Characterization of a dual BET/HDAC inhibitor for treatment of pancreatic ductal adenocarcinoma

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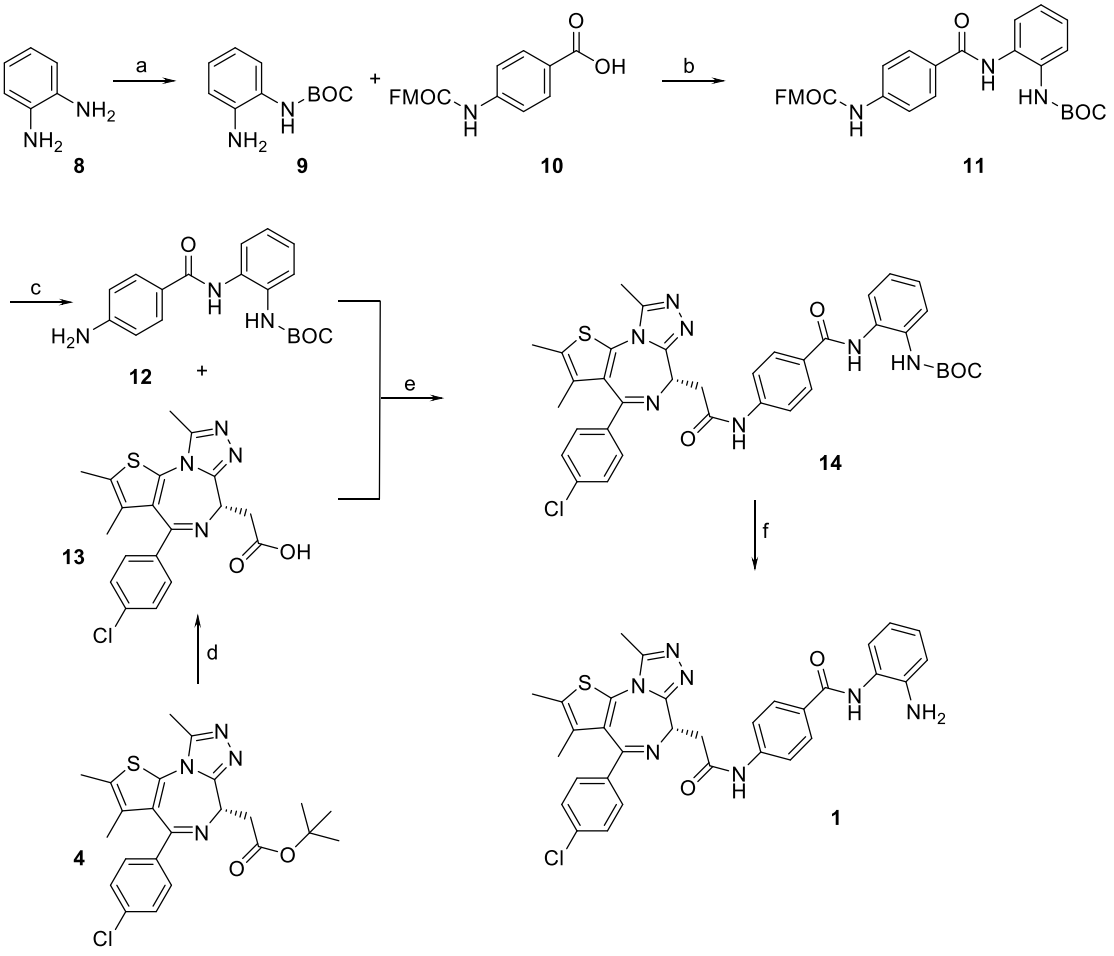
Supplementary Materials and Methods

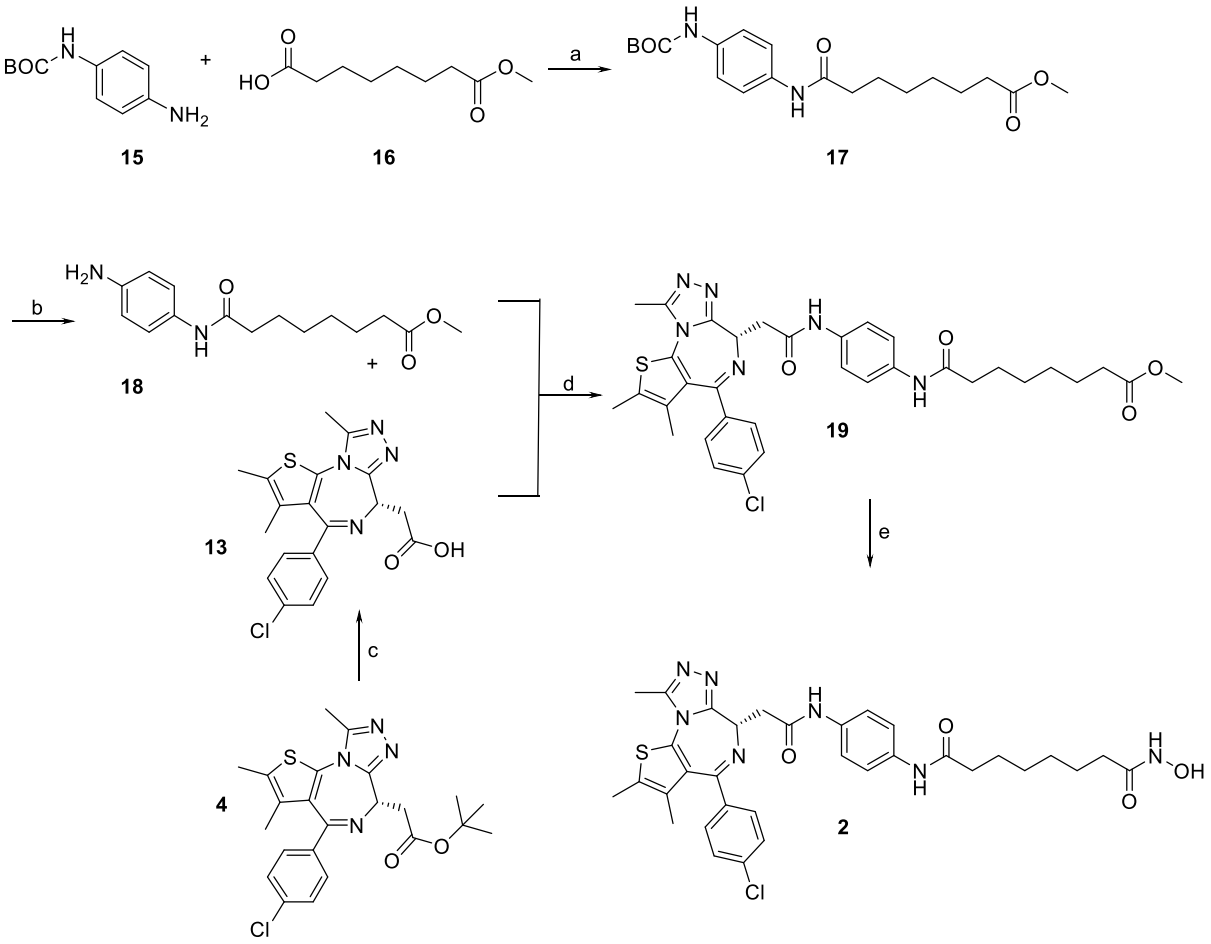
Synthesis of TW9 (1), TW12 (2) and TW22 (3).

Dual inhibitors TW9 **(1)**, TW12 **(2)** and TW22 **(3)** were synthesized as outlined in Schemes 1-3.



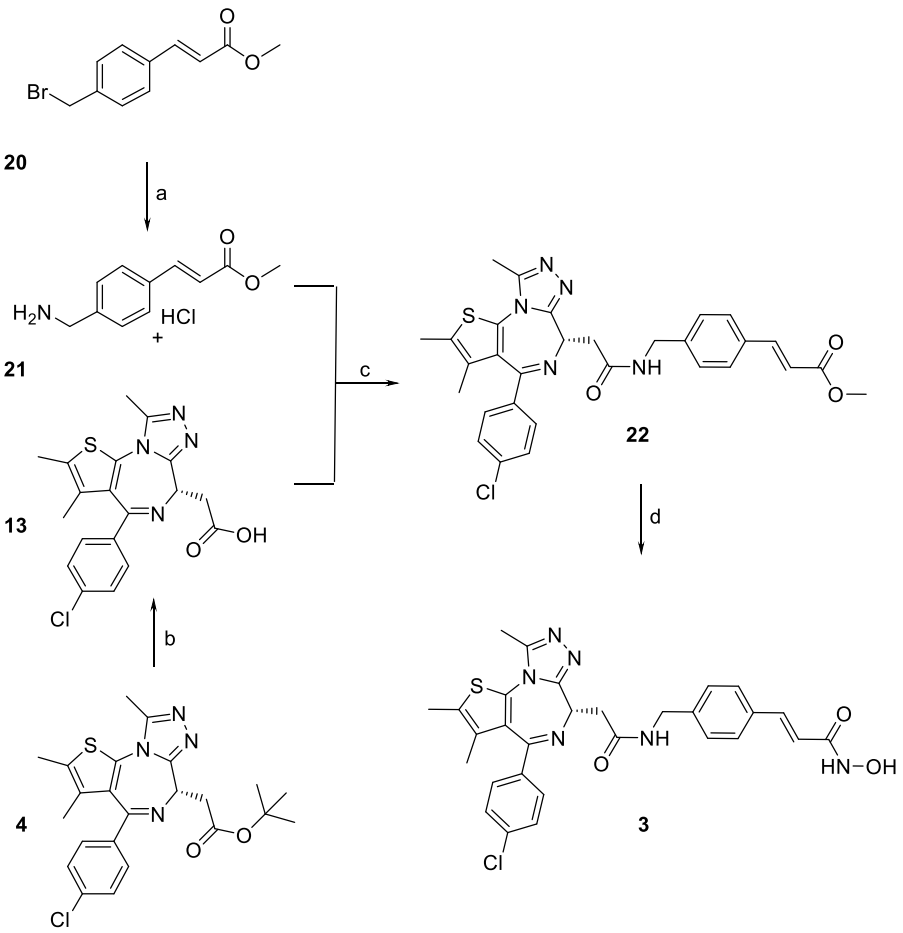
Scheme 1. Synthesis route TW9 **(1)**.

Reagents and conditions : (a) CH_2Cl_2, BOC_2O, RT, 16h, 52% ; (b) CH_2Cl_2/DMF, HATU, DIPEA, RT, 16h, 60%; (c) morpholine, RT, 30 min, 70% ; (d) THF/MeOH/H_2O, LiOH, 16h, 99% ; (e) CH_2Cl_2/DMF, HATU, DIPEA, RT, 16 h, 38% ; (f) CH_2Cl_2, TFA, RT, 45h, 64%.



Scheme 2. Synthesis route TW12 **(2)**.

Reagents and conditions : (a) CH_2Cl_2/DMF, HATU, DIPEA, RT, 16 h, 88% ; (b) CH_2Cl_2, TFA, RT, 45 min, 52%; (c) THF/MeOH/H_2O, LiOH, 16 h, 99% ; (d) CH_2Cl_2/DMF, HATU, DIPEA, RT, 16 h; 45% (e) MeOH, [NH_3OH]Cl, CH_3NaO, -78 °C, 3 h, 26%.



Scheme 3. Synthesis route TW22 **(3)**.

Reagents and conditions: (a) DMF, NaN_3, 80 °C, 2 h, THF, H_2O, TPP, 87% ; (b) THF/MeOH/H_2O, LiOH, 16 h, 99% ; (c) CH_2Cl_2/DMF, HATU, DIPEA, RT, 16 h; 73% (d) MeOH, $[NH_3OH]Cl$, CH_3NaO, -78 °C, 3 h, 29%.

If not mentioned otherwise, all reactions were carried out under a dry argon atmosphere. If reactions were heated in an oil bath, the stated reaction temperature was 10 °C below the temperature of the oil bath. Anhydrous solvents were bought from Sigma-Aldrich and stored over molecular sieves (3 Å).

Flash chromatography purification was performed with a PURIFLASH XS 420 system from Interchim using puriFlash cartridges (15 µm, 30 µm or 50 µm spherical silica) and technical grade solvents. UV absorption was detected at 254 and 280 nm.

ESI spectra were measured using a ThermoFisher Surveyor MSQ spectrometer. TLC-MS was measured using a TLC-MS interface 2 from Camag. High-resolution mass spectra were measured in positive mode using a Thermo Fisher Scientific MALDI LTQ Orbitrap XL spectrometer, and α-cyano-4-hydroxycinnamic acid (HCCA) was used as the matrix.

NMR spectra were recorded at 298 K using the following spectrometers: Bruker DPX-250, Avance-400, Avance-300, Avance-500, or DRX-600. Chemical shift values are referenced to (residual) solvent signals (DMSO: δ = 2.50/39.5 ppm [$^1H/^{13}C\{^1H\}$]).

Abbreviations: s = singlet, br = broad signal, d = doublet, dd = doublet of doublets, ddd = doublet of doublets of doublets, dt = doublet of triplets, t = triplet, td = triplet of doublets, q = quartet, m = multiplet, u = undefined, $\{^1H\}$ = ^1H-decoupled

HPLC analysis was performed on an Agilent system with a 1260 Infinity II MWD (Multiple Wavelength Detector) with an Eclipse XDB-C18 (5 µm, 4.6x250 mm) column or InfinityLab Poroshell 120 EC-C18 (2.7µm, 4.6 x 100mm) column (both purchased from Agilent). UV absorption was detected at 254 nm and 280 nm. The solvents used were H_2O and MeCN (both supplemented with 0.1 % TFA).

Procedures:

HATU coupling reaction:

HATU (1.2 eq) was added to a solution of the corresponding carboxylic acid (1 eq) with DIPEA (1.2 eq) in CH_2Cl_2/DMF (2:1). The mixture was stirred for one hour, then the corresponding amine (1 eq) was added and stirred overnight. After removing the solvent under reduced pressure, the remaining residue was partitioned between EtOAc and water. The aqueous phase was extracted three times with EtOAc and the combined organic fractions were washed with brine and dried over $MgSO_4$. The residue was purified via flash chromatography.

FMOC deprotection:

The Fmoc-protected compound (1 eq) was dissolved in CH_2Cl_2/DMF (1:1) and piperidine (20% of total volume) was added. The reaction (monitored via TLC) was complete after 45 minutes. The solvent was evaporated under reduced pressure, and the residue was purified via flash chