminary optimization led to the discovery of several potent compounds with good solubility, in which SKLB1049 was selected for subsequent studies for mechanism of action (MOA), selectivity and cellular assays. Biological evaluation indicated that SKLB1049 was a highly potent candidate for EZH2 wild-type, Y641 mutant and A677 mutant enzymes with improved solubility than EPZ6438, through a SAM-competitive manner. In cell-based assays, SKLB1049 remarkably reduced global H3K27me3 and H3K27me2 levels in a concentration- and time-dependent manner, with no affection on EZH2 levels. SKLB1049 could selectively killed two DLBCL cell lines harboring the EZH2 mutants, SU-DHL-6 and Pfeiffer cells in a concentration- and time-dependent manner, but with no apparent impact on normal cell lines. Further study indicated that SKLB1049 caused cell arrest in G_0/G_1 phase. Together, our study may contribute to the further design of novel scaffolds against EZH2. Further studies of the biological mechanisms and anti-tumor research *in vivo* are in progress.

Experimental

Chemistry

Unless otherwise noted, all materials were obtained from commercial suppliers and used without purification. The ^1H and ^{13}C NMR spectra were recorded on a Bruker AVANCE^{III} 400 spectrometer using DMSO-d_6 or $CDCl_3$ as the solvent. Chemical shifts (δ) were reported in ppm relative to Me_4Si (internal standard), and coupling constants (*J*) were reported in Hz. Mass Spectra (MS) were performed on a Waters Q-TOF Premier mass spectrometer. Thin layer chromatography (TLC) used Qingdao Haiyang Silica gel F-254 plates, and column chromatography were performed using Qingdao Haiyang Silica gel 60 (300-400 mesh). HPLC analysis was performed on an UltiMate 3000 HPLC system (Dionex, USA). All tested compounds were purified until the purity was ≧95%, detected by HPLC under UV 254 nm wavelength, NMR and ESI-MS.

General Procedure for the Preparation of 1,3-diketones 2a-2h. **Method A for 2a-2b, 2e-2h**. A mixture of cyclic ketone (14 mmol, 1.0 equiv) and toluene (0.5 mL) was added dropwise to a suspension of 60% sodium hydride (28 mmol, 2.0 equiv) in ethyl acetate (28 mmol, 2.0 equiv). After hydrogen evolution ceased, the reaction mixture was heated at 40 °C for 3 h. Additional stirring at room temperature was continued and excess sodium hydride was quenched with methanol. The reaction mixture was poured into water, neutralized with concentrated HCl, and extracted with CH_2Cl_2. The combined extracts were washed with brine, dried, and concentrated. Purification by silica flash chromatography (hexanes/EtOAc) giving 2a-2b, 2e-2e. Yeild: 30-60%.

Method B for 2c-2d. A solution of cyclic ketone (1.0 equiv) in dry THF (0.67 M) was added to a stirred solution of freshly prepared LDA (lithium diisopropylamide) (1.0 equiv) in THF (1.0 M) at -78 °C. The resulting solution was stirred for 2 h. A solution of acetyl chloride (1.0 equiv) in THF (1.2 M) was added to the above solution. After stirring at -78 °C was continued for 1 h, the cooling bath was removed and stirring was continued for 3 h. The reaction mixture was poured into a saturated aqueous NH_4Cl (25 mL) and then extracted with CH_2Cl_2 (3×). The combined extracts were washed with brine, dried, and concentrated. Purification by silica flash chromatography (hexanes/EtOAc) giving 2c-2d. Yeild: 50-60%.

General procedure for the synthesis of 3. 1,3-diketones (14 mmol, 1.0 equiv), cyanoacetamide (14 mmol, 1.0 equiv) and DABCO (14 mmol, 1.0 equiv) was added to a solution of EtOH (25 mL), then the resulting mixture was stirred at 90°C for 10 h. The reaction mixture was cooled to rt and the solid precipitate was filtered, washed with EtOH and dried to afford the desired compound. Yeild: 20-60%.

General procedure for the synthesis of 4. To an ice-bath cooled THF (100 mL) solution of **3** (10 mmol, 1.0 equiv) were added $NaBH_4$ (11 mmol, 1.1 equiv) and I_2 (10 mmol, 1.0 equiv) and the mixture stirred for 30 min. The reaction mixture was then heated at reflux for 10 h and then allowed to cool to room temperature. After cooling to 0°C, the reaction mixture was acidified by slow addition of 3N HCl (l mL). The reaction mixture was concentrated in vacuo and the crude product 4 used directly in the next step without further purification.

General procedure for the synthesis of 6. The starting material methyl 3-amino-5-bromo-2-methylbenzoate (**5**) was synthesized based on a literature method. To a stirred solution of **5** (10 mmol, 1.0 equiv) and cyclic ketone (15 mmol, 1.5 equiv) in dichloroethane (50 mL) was added acetic acid (60 mmol, 6.0 equiv) and the reaction mixture stirred at room temperature for 15 minutes, then the reaction mixture was cooled to 0 °C and sodium triacetoxyborohydride (30 mmol, 3.0 equiv) was added. The reaction mixture was stirred overnight at room temperature. Upon completion of the reaction as determined by TLC, aqueous sodium bicarbonate solution was added to the reaction mixture until a pH of 7-8 was obtained. The organic phase was separated and the aqueous phase was extracted with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude compound was purified by column chromatography eluting with ethyl acetate: hexane to afford the desired compound 6 as a solid. Yeild: 70%.

General procedure for the synthesis of 7. To a stirred solution of **6** (10 mmol, 1.0 equiv) and acetaldehyde (15 mmol, 1.5 equiv) in dichloroethane (50 mL) was added acetic acid (60 mmol, 6.0 equiv) and the reaction mixture stirred at room temperature for 15 minutes, then the reaction mixture was cooled to 0 °C and sodium triacetoxyborohydride (30 mmol, 3.0 equiv) was added. The reaction mixture was stirred overnight at room temperature. Upon completion of the reaction as determined by TLC, aqueous sodium bicarbonate solution was added to the reaction mixture until a pH of 7-8 was obtained. The organic phase was separated and the aqueous phase was extracted with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude compound was purified by column chromatography eluting with ethyl acetate: hexane to afford the desired compound **7** as a liquid. Yeild: 60%.

General procedure for the synthesis of 8. Ester **7** (10 mmol, 1.0 equiv) in ethanol (25 mL) was added aqueous NaOH (15 mmol, 1.5 equiv in 5 mL water) and the resulting mixture was stirred at 60 °C for 1 h. Upon completion of the reaction as determined by TLC, the solvent was removed under reduced pressure and the residue obtained was acidified with 1N HCl until a pH 4 was obtained. The precipitate was filtered off, washed with water and dried to afford the desired acid **8**. Yeild: 80%.

General procedure for the synthesis of 9. The above acid **8** (1.0 mmol, 1.0 equiv) and amino **4** (2 mmol, 2.0 equiv) were dissolved in DMSO (10 mL), then HOAT (1.5 mmol, 1.5 equiv), EDCI (1.5 mmol, 1.5 equiv) and *N*-methylmorpholine (3 mmol, 3 equiv) were added to it. The reaction mixture was stirred at room temperature for 20 h. Upon completion of the reaction as determined by TLC, the reaction mixture was poured onto ice-cold water (100 mL), stirred for 30 minutes and the precipitated solid was collected by filtration, washed with water (50 mL) and air dried. The crude compound was purified by column chromatography eluting to afford the desired compound **9** as a solid. Yeild: 65-78%.

General procedure for the synthesis of 10. To a stirred solution of **9** (1.0 mmol, 1.0 equiv) in dioxane/ water mixture (20 mL/2 mL) was added boronic ester derivatives (1.08 mmol, 1.08 equiv) followed by addition of Na_2CO_3(3.6 mmol, 3.6 equiv). The solution was purged with argon for 15 minutes and then $PdCl_2(dppf)\cdot CH_2Cl_2$ (0.1 mmol, 0.1 equiv) was added and the solution was again purged with argon for a further 10min. The reaction mixture was heated at 100°C for 4 h. After completion (monitored by TLC), the reaction mixture was diluted with water and extracted with 10% MeOH/DCM. The combined organic layers were dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure. The crude compound was purified by column chromatography eluting with methanol: DCM to the title compound as a solid.

5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)-4'-(morpholinomethyl)biphenyl-3-carboxamide (**10a**). Yield: 85%; ^{1}H NMR (400 MHz, DMSO) δ 11.50 (s, 1H), 8.17 (s, 1H), 7.58 (d, J = 6.8 Hz, 2H), 7.38 (m, 3H), 7.22 (s, 1H), 4.32 (s, 2H), 3.83 (d, J = 9.6 Hz, 2H), 3.58 (s, 4H), 3.49 (s, 2H), 3.25 (t, J = 10.9 Hz, 2H), 3.08 (m, 2H), 3.02 (m, 1H), 2.75 (s, 2H), 2.37 (s, 6H), 2.25 (s, 3H), 2.10 (s, 3H), 1.65 (s, 6H), 1.53 (m, 2H), 0.84 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 169.03, 161.53, 150.04, 148.84, 140.62, 139.60, 138.56, 136.95, 132.56, 129.48, 126.37, 122.78, 120.76, 111.76, 66.29, 66.16, 62.04, 57.81, 53.14, 41.05, 34.57, 30.23, 26.60, 24.09, 22.15, 21.89, 15.93, 14.55, 12.67. ESI-MS m/z 613.2 $(M + H)^+$.

5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-N-((1-methyl-3-oxo-3,5,6,7,8,9-hexahydro-2H-cyclohepta[c]pyridin-4-yl)methyl)-4'-(morpholinomethyl)biphenyl-3-carboxamide (**10b**). Yield: 74%; 1H NMR (400 MHz, DMSO) δ 11.40 (s, 1H), 8.19 (s, 1H), 7.58 (d, J = 7.7 Hz, 2H), 7.39 (d, J = 4.0 Hz, 2H), 7.36 (s, 1H), 7.22 (s, 1H), 4.38 (d, J = 4.0 Hz, 2H), 3.83 (d, J = 10.6 Hz, 2H), 3.58 (s, 4H), 3.48 (s, 2H), 3.25 (t, J = 11.2 Hz, 2H), 3.16 (m, 2H), 3.02 (m, 1H), 2.65 (d, J = 9.4 Hz, 2H), 2.36 (s, 4H), 2.26 (s, 3H), 2.19 (s, 3H), 1.74 (s, 2H), 1.67 (m, 2H), 1.53 (m, 4H), 1.45 (s, 2H), 1.24 (s, 2H), 0.83 (t, J = 6.9 Hz, 3H). 13C NMR (100 MHz, CDCl3) δ 170.16, 163.55, 150.05, 149.57, 147.42, 139.30, 137.89, 133.62, 131.73, 131.34, 131.24, 130.16, 128.37, 128.25, 126.84, 123.98, 121.93, 121.15, 120.73, 67.33, 66.23, 62.44, 58.41, 53.06, 41.54, 37.07, 33.29, 31.89, 30.47, 29.70, 29.37, 27.45, 26.49, 25.63, 22.70, 20.79, 16.94, 14.78, 14.15, 12.84. ESI-MS m/z 627.2 $(M + H)^+$.

5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-N-((1-methyl-3-oxo-2,3,5,6,7,8,9,10-octahydrocycloocta[c]pyridin-4-yl)methyl)-4'-(morpholinomethyl)biphenyl-3-carboxamide (**10c**). Yield: 84%; ^{1}H NMR (400 MHz, DMSO) δ 11.35 (s, 1H), 8.18 (t, *J* = 4.8 Hz, 1H), 7.57 (d, *J* = 8.0 Hz, 2H), 7.39 (d, *J* = 4.4 Hz, 2H), 7.36 (s, 1H), 7.21 (s, 1H), 4.37 (d, *J* = 4.6 Hz, 2H), 3.83 (d, *J* = 10.4 Hz, 2H), 3.57 (s, 4H), 3.48 (s, 2H), 3.25 (t, *J* = 11.3 Hz, 1H), 3.08 (m, 2H), 3.02 (m, 1H), 2.60 (m, 2H), 2.36 (s, 4H), 2.24 (s, 3H), 2.21 (s, 3H), 1.66 (m, 2H), 1.58 (s, 2H), 1.54 (d, *J* = 8.0 Hz, 2H), 1.39 (s, 2H), 1.27 (s, 2H), 0.83 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, CDCl3) δ 170.16, 164.15, 149.94, 149.52, 144.99, 144.42, 139.41, 137.81, 133.72, 130.18, 126.94, 123.96, 122.71, 120.71, 117.96, 67.34, 66.19, 62.48, 58.40, 53.09, 41.51, 37.07, 31.93, 30.80, 30.47, 29.80, 29.71, 29.57, 26.18, 25.65, 25.52, 16.18, 14.75, 14.15, 12.83. ESI-MS m/z 641.2 $(M + H)^+$.

N-((1,7-dimethyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)-5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-4'-(morpholinomethyl)biphenyl-3-carboxamide (**10d**). Yield: 79%; ^{1}H NMR (400 MHz, DMSO) δ 11.49 (s, 1H), 8.16 (t, *J* =

4.4 Hz, 1H), 7.57 (d, *J* = 8.0 Hz, 2H), 7.45 – 7.26 (m, 1H), 7.21 (s, 1H), 4.31 (t, *J* = 4.0 Hz, 2H), 3.83 (d, *J* = 10.2 Hz, 2H), 3.64 – 3.53 (m, 4H), 3.48 (s, 2H), 3.25 (t, *J* = 11.2 Hz, 2H), 3.14 – 3.04 (m, 2H), 3.04 – 2.87 (m, 1H), 2.61 (m, 2H), 2.36 (s, 4H), 2.25 (s, 3H), 2.10 (s, 3H), 1.90 (m, 1H), 1.80 (m, 1H), 1.66 (m, 4H), 1.52 (m, 2H), 1.19 (m, 1H), 1.01 (d, *J* = 6.4 Hz, 3H), 0.83 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.17, 163.60, 150.66, 149.63, 140.58, 139.36, 137.71, 133.75, 130.28, 126.81, 123.92, 121.10, 120.76, 114.88, 67.33, 66.01, 58.39, 52.88, 41.50, 36.12, 33.48, 30.47, 30.39, 28.52, 27.37, 21.91, 16.58, 14.78, 12.84. ESI-MS m/z 627.2 $(M + H)^+$.

5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-N-((7-ethyl-1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)-4-methyl-4'-(morpholinomethyl)biphenyl-3-carboxamide (**10e**). Yield: 74%. ^{1}H NMR (400 MHz, DMSO) δ 11.49 (s, 1H), 8.16 (s, 1H), 7.57 (s, 1H), 7.38 (s, 2H), 7.21 (s, 1H), 4.31 (s, 2H), 3.83 (s, 2H), 3.57 (s, 4H), 3.48 (s, 4H), 3.25 (s, 2H), 3.08 (s, 2H), 2.93 (m, 1H), 2.56 (s, 4H), 2.36 (s, 3H), 2.25 (s, 3H), 2.11 (s, 2H), 1.88 (m, 2H), 1.65 (m, 2H), 1.53 (m, 2H), 1.34 (m, 2H), 1.14 (m, 1H), 0.93 (s, 3H), 0.83 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.17, 163.60, 150.97, 149.64, 140.66, 140.56, 139.39, 133.79, 130.30, 126.80, 123.91, 121.05, 120.75, 114.85, 67.33, 58.39, 52.80, 41.48, 36.13, 35.16, 31.33, 30.48, 29.71, 29.38, 29.00, 28.00, 27.32, 22.71, 16.59, 14.78, 14.15, 12.84, 11.44. ESI-MS m/z 641.2 $(M + H)^+$.

N-((7-tert-butyl-1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)-5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-4'-(morpholinomethyl)biphenyl-3-carboxamide (**10f**). Yield: 67%. ^{1}H NMR (400 MHz, DMSO) δ 11.49 (s, 1H), 8.16 (t, *J* = 4.4 Hz, 1H), 7.57 (d, *J* = 8.0 Hz, 2H), 7.45 – 7.26 (m, 1H), 7.21 (s, 1H), 4.31 (t, *J* = 4.0 Hz, 2H), 3.83 (d, *J* = 10.2 Hz, 2H), 3.64 – 3.53 (m, 4H), 3.48 (s, 2H), 3.25 (t, *J* = 11.2 Hz, 2H), 3.14 – 3.04 (m, 2H), 3.04 – 2.87 (m, 1H), 2.61 (m, 2H), 2.36 (s, 4H), 2.25 (s, 3H), 2.10 (s, 3H), 1.90 (m, 1H), 1.80 (m, 1H), 1.66 (m, 4H), 1.52 (m, 2H), 1.19 (m, 1H), 1.01 (s, 9H), 0.83 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, DMSO) δ170.04, 159.80, 155.87, 142.77, 141.48, 140.30, 139.71, 138.87, 136.80, 129.17, 128.90, 128.52, 128.20, 127.99, 116.49, 114.00, 67.12, 67.08, 63.42, 54.66, 51.93, 46.90, 46.37, 36.13, 32.67, 29.95, 29.31, 28.02, 26.85, 24.74, 16.01, 15.61, 14.39, 12.58. ESI-MS m/z 669.2 $(M + H)^+$.

5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-4'-(morpholinomethyl)-N-((1,7,7-trimethyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)biphenyl-3-carboxamide (**10g**). Yield: 69%. ^{1}H NMR (400 MHz, DMSO) δ 11.51 (s, 1H), 8.21 (s, 1H), 7.57 (s, 2H), 7.45 – 7.26 (m, 1H), 7.21 (s, 1H), 4.31 (t, J = 4.0 Hz, 2H), 3.83 (d, J = 10.2 Hz, 2H), 3.64 – 3.53 (m, 4H), 3.48 (s, 2H), 3.25 (t, J = 11.2 Hz, 2H), 3.14 – 3.04 (m, 2H), 3.04 – 2.87 (m, 1H), 2.61 (m, 2H), 2.36 (s, 4H), 2.25 (s, 3H), 2.10 (s, 3H), 1.90 (m, 1H), 1.80 (m, 1H), 1.66 (m, 4H), 1.52 (m, 2H), 1.19 (m, 1H), 1.1 (s, 6H), 0.83 (t, J = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, CDCl3) δ 170.18, 150.25, 149.62, 141.12, 140.84, 139.39, 133.80, 130.37, 126.85, 123.89, 120.75, 114.48, 67.33, 65.87, 58.37, 52.81, 41.47, 38.56, 36.25, 34.73, 30.46, 28.75, 28.01, 24.61, 16.65, 14.76, 12.84. ESI-MS m/z 641.2 $(M + H)^+$.

N-((1,6-dimethyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)-5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-4'-(morpholinomethyl)biphenyl-3-carboxamide (**10h**). Yield: 80%. ^{1}H NMR (400 MHz, DMSO) δ 11.49 (s, 1H), 8.15 (t, *J* = 4.8 Hz, 1H), 7.54 (m, 2H), 7.48 – 7.30 (m, 3H), 7.22 (s, 1H), 4.33 (d, *J* = 4.8 Hz, 2H), 3.83 (d, *J* = 10.0 Hz, 2H), 3.70 – 3.53 (m, 4H), 3.48 (s, 2H), 3.25 (t, *J* = 11.2Hz, 2H), 3.09 (m, 2H), 3.04 – 2.90 (m, 2H), 2.36 (s, 6H), 2.25 (s, 3H), 2.10 (s, 3H), 1.81 (m, 1H), 1.66 (m, 3H), 1.52 (m, 2H), 1.21 (m, 2H), 1.01 (d, *J* = 6.4 Hz, 3H), 0.83 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.17, 163.53, 151.06, 149.56, 140.62, 139.90, 139.40, 137.90, 133.59, 130.05, 126.82, 123.92, 120.93, 120.78, 114.46, 77.39, 77.07, 76.75, 67.33, 66.32, 62.53, 58.41, 53.14, 41.51, 36.07, 35.78, 30.47, 29.71, 28.51, 24.77, 21.91, 16.62, 14.78, 12.85. ESI-MS m/z 627.2 $(M + H)^+$.

5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-4'-(morpholinomethyl)-N-((2-oxo-2,3,5,6-tetrahydrobenzo[*f*]isoquinolin-1-yl)methyl)biphenyl-3-carboxamide (**10i**) Yield: 74%. ^{1}H NMR (400 MHz, DMSO) δ 11.65 (s, 1H), 8.33 (s, 1H), 7.92 (s, 1H), 7.58 (d, *J* = 7.6 Hz, 3H), 7.46 (t, *J* = 8.0 Hz, 3H), 7.30 (s, 3H), 7.25 (s, 1H), 4.47 (d, *J* = 4.0 Hz, 2H), 3.83 (d, *J* = 10.0 Hz, 2H), 3.57 (s, 4H), 3.48 (s, 2H), 3.25 (t, *J* = 11.3 Hz, 2H), 3.09 (d, *J* = 6.7 Hz, 2H), 3.02 (m, 1H), 2.80 (m, 2H), 2.64 (m, 2H), 2.36 (s, 4H), 2.28 (s, 6H), 1.66 (m, 2H), 1.53 (m, 2H), 1.09 (t, *J* = 7.0 Hz, 3H), 0.83 (t, *J* = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.32, 164.21, 149.58, 149.47, 139.64, 139.26, 138.20, 137.74, 135.98, 133.10, 129.81, 129.50, 128.26, 128.01, 126.86, 124.58, 123.97, 123.19, 120.79, 116.36, 67.29, 66.70, 62.88, 58.38, 53.41, 41.45, 36.79, 30.44, 28.17, 22.81, 16.40, 14.79, 12.86. ESI-MS m/z 661.2 $(M + H)^+$.

5-bromo-3-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-2-methyl-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)benzamide (**10j**). Yield: 74%. ^{1}H NMR (400 MHz, DMSO) δ 11.38 (s, 1H), 8.24 (s, 1H), 7.31 (s, 1H), 7.09 (s, 1H), 4.33 (d, *J* = 4.6 Hz, 2H), 3.83 (d, *J* = 10.0 Hz, 2H), 3.24 (t, *J* = 10.8 Hz, 2H), 3.09 – 2.97 (m, 2H), 2.97 – 2.83 (m, 1H), 2.72 (s, 2H), 2.38 (s, 2H), 2.15 (s, 3H), 2.09 (s, 3H), 1.59 (m, 6H), 1.51 (m, 2H), 0.79 (t, *J* = 6.8 Hz, 2H). ^{13}C NMR (100 MHz, DMSO) δ 167.74, 161.55, 150.38, 141.24, 140.33, 132.96, 127.06, 124.75, 120.87, 117.38, 111.76, 66.09, 58.15, 40.75, 34.55, 29.83, 26.61, 24.20, 22.17, 16.00, 14.82, 12.42. ESI-MS m/z 516.1 $(M + H)^+$.

5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)biphenyl-3-carboxamide (**10k**). Yield: 77%. ^{1}H NMR (400 MHz, CDCl3) δ 13.30 (s, 1H) , 7.46 (d, J = 6.6 Hz, 2H), 7.36 – 7.21 (m, 5H), 4.57 (s, 2H), 3.94 (d, J = 10.2 Hz, 2H), 3.31 (t, J = 9.6 Hz, 2H), 3.10 (d, J = 6.4 Hz, 2H), 3.02 (m, 1H), 2.95 (s, 2H), 2.30 (s, 2H), 1.97 (s, 3H), 1.71 (m, 7H), 1.26 (s, 4H), 0.89 (t, J = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.72, 163.52, 151.73, 140.90, 140.21, 139.50, 138.74, 128.80, 127.51, 126.88, 123.88, 120.97, 72.79, 67.23, 42.50, 31.94, 30.19, 29.71, 29.37, 27.43, 24.85, 22.71, 22.19, 16.49, 15.07, 14.15, 12.60. ESI-MS m/z 514.2 $(M + H)^+$.

4'-chloro-5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)biphenyl-3-carboxamide (**10l**). Yield: 80%. ^{1}H NMR (400 MHz, DMSO) δ 11.59 (s, 1H), 8.19 (s, 1H), 7.66 (s, 2H), 7.49 (d, *J* = 5.3 Hz, 2H), 7.42 (s, 1H), 7.25 (s, 1H), 4.33 (s, 2H), 3.82 (s, 2H), 3.25 (s, 2H), 3.05 (m, 3H), 2.75 (s, 2H), 2.37 (s, 2H), 2.27 (s, 3H), 2.10 (s, 3H), 1.64 (s, 6H), 1.53 (m, 2H), 0.83 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 169.79, 163.47, 151.54, 140.86, 139.64, 138.67, 137.35, 133.81, 133.52, 128.90, 128.09, 123.66, 121.38, 120.95, 115.38, 72.83, 67.24, 59.30, 42.19, 35.95, 31.93, 30.23, 29.70, 29.37, 27.43, 24.87, 22.70, 22.17, 16.51, 15.05, 14.15, 12.71. ESI-MS m/z 548.2 $(M + H)^+$.

5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)-4'-(trifluoromethyl)biphenyl-3-carboxamide (**10m**). Yield: 64%. ^{1}H NMR (400 MHz, DMSO) δ 11.48 (s, 2H), 8.19 (s, 1H), 7.86 (m, 2H), 7.80 (m, 2H), 7.48 (s, 1H), 7.30 (s, 1H), 4.32 (s, 2H), 3.84 (d, *J* = 7.5 Hz, 2H), 3.26 (m, 2H), 3.11 (m, 2H), 3.03 (m, 1H), 2.75 (s, 2H), 2.38 (s, 2H), 2.27 (s, 3H), 2.10 (s, 3H), 1.65 (s, 6H), 1.53 (m, 2H), 0.84 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.57, 165.86, 165.24, 163.10, 151.87, 143.76, 140.96, 139.83, 137.26, 129.70, 129.46, 128.31, 127.40, 127.21, 125.73, 125.50, 123.93, 122.80, 120.86, 115.87, 67.18, 35.91, 31.94, 30.12, 29.71, 29.37, 27.45, 24.89, 22.71, 22.13, 22.09, 16.59, 15.17, 14.14, 12.58. ESI-MS m/z 582.2 $(M + H)^+$.

5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4'-methoxy-4-methyl-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)biphenyl-3-carboxamide (**10n**). Yield: 77%. ^{1}H NMR (400 MHz, DMSO) δ 11.55 (s, 1H), 8.15 (t, *J* = 4.8 Hz, 1H), 7.55 (d, *J* = 8.8 Hz, 2H), 7.35 (s, 1H), 7.18 (s, 1H), 7.00 (d, *J* = 8.8 Hz, 2H), 4.32 (d, *J* = 4.4 Hz, 2H), 3.82 (d, *J* = 10.8 Hz, 2H), 3.78 (s, 3H), 3.24 (t, *J* = 11.2 Hz, 2H), 3.07 (m, 2H), 3.00 (m, 1H), 2.74 (s, 2H), 2.37 (s, 2H), 2.23 (s, 3H), 2.10 (s, 3H), 1.64 (s, 6H), 1.53 (m, 2H), 0.83 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, DMSO) δ 169.11, 161.52, 158.78, 150.38, 148.77, 139.54, 136.82, 132.20, 127.64, 122.36, 120.29, 114.29, 66.30, 57.80, 55.15, 35.10, 30.26, 26.60, 24.08, 22.15, 21.89, 15.93, 14.49, 12.66. ESI-MS m/z 555.2 $(M + H)^+$.

5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)-4'-morpholinobiphenyl-3-carboxamide (**10o**). Yield: 87%. ^{1}H NMR (400 MHz, DMSO) δ 11.49 (s, 1H), 8.12 (t, *J* = 4.6 Hz, 1H), 7.49 (d, *J* = 8.0 Hz, 2H), 7.34 (s, 1H), 7.16 (s, 1H), 7.01 (d, *J* = 8.4 Hz, 2H), 4.31 (d, *J* = 4.4 Hz, 2H), 3.83 (d, *J* = 10.8 Hz, 2H), 3.79 – 3.68 (m, 4H), 3.25 (t, *J* = 11.2 Hz, 2H), 3.13 (m, 4H), 3.08 (m, 2H), 3.05 (s, 1H), 2.74 (s, 2H), 2.38 (s, 2H), 2.22 (s, 3H), 2.10 (s, 3H), 1.64 (m, 6H), 1.52 (m, 2H), 0.83 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.07, 163.26, 151.46, 150.59, 140.83, 138.17, 127.53, 120.83, 115.70, 115.21, 77.39, 77.07, 76.75, 67.17, 66.76, 49.10, 36.24, 30.11, 29.71, 27.41, 24.89, 22.25, 22.19, 16.57, 15.04, 12.65. ESI-MS m/z 599.0 $(M + H)^+$.

5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)-3'-morpholinobiphenyl-3-carboxamide (**10p**). Yield: 70%; ^{1}H NMR (400 MHz, DMSO) δ 11.47 (s, 1H), 8.13 (t, 1H), 7.36 (s, 1H), 7.30 (t, *J* = 8.0 Hz, 1H), 7.20 (s, 1H), 7.09 (s, 1H), 7.02 (d, *J* = 8.0 Hz, 1H), 6.94 (d, *J* = 8.0 Hz, 1H), 4.32 (d, *J* = 4.0Hz, 2H), 3.83 (d, *J* = 12.0Hz, 2H), 3.76 (s, 4H), 3.25 (t, *J* = 12.0 Hz, 2H), 3.17 (m, 4H), 3.12 – 3.05 (m, 1H), 3.02 (m, 1H), 2.75 (s, 2H), 2.38 (s, 2H), 2.24 (s, 3H), 2.10 (s, 3H), 1.64 (m, 6H), 1.59 – 1.43 (m, 2H), 0.83 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, DMSO) δ 169.10, 161.52, 151.60, 150.09, 148.71, 140.79, 139.53, 137.84, 132.50, 129.45, 123.00, 121.03, 120.70, 117.74, 114.25, 113.23, 111.51, 66.28, 66.11, 57.83, 48.45, 41.14, 34.56, 30.27, 26.63, 24.08, 22.16, 21.90, 15.92, 14.54, 12.70. ESI-MS m/z 599.0 $(M + H)^+$.

5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)-4'-((4-methylpiperazin-1-yl)methyl)biphenyl-3-carboxamide (**10q**). Yield: 72%; ^{1}H NMR (400 MHz, DMSO) δ 11.49 (s, 1H), 8.15 (s, 1H), 7.56 (d, *J* = 8.0 Hz, 2H), 7.48 – 7.28 (m, 3H), 7.22 (s, 1H), 4.32 (s, 2H), 3.83 (d, *J* = 8.4 Hz, 2H), 3.47 (s, 2H), 3.27 (m, 6H), 3.09 (m, 3H), 2.75 (s, 2H), 2.37 (s, 6H), 2.25 (s, 3H), 2.17 (s, 3H), 2.10 (s, 3H), 1.65 (m, 6H), 1.53 (m, 2H), 0.83 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 169.04, 161.52, 150.04, 148.84, 139.59, 138.49, 136.96, 132.53, 129.38, 126.35, 122.77, 120.75, 111.51, 66.29, 61.59, 57.81, 54.53, 52.28, 45.45, 34.57, 30.24, 26.60, 24.09, 22.15, 21.89, 15.93, 14.54, 12.67. ESI-MS m/z 626.2 $(M + H)^+$.

3-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-2-methyl-5-(1-methyl-1H-pyrazol-4-yl)-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)benzamide (**10r**). Yield: 60%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.53 (s, 1H), 7.65 (s, 1H), 7.51 (s, 1H), 7.36 (s, 1H), 7.20 (s, 1H), 7.17 (s, 1H), 4.58 (d, *J* = 5.2 Hz, 2H), 3.94 (d, *J* = 10.4 Hz, 2H), 3.86 (s, 3H), 3.31 (t, *J* = 10.0 Hz, 2H), 3.07 (d, *J* = 6.8 Hz, 2H), 2.95 (m, 3H), 2.38 (s, 2H), 2.32 (s, 3H), 2.10 (s, 3H), 1.73 (m, 4H), 0.87 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, DMSO) δ 169.19, 161.55, 150.03, 148.73, 140.38, 139.69, 135.89, 130.76, 129.64, 127.78, 121.54, 121.24, 120.71, 119.02, 111.54, 66.31, 57.80, 41.08, 38.54, 34.55, 30.28, 26.60, 24.09, 22.14, 21.89, 15.93, 14.46, 12.71. ESI-MS m/z 518.1 $(M + H)^+$.

3-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-5-(6-fluoropyridin-3-yl)-2-methyl-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)benzamide (**10s**). Yield: 85%; ^{1}H NMR (400 MHz, DMSO) δ 11.51 (s, 1H), 8.53 (s, 1H), 8.27 (t, *J* = 7.2 Hz, 1H), 8.18 (s, 1H), 7.48 (s, 1H), 7.28 (s, 1H), 7.26 (s, 1H), 4.32 (d, *J* = 4.0 Hz, 2H), 3.83 (d, *J* = 10.5 Hz, 2H), 3.25 (t, *J* = 11.2 Hz, 2H), 3.10 (d, *J* = 6.4 Hz, 2H), 3.03 (m, 1H), 2.75 (s, 2H), 2.37 (s, 2H), 2.21 (s, 3H), 2.10 (s, 3H), 1.64 (s, 6H), 1.52 (m, 2H), 0.82 (t, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.60, 164.35, 163.12, 161.88, 158.63, 151.99, 148.49, 145.63, 145.45, 140.94, 139.95, 139.62, 134.18, 133.96, 123.72, 120.96, 115.86, 109.66, 109.39, 67.20, 59.49, 42.25, 35.85, 30.20, 27.46, 24.92, 22.41, 16.50, 15.12, 12.91. ESI-MS m/z 533.0 $(M + H)^+$.

5-(6-chloropyridin-3-yl)-3-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-2-methyl-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)benzamide (**10t**). Yield: 72%; ^{1}H NMR (400 MHz, DMSO) δ 11.48 (s, 1H), 8.71 (d, *J* = 2.4 Hz, 1H), 8.34 – 7.91 (m, 2H), 7.58 (d, *J* = 8.4 Hz, 1H), 7.50 (s, 1H), 7.30 (s, 1H), 4.32 (d, *J* = 4.8 Hz, 2H), 3.83 (d, *J* = 9.6 Hz, 2H), 3.25 (t, *J* = 11.2 Hz, 2H), 3.15 – 3.06 (m, 2H), 3.03 (m, 1H), 2.75 (s, 2H), 2.38 (s, 2H), 2.27 (s, 3H), 2.10 (s, 3H), 1.66 (m, 6H), 1.57 – 1.44 (m, 2H), 0.82 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, DMSO) δ 168.71, 161.53, 150.10, 149.31, 149.06, 147.70, 140.37, 139.81, 137.75, 134.67, 133.92, 132.61, 124.25, 123.11, 120.96, 120.67, 111.52, 66.31, 57.78, 40.95, 34.58, 30.24, 26.61, 24.08, 22.13, 21.89, 15.93, 14.68, 12.70. ESI-MS m/z 549.2 $(M + H)^+$.

3-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-2-methyl-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)-5-(6-(4-methylpiperazin-1-yl)pyridin-3-yl)benzamide (**10u**). Yield: 82%; ^{1}H NMR (400 MHz, DMSO) δ 11.48 (s, 1H), 8.39 (d, *J* =

2.4 Hz, 1H), 8.12 (t, J = 4.8 Hz, 1H), 7.75 (m, 1H), 7.36 (s, 1H), 7.17 (s, 1H), 6.89 (d, J = 8.8 Hz, 1H), 4.31 (d, J = 4.8 Hz, 2H), 3.83 (d, J = 10.2 Hz, 2H), 3.52 (s, 4H), 3.25 (t, J = 11.2 Hz, 2H), 3.08 (m, 2H), 3.00 (m, 1H), 2.71 (s, 2H), 2.41 (m, 8H), 2.23 (s, 3H), 2.11 (s, 3H), 1.64 (s, 7H), 1.51 (m, 2H), 0.82 (t, J = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.10, 163.57, 158.36, 151.14, 149.59, 145.90, 140.75, 139.50, 135.86, 135.42, 132.93, 125.77, 123.08, 121.04, 119.94, 114.94, 106.84, 67.32, 58.38, 54.53, 45.80, 44.79, 41.52, 35.88, 30.45, 27.39, 24.85, 22.27, 22.19, 16.52, 14.74, 12.76. ESI-MS m/z 613.2 $(M + H)^+$.

Enzyme inhibitory assays

The enzyme inhibitory assays were performed according to the AlphaLISA assay protocols (PerkinElmer Inc.).

Western blot analysis

After treatment with a series of concentrations of SKLB1049 for various days at 37 °C, cells were harvested, washed in ice-cold PBS, and lysed with RIPA buffer (Beyotime, China). And the protein concentrations were determined by the Bradford method. Proteins were separated by gel electrophoresis on 5–10% SDS-PAGE gels and probed with specific antibodies (Cell Signaling Technology, USA) including anti-H3K27me3, anti-H3K27me2, anti-EZH2, anti-H3 and anti-β-actin. All of the antibodies were used at a 1:1,000 dilution, and the horseradish peroxidase-coupled secondary antibodies (Zhong Shan Golden Bridge Bio-technology, China) were used at 1:5,000.

Cell viability assay

Human cancer cell lines used in this investigation were obtained from American Type Culture Collection (ATCC, Rockville, MD). The MTT assay was used to measure the cell viability after compounds treatment. Cells were seeded in 96-well microplates at a density of 2-5*10^3 cells per well, and then, cultured for 24 h. After treatment with various concentrations of compound for 4 or 6 days, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated 2-4 h at 37°C, the formazan crystals were dissolved with 50 μL of acidified SDS (20%, w/v). The absorbance of each well was measured with Spectra MAX M5 microplate spectrophotometer at 570 nm wavelength, and the median inhibitory concentration (IC_{50}) of each cell line was calculated.

Flow cytometry for cell cycle analysis

The cell cycle was measured by FCM assay. Approximately 1*10^5 cells were collected after treatment with vehicle or SKLB1049 in various concentrations for 14 d and fixed with 70% ethanol overnight. The cells were then washed with cold PBS and stained with 50 μg/ml propidium iodide containing 100 μg/ml RNase, and 0.1% Triton X-100. The cell cycle profiles were determined on a FACS Calibur flow cytometer (Becton Dicknson, USA) and analyzed using the ModFit LT 3.2 software (Verity Software House, USA).

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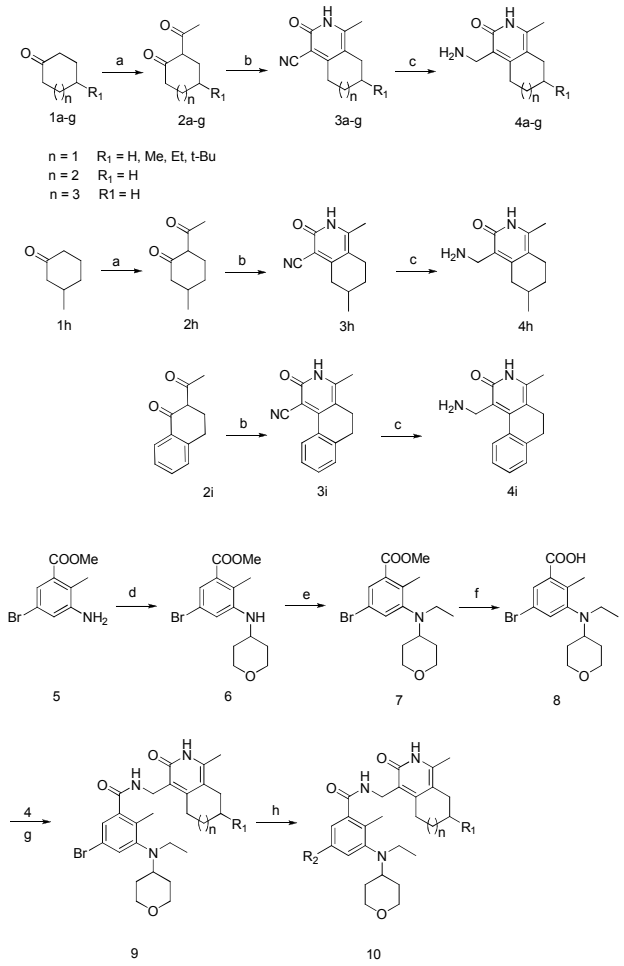


Scheme 1. Reagents and conditions: (a) NaH, EA, PhMe, 0°C- RT or LDA, acetyl chloride, THF, -78°C; (b) cyanothioacetamide, DABCO, ethanol, reflux; (c) $NaBH_4$, I_2, THF, 0°C- reflux; (d) sodium triacetoxyborohyride, acetic acid, trichloromethane, 0 °C- RT; (e) sodium triacetoxyborohyride, acetic acid, 1,2-dichloroethane, 0° C- RT; (f) 3 M NaOH, ethanol, RT, 3 h, then adjust to pH 4- 5 with 1 N HCl; (g) EDC, HOAT, NMM, DMSO, RT, 12- 48 h; (h) $PdCl_2(dppf)\cdot CH_2Cl_2$, Na_2CO_3 , 10:1 dioxane/H_2O, 110°C.

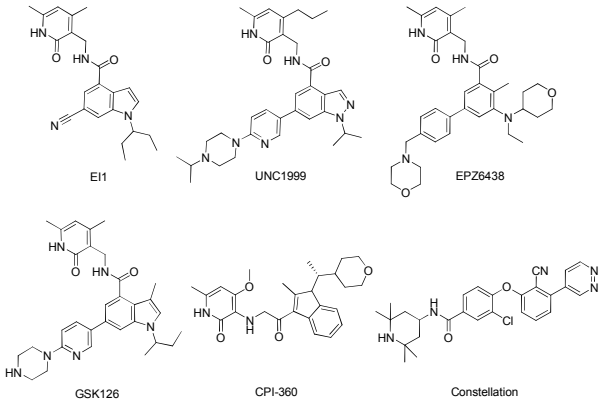


Fig. 1. Disclosed EZH2 inhibitors.

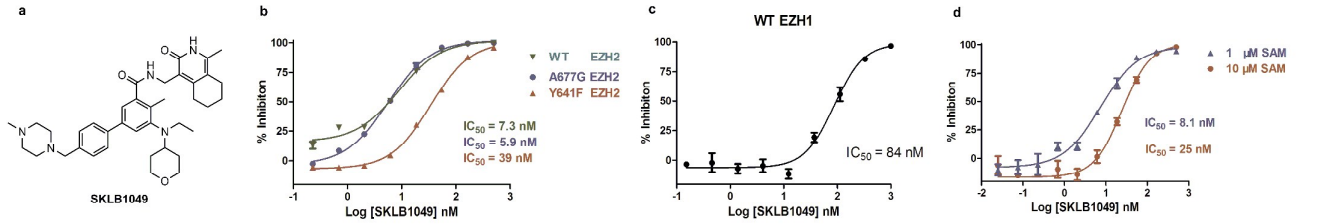


Fig. 2. SKLB1049 was a potent, SAM-competitive and selective inhibitor of EZH2. (a) Chemical structures of SKLB1049. (b) Determination of the inhibitory potential of SKLB1049 on WT EZH2 (green), Y641F mutant EZH2 (red), and A677G EZH2 (blue). (c) Determination of the inhibitory potential of SKLB1049 on WT EZH1. (d) Determination of the mechanism of action of SKLB1049. Data were represented as mean values ± SEM (n = 2).

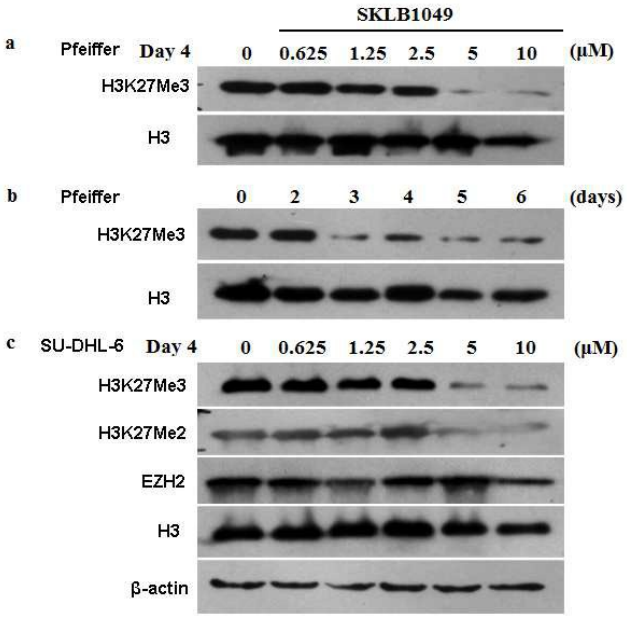


Fig. 3. Compound SKLB1049 reduced H3K27me3 in Pfeiffer and SU-DHL-6 cells in a dose- and time-dependent manner. (a) Pfeiffer cells was treated with 0.625 μM, 1.25 μM, 2.5 μM , 5 μM or 10 μM of SKLB1049 for 4 days and whole cell extracts were analyzed by western blotting for levels of H3K27me3. H3 and β-actin levels served as loading controls. (b) Similarly effect of H3K27me3 levels with 5 μM SKLB1049 in Pfeiffer cells over 6 days were determined. (c) SU-DHL-6 cells was treated of SKLB1049 for 4 days and effect of H3K27me3, H3K27me2 and EZH2 levels were determined. H3 and β-actin levels served as loading controls.

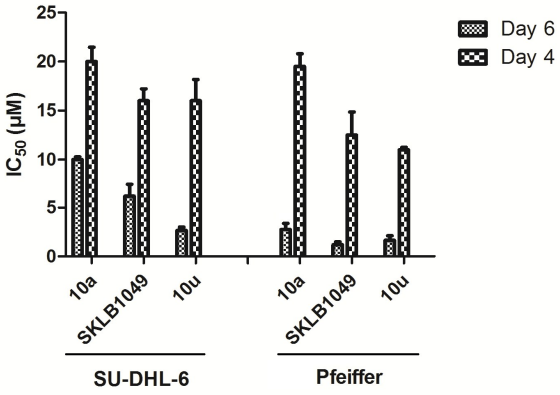


Fig. 4. Viability across SU-DHL-6 and Pfeiffer cell lines treated with **10a**, SKLB1049 and **10u** for 4 and 6 days was assessed. Data were represented as mean values ± SEM (n = 3).

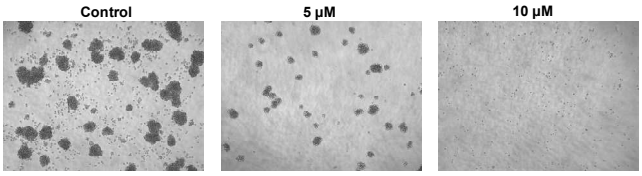


Fig. 5. Bright-field microscopy images of SU-DHL-6 cancer cells, after incubation with SKLB1049 for 6-days at varying concentrations: 5 μM, 10 μM.

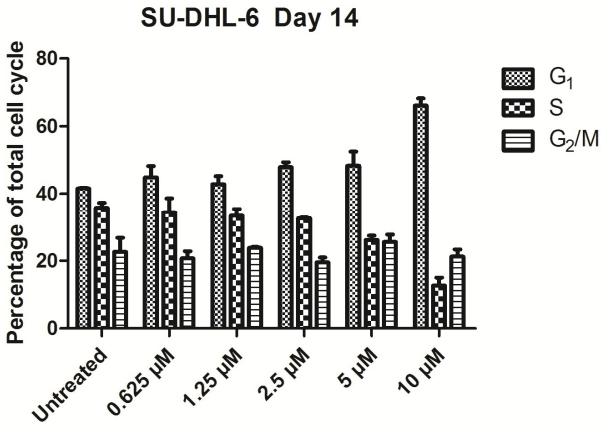
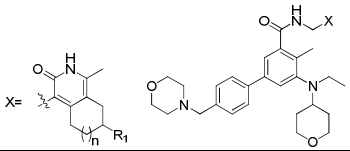
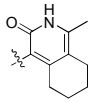
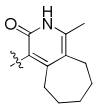
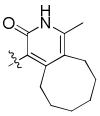
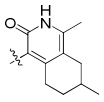
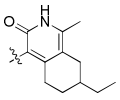
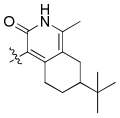
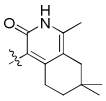
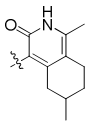
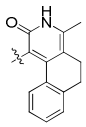


Fig. 6. Compound SKLB1049 induced G_0/G_1 arrest in EZH2 mutant SU-DHL-6 cells. Cell cycle analysis (by flow cytometry) was assessed in SU-DHL-6 cells during incubation with either vehicle or 0.625 μM, 1.25 μM, 2.5 μM , 5 μM, 10 μM SKLB1049 for 14 days. Data were represented as mean values ± SEM (n = 2).

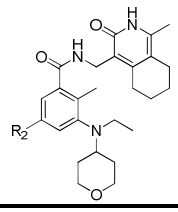
Table 1. *In Vitro* Potencies of Newly Synthesized Inhibitors **10a-10i.**

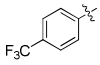
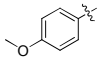
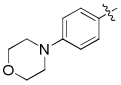
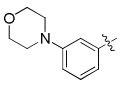
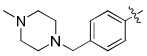


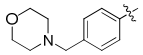
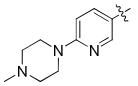
Compd	X	EZH2 IC_{50} (nM) [a]			cells IC_{50} (μM)	
		$EZH2^{WT}$	$EZH2^{Y641F}$	$EZH2^{A677G}$	SU-DHL-6	Pfeiffer
10a		14	27.2	7.0	20	19.5
10b		600	>500	NT[b]	>30	27
10c		610	>500	NT	>30	21
10d		0.66	24	2.9	13.57	19
10e		1.3	23	4.4	18	9
10f		6.4	94	20	23.5	20
10g		3.3	NT	NT	NT	NT
10h		20	63	12	28	22
10i		>5000	NT	NT	>30	>30
EPZ6438		0.27	2.3	0.9	NT	NT
GSK126		1.2	1.0	0.56	NT	NT

[a] The IC_{50} values were averaged from two independent dose-response curves. [b] Not tested.

Table 2. *In Vitro* Potencies of Newly Synthesized Inhibitors **10j-10u.**



Compd	R_2	EZH2 IC_{50} (nM)[a]			cells IC_{50} (μM)	
		$EZH2^{WT}$	$EZH2^{Y641F}$	$EZH2^{A677G}$	SU-DHL-6	Pfeiffer
10j	-Br	19	NT[b]	NT	>30	>30
10k		75	NT	NT	>30	>30
10l		140	1266	NT	>30	>30
10m		9.6	455	NT	>30	>30
10n		55	875	NT	>30	>30
10o		17	NT	38	>30	>30
10p		10	NT	161	>30	>30
10q (SKLB1049)		7.3	39	5.9	16	12.5

10a		14	27.2	7.0	20	19.5
10r		25	NT	45	>30	>30
10s		15.5	NT	NT	>30	>30
10t		19.5	165	NT	>30	>30
10u		4.0	39	5.9	16	11

[a] The IC_{50} values were averaged from two independent dose-response curves. [b] Not tested.

Table 3. The solubility of compound **10a**, **10q** (SKLB1049), **10u** and EPZ6438.

Compd	Solution (μg/ml)						
	water	normal saline	PH=6.5	methanol	ethanol	acetone	dichloromethane
10a	29.7	41.1	29.9	36926.3	265779	44339.3	443393
SKLB1049	185.3	87.6	243.0	>30000	>200000	>30000	>400000
10u	41.3	3.5	0.6	>30000	>200000	>30000	>400000
EPZ6438	3.0	6.8	4.6	10252.3	2121.3	284.9	186437

Table 4. The MTT data (6 days).[a]

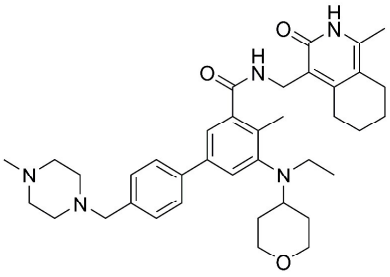
Compd	IC_{50} (μM)				
	SU-DHL-6	Pfeiffer	HEK-293	LO2	VERO
10a	10.02	2.76	>40	>40	>40
SKLB1049	6.17	1.19	>40	>40	>40
10u	2.65	1.65	>40	>40	>40
EPZ6438	6.12	1.47	>40	>40	10.81

[a] IC_{50} value was average of three determinations and deviation from the average was < 5% of the average value.

Design, synthesis and biological evaluation of novel 1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolins as potential EZH2 inhibitors

Abstract

The histone lysine methyltransferase EZH2 has been implicated as a key component in cancer aggressiveness, metastasis and poor prognosis. This study discovered a new class of hexahydroisoquinolin derivatives as EZH2 inhibitors. Structure activity relationship study showed that the steric hindrace was important to the activity for EZH2. Preliminary optimization study led to the discovery of several potent compounds with low nanomolar to sub-nanomolar potency for EZH2. Biological evaluation indicated that SKLB1049 was a highly potent with improved solubility than EPZ6438, SAM-competitive, and cell-active EZH2 inhibitor that decreased global H3K27me3 in SU-DHL-6 and Pfeiffer lymphoma cells in a concentration- and time-dependent manner. Further study indicated that SKLB1049 caused cell arrest in G_0/G_1 phase. These compounds would be useful as chemical tools to further explore the biology of EZH2 and provided us a start point to develop new EZH2 inhibitors.



SKLB1049