Supplementary Material

Enhancement of Histone Deacetylase Inhibitor Sensitivity in Combination with Cyclin-Dependent Kinase Inhibition for the Treatment of Oral Squamous Cell Carcinoma

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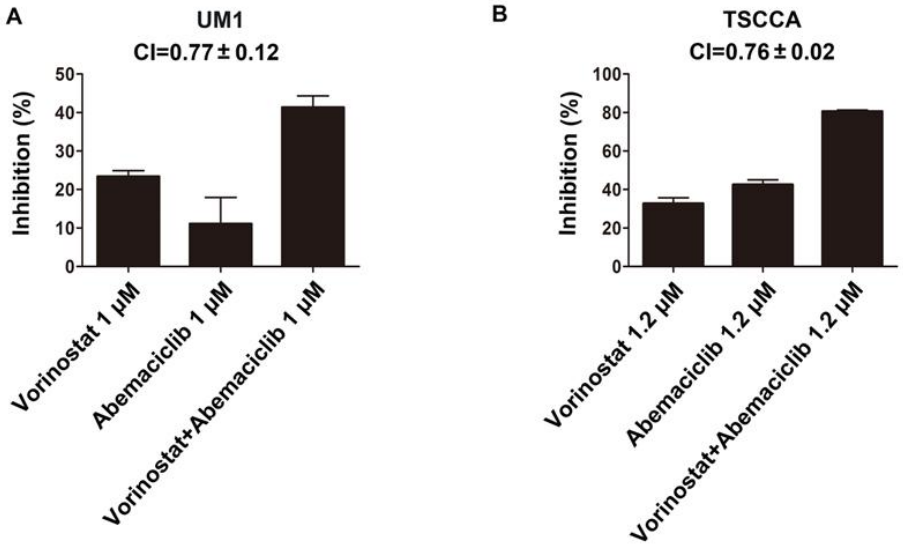


Figure S1. Synergic effect between Vorinostat and Abemaciclib *in vitro*. Synergic effect of HDAC inhibitor (Vorinostat,) and CDK4/6 inhibitor (Abemaciclib) on growth of **(A)** UM1 cell line and **(B)** TSCCA cell line.

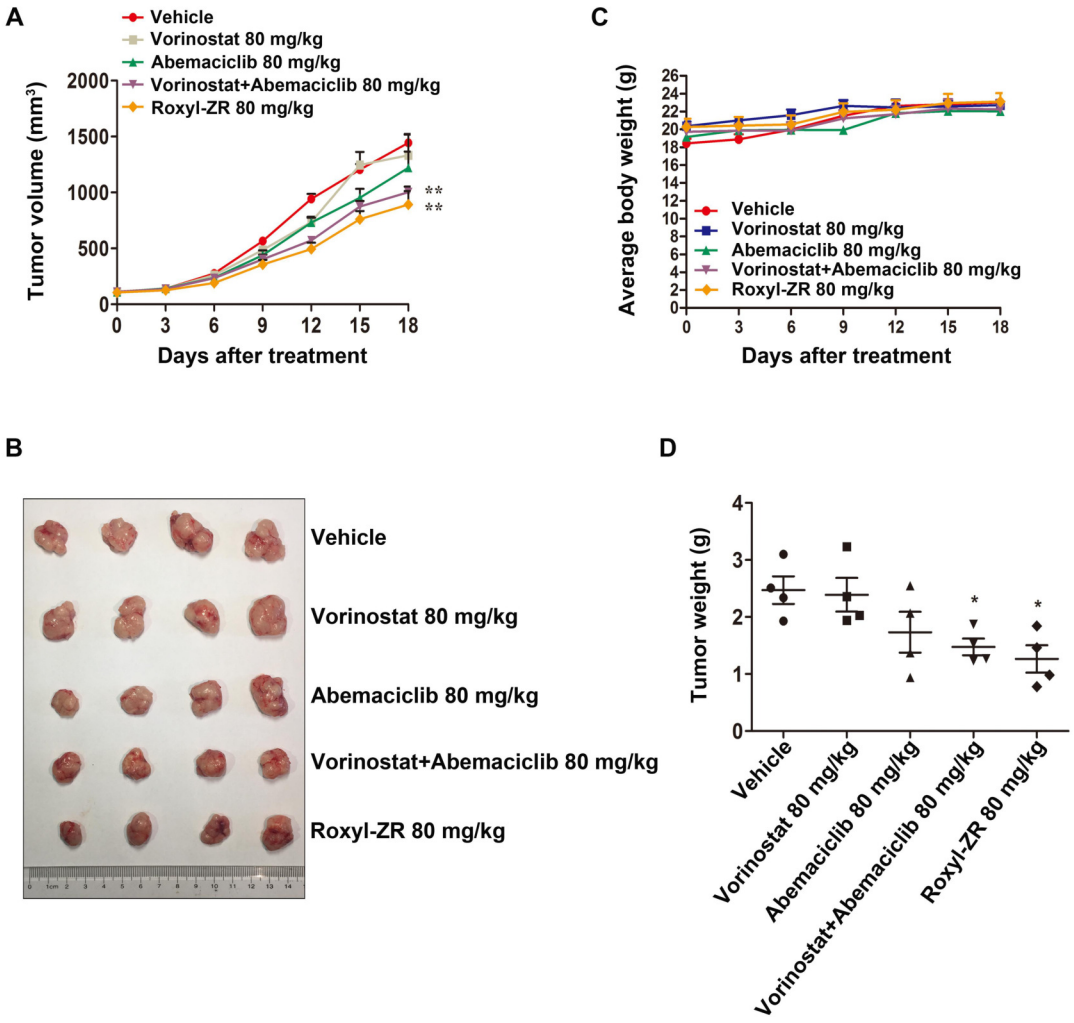
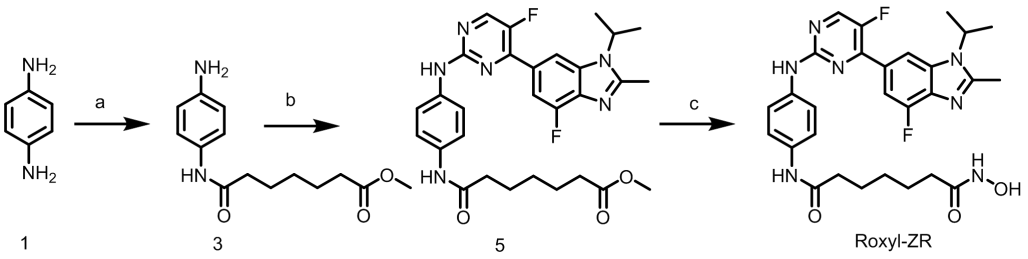


Figure S2. Synergic effect between Vorinostat and Abemaciclib *in vivo*. A. Tumor growth curve of UM1 subcutaneous xenograft models. Mice were treated with Vorinostat (80 mg/kg), Abemaciclib (80 mg/kg) alone or combinations daily for 18 days. Growth curve was plotted by measuring the relative tumor volume every 3 days. **B**. The average body weight of xenograft tumor mice after treated with Vorinostat, Abemaciclib alone or combinations, Roxyl-ZR or vehicle. **C**. Photograph of excised tumors with respective mice at day 18. **D**. Tumor weight of final excised tumors for each treatment group at day 18. Combination index (CI=0.84) was labeled to show the synergic effect *in vivo*.

Scheme 1. Synthesis of the compound Roxyl-ZR.



Reagents and conditions: (a) EDCI, HOBt, DIEA, rt, 12 h, 77% yield; (b) $Pd(OAc)_2$, BINAP, Cs_2CO_3, 100 °C, 12 h, 63% yield; (c) NH_2OH, CH_3OH, reflux, 18 h, 63% yield.

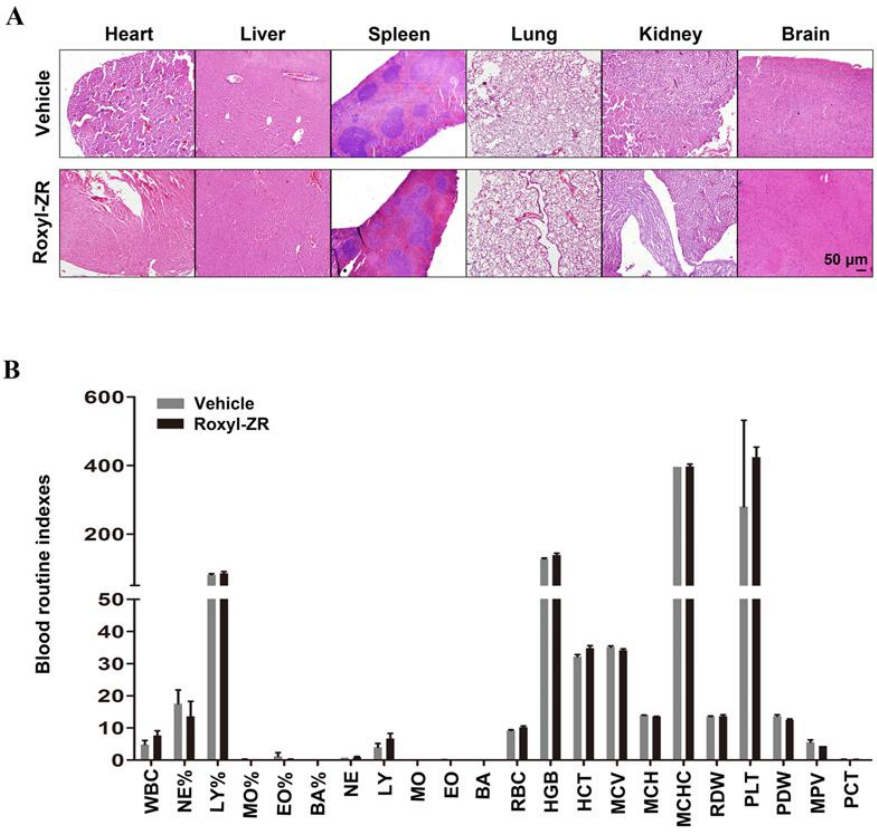


Figure S3. Roxyl-ZR showed no acute toxic effects on mice *in vivo*. (A) Balb/c mice were treated with Roxyl-ZR 80 mg/kg for 48 h, organs were excised. HE staining indicated that Roxyl-ZR had no general toxicity on fatal organs as heart, liver, spleen, lung, kidney and brain, which compared to Control group. Scale bar, 50 μm. (B) Balb/c mice were treated with Roxyl-ZR 80 mg/kg once two days for 4 times and then sacrificed. The blood routine examination demonstrated that Roxyl-ZR was no harmful to blood cells compared to Control group.

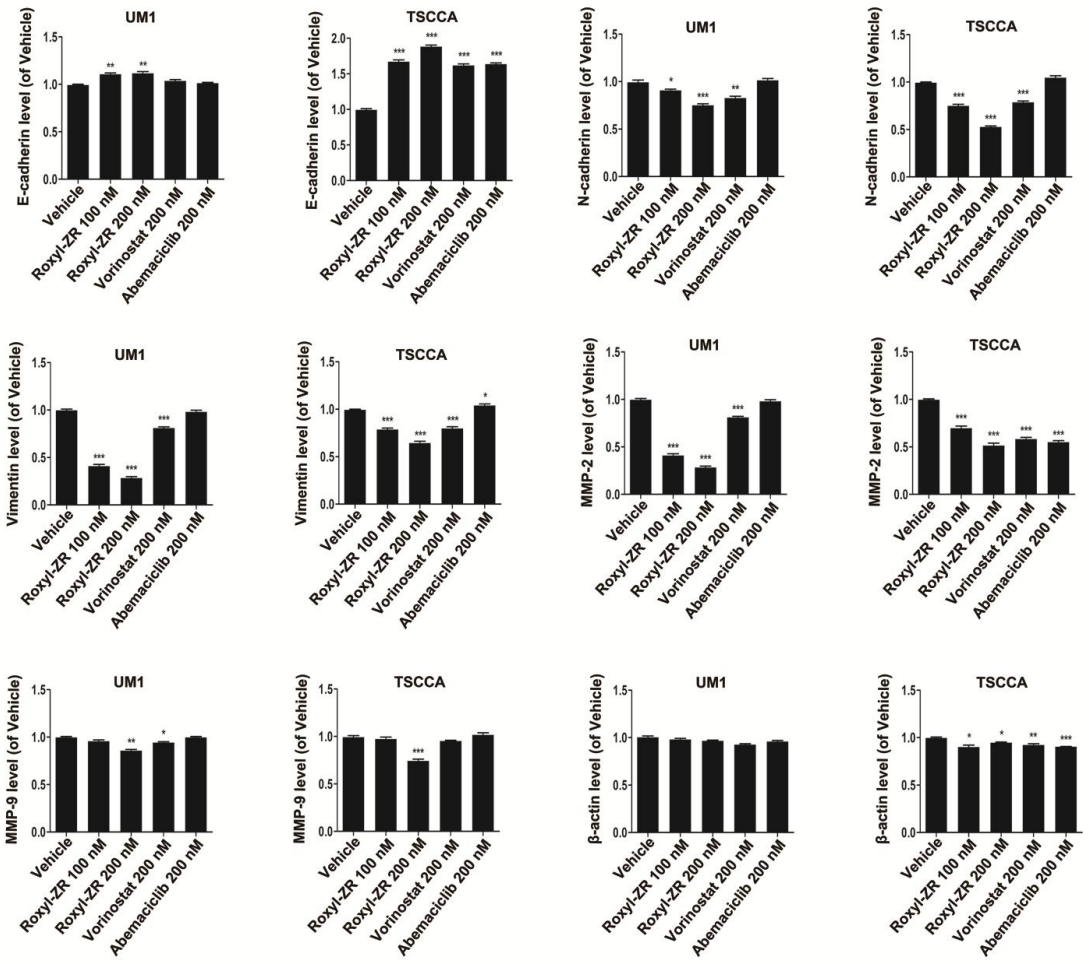


Figure S4. Bar graph for the quantitative comparison among protein expression levels. Roxyl-ZR up-regulated E-cadherin abundance and down-regulated N-cadherin, Vimentin, MMP-2 or MMP-9 abundance with anti-β-actin as a loading control by Western Blot assay.

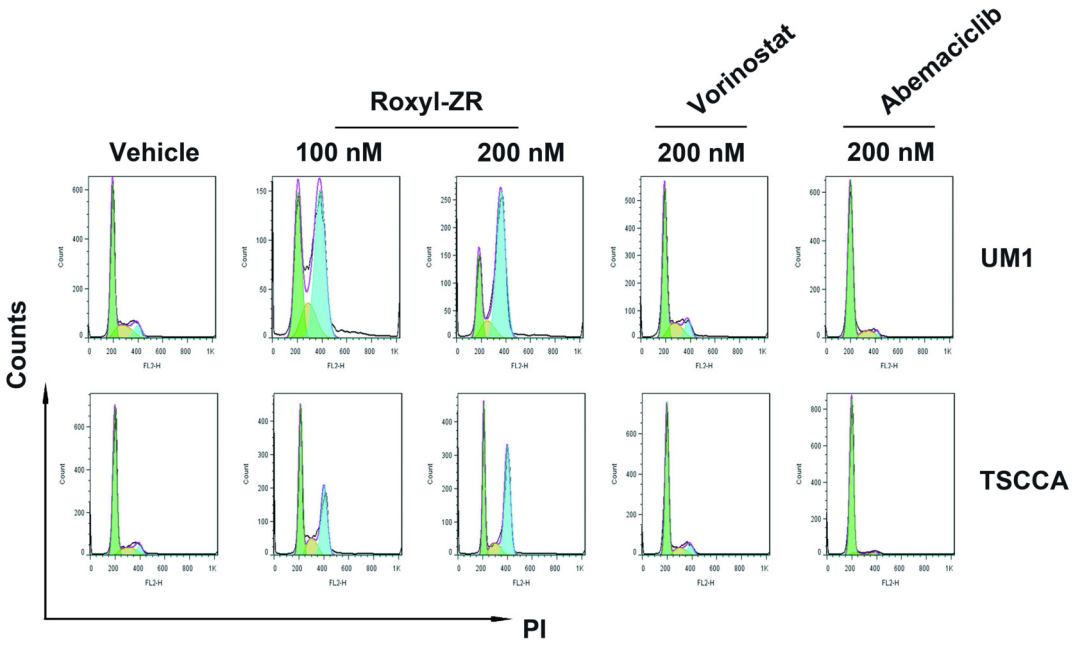


Figure S5. Roxyl-ZR induces the cell cycle arrest of OSCC. Roxyl-ZR induced G2 arrest was accompanied by reduction of G0/G1 and the S phase, as determined by FACS.

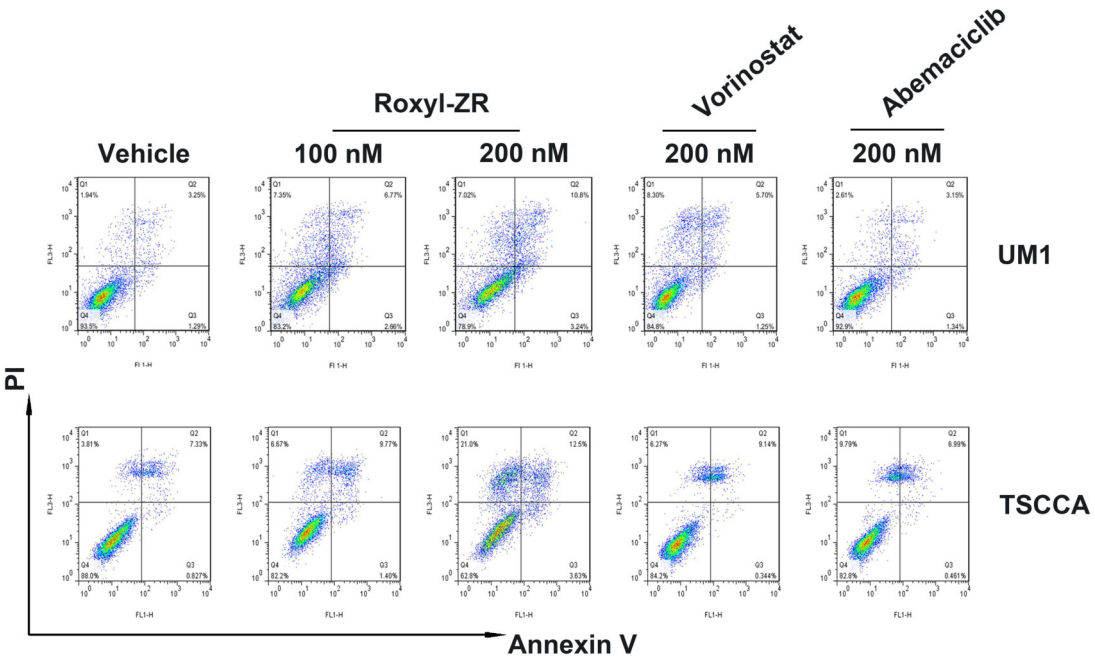


Figure S6. Roxyl-ZR induces the cell apoptosis of OSCC. Roxyl-ZR mediated an increase in apoptosis compared to other groups by AV/PI dual staining.

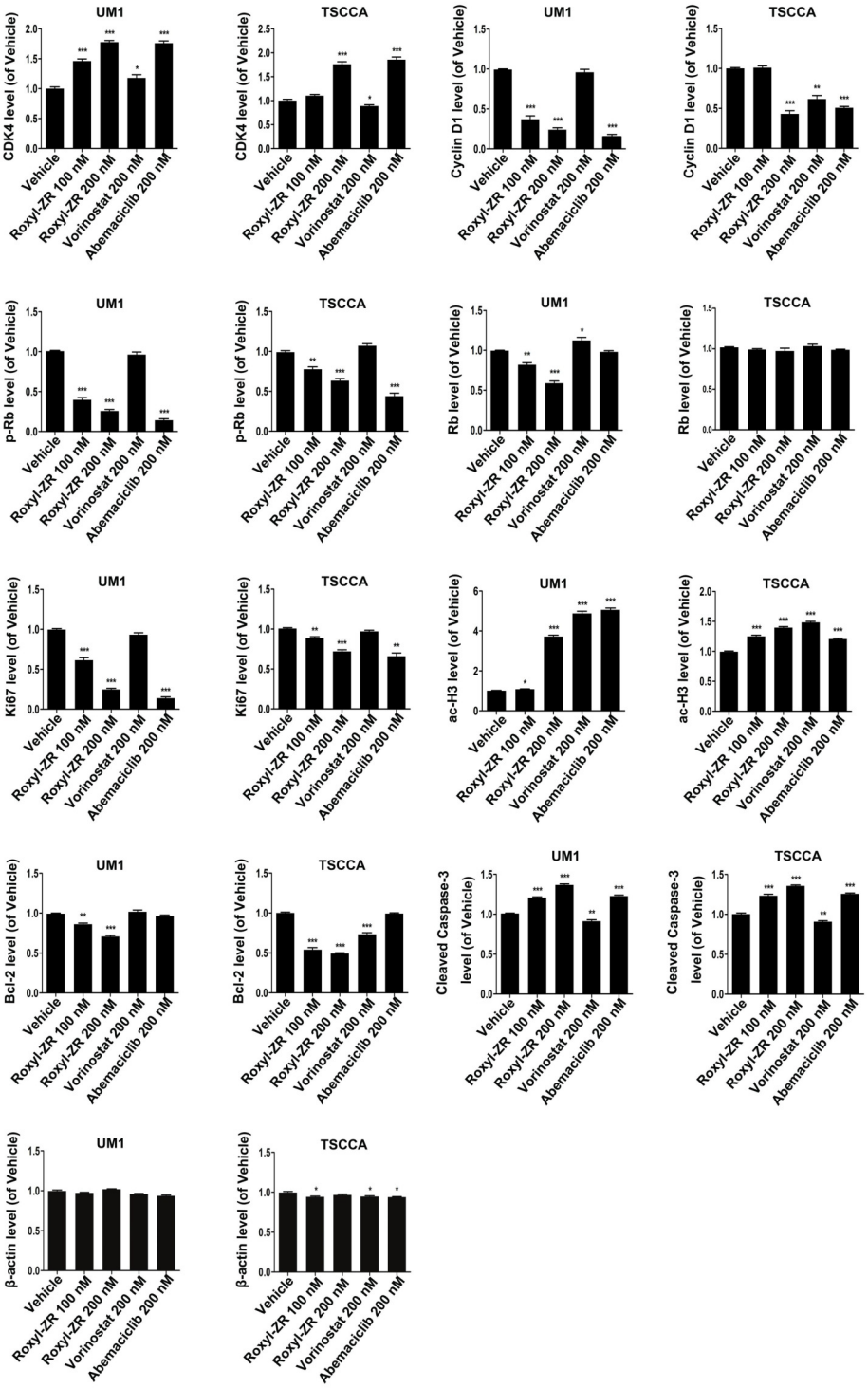


Figure S7. Bar graph for the quantitative comparison among protein expression levels. After cells were treated with Roxyl-ZR, Vorinostat, Abemaciclib or vehicle, the level of Cyclin D1, p-Rb, Ki67 and Bcl-2 abundance were significantly down-regulated, while CDK4, ac-H3 and Cleaved Caspase-3 abundance were up-regulated with anti-β-actin as a loading control by Western Blot assay.

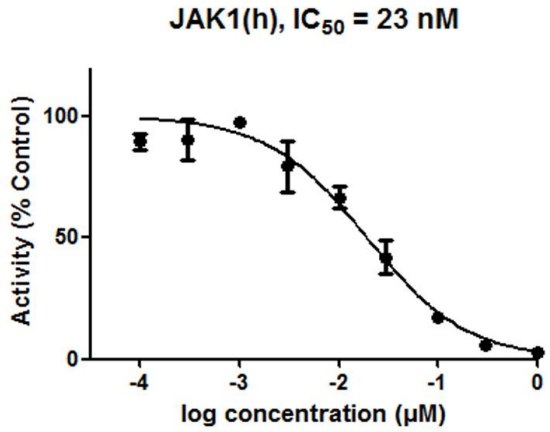


Figure S8. Kinase activity on JAK1 of Roxyl-ZR.

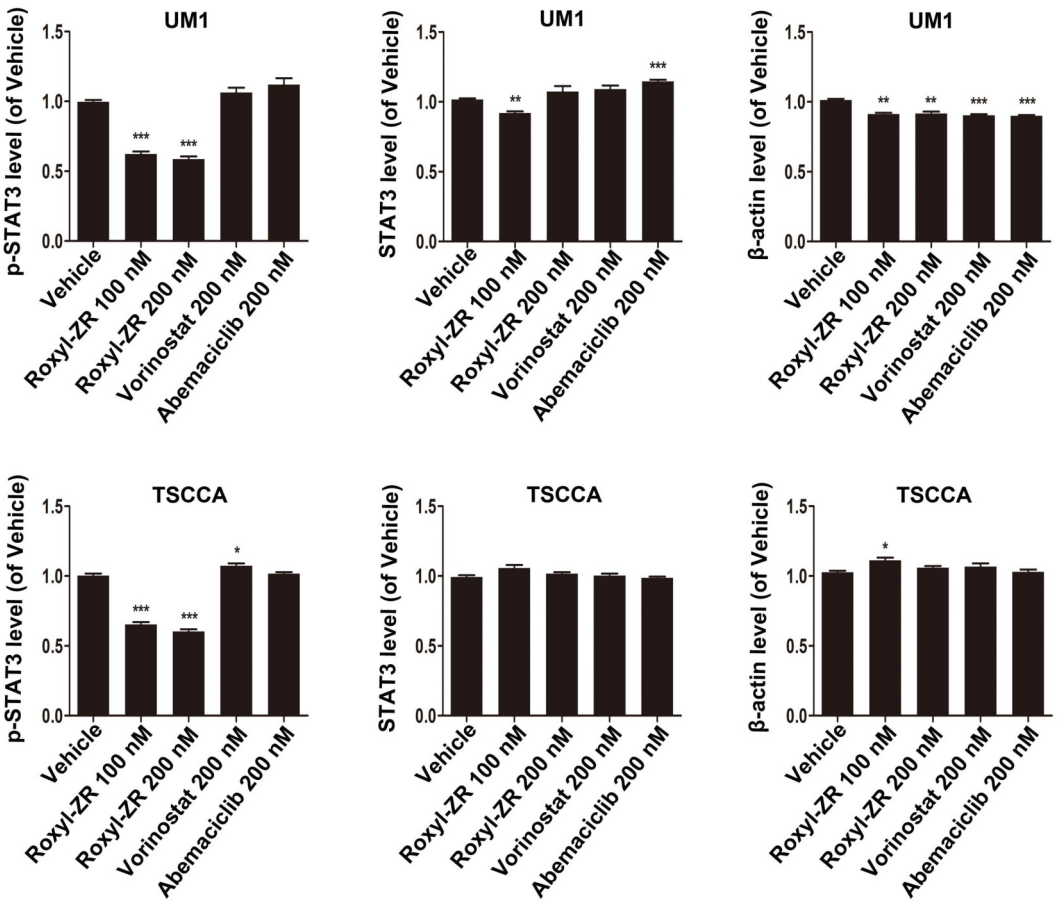


Figure S9. Bar graph for the quantitative comparison among protein expression levels. Roxyl-ZR decreased the level of p-STAT3 abundance in dose-dependence with anti-β-actin as a loading control by Western Blot assay.

Materials and Methods

Molecular docking

Since there is no 3D structure of CDK4-ligand complex reported at present, the structure of CDK6 in complex (PDB entry: 4EZ5) was used as a template for homology modeling. MODELLER (Eswar et al, 2008) in the Discovery Studio (Accelrys, San Diego, CA) (DS) was employed for the homology modeling. The compounds were docked to CDK4 and HDAC1 (PDB code: 4BKX) by GOLD (version 5.0). The crystal structure of HDAC1 was taken from the RCSB Protein Data Bank (PDB entry: 4BKX). Hydrogen atoms were added to the proteins by using Discovery Studio 3.1. GoldScore was selected as the scoring function, and other parameters were set as default. The image was created using PyMOL (Delano, 2014).

Enzyme Inhibition Assays

The HDAC activity assay assays were performed by BPS Bioscience Inc. All of the compounds are dissolved in DMSO. The serial dilution of the compounds was first performed in 100% DMSO with the highest concentration at 1mM. Each intermediate compound dilution (in 100% DMSO) will then get directly diluted 10x fold into assay buffer for an intermediate dilution of 10% DMSO in HDAC assay buffer and 5 µL of the dilution was added to a 50 µL reaction so that the final concentration of DMSO is 1% in all of reactions. The enzymatic reactions for the HDAC enzymes were conducted in duplicate at 37ºC for 30 minutes in a 50 µL mixture containing HDAC assay buffer, 5 µg BSA, an HDAC substrate, a HDAC enzyme and a test compound. After enzymatic reactions, 50 µL of 2 x HDAC Developer was added to each well for the HDAC enzymes and the plate was incubated at room temperature for an additional 15 minutes. Fluorescence intensity was measured at an excitation of 360 nm and an emission of 460 nm using a Biotek Synergy microplate reader.

The *in vitro* kinase assays were performed by Eurofins Pharma Discovery Services UK Limited (Eurofins). CDK4/Cyclin D3(h) is incubated with 8 mM MOPS pH 7.0, 0.2 mM EDTA, 0.03% BSA, 0.03% Tween 20, 20 mM DTT, and 0.2 mg/ml Rb fragment, 10 mM MgAcetate and [gamma-33P-ATP] (specific activity and concentration as required). The reaction is initiated by the addition of the Mg/ATP mix. After incubation for 40 minutes at room temperature, the

reaction is stopped by the addition of phosphoric acid to a concentration of 0.5%. 10 µL of the reaction is then spotted onto a P30 filtermat and washed four times for 4 minutes in 0.425% phosphoric acid and once in methanol prior to drying and scintillation counting.

JAK1 (h) is incubated with 20 mM Tris/HCl pH 7.5, 0.2 mM EDTA, 500 µM GEEPLYWSFPAKKK, 10 mM MgAcetate and [gamma-33P]-ATP (specific activity and concentration as required). The reaction is initiated by the addition of the Mg/ATP mix. After incubation for 40 minutes at room temperature, the reaction is stopped by the addition of phosphoric acid to a concentration of 0.5%. 10 µL of the reaction is then spotted onto a P30 filtermat and washed four times for 4 minutes in 0.425% phosphoric acid and once in methanol prior to drying and scintillation counting.

Chemistry

DMSO as vehicle, Abemaciclib and Vorinostat are purchased from commercial supplier Solarbio Science & Technology, Jinan heropharma pharmaceutical Co., Ltd. and Tokyo Chemical Industry (TCI), respectively.

^{1}H NMR (400 MHz), ^{13}C NMR (101 MHz) and ^{19}F NMR (376 MHz) spectra were taken on a Bruker AV-400 MHz spectrometer. High-resolution mass spectra (HRMS) were recorded on a VG ZAB-HS mass spectrometer under electron spray ionization (ESI). All of the solvents were purified and distilled according to the standard procedure. The purity of tested compound was assessed to be >95% by HPLC analysis on a Shimadzu Prominence-i LC-2030C 3D system.

methyl 7-((4-aminophenyl)amino)-7-oxoheptanoate(3). To a two-necked flask, 7-methoxy-7-oxoheptanoic acid (2) (1.38 g, 7.92 mmol), *p*-Phenylenediamine (1) (2.98 g, 15.84 mmol), EDCI (2.28 g, 11.9 mmol), HOBt (1.28 g, 9.5 mmol), DIEA (2.05 g, 15.84 mmol) and DMF (15 mL) were charged. The mixture was stirred at room temperature for 12 hours, then quenched by water. The mixture was extracted by ethyl acetate, and the combined organic layer was washed with brine solution and dried by anhydrous magnesium sulphate. The solvent was evaporated, and the residue was purified by silica gel column chromatography to obtain 1.614 g (77%) of 3 as a grey solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.26 – 7.19 (m, 2H), 6.62 – 6.54 (m, 2H), 3.63 (s, 3H), 3.57 (br, 2H), 2.33 – 2.21 (m, 4H), 1.74 – 1.55 (m, 4H), 1.39 – 1.29 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.28, 171.23, 143.18, 129.42, 122.08, 115.37, 51.56, 37.05, 33.84, 28.65, 25.32, 24.55.

methyl7-((4-((5-fluoro-4-(4-fluoro-1-isopropyl-2-methyl-1H-benzo[d]imidazol-6-yl)pyrimidin-2-yl)amino)phenyl)amino)-7-oxoheptanoate (5). To a suspension of 6-(2-chloro-5-fluoropyrimidin-4-yl)-4-fluoro-1-isopropyl-2-methyl-1H-benzo[d]imida zole (4) (323 mg, 1 mmol) in 15 mL 1,4-dioxane were added compound 3 (264 mg, 1

mmol), $Pd(OAc)_2$ (5.6 mg, 0.025 mmol), BINAP (31 mg, 0.05 mmol) and Cs_2CO_3 (489 mg, 1.5 mmol) and the flask was purged with Ar. Then the flask was sealed and the mixture was heated for 12 h at 100 ℃. The reaction was cooled to room temperature, the solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography to obtain 347 mg (63%) of 5 as a solid. ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 9.79 (s, 1H), 9.68 (s, 1H), 8.58 (d, *J* = 4.0 Hz, 1H), 8.26 (d, *J* = 1.3 Hz, 1H), 7.71 (d, *J* = 9.1 Hz, 2H), 7.65 (dd, *J* = 12.0, 1.2 Hz, 1H), 7.56 (d, *J* = 9.0 Hz, 2H), 4.85 (p, *J* = 6.8 Hz, 1H), 3.58 (s, 3H), 2.64 (s, 3H), 2.31 (t, *J* = 7.6 Hz, 4H), 1.60 (dd, *J* = 24.2, 7.4 Hz, 10H), 1.32 (dd, *J* = 7.0, 3.1 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 173.28, 170.63, 156.36, 154.42, 153.48, 151.07 (d, *J* = 11.3 Hz), 149.64, 148.62, 147.78 (d, *J* = 26.5 Hz), 136.28 (d, *J* = 9.8 Hz), 135.75, 133.61, 133.13 (d, *J* = 16.6 Hz), 126.80 (t, *J* = 6.7 Hz), 119.21(d, *J* = 20.8 Hz), 108.69 (d, *J* = 4.2 Hz), 106.95(dd, *J* = 20.1, 8.9 Hz), 51.11, 48.08, 36.14, 33.14, 28.12, 24.83, 24.19, 20.89, 14.49. ^{19}F NMR (376 MHz, DMSO-*d*$_6$) δ -128.68 (s, 1F), -150.83 (s, 1F). ESI-HRMS m/z calcd for $C_{29}H_{32}F_2N_6O_3$ 550.2504, found 551.2576 $[M + H]^+$. HPLC purity 96%.

N1-(4-((5-fluoro-4-(4-fluoro-1-isopropyl-2-methyl-1H-benzo[d]imidazol-6-yl)pyrimidin-2-yl)amino)phenyl)-N7-hydroxyheptanediamide(6). To a stirred solution of the compound 5 (276 mg, 0.5 mmol) in methanol (10 mL) was added a solution of hydroxylamine (50% in water, 3 mL). The resulting solution was stirred for 18 h under reflux. The solvent was removed under vacuum and the crude residue purified by chromatography on a silica gel column to obtain 174 mg (63%) of 6 as a solid. ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 10.38 (s, 1H), 9.81 (s, 1H), 9.70 (s, 1H), 8.71 (d, *J* = 1.7 Hz, 1H), 8.58 (d, *J* = 3.9 Hz, 1H), 8.26 (s, 1H), 7.70 (d, *J* = 8.6 Hz, 2H), 7.64 (d, *J* = 12.1 Hz, 1H), 7.56 (d, *J* = 8.6 Hz, 2H), 4.84 (p, *J* = 6.9 Hz, 1H), 2.63 (s, 3H), 2.28 (t, *J* = 7.3 Hz, 2H), 1.96 (t, *J* = 7.3 Hz, 2H), 1.68 – 1.47 (m, 10H), 1.29 (q, *J* = 8.1 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 171.19, 169.54, 156.84, 155.54 – 154.55 (m), 153.96, 151.55 (d, *J* = 12.6 Hz), 150.07 (d, *J* = 10.3 Hz), 149.11, 148.30 (d, *J* = 26.2 Hz), 136.76 (d, *J* = 10.1 Hz), 136.24, 134.11, 133.60 (d, *J* = 17.2 Hz), 127.28 (t, *J* = 6.5 Hz), 119.70 (d, *J* = 19.0 Hz), 109.17, 107.46 (dd, *J* = 19.8, 8.8 Hz), 48.58, 36.69,

32.64, 28.76, 25.43, 21.40, 15.00. ^{19}F NMR (376 MHz, DMSO-d_6) δ -128.24(s, 1F), -150.39(s, 1F). ESI-HRMS m/z calcd for $C_{28}H_{31}F_2N_7O_3$ 551.2456, found 552.2521 [M + H]$^+$. HPLC purity 98%.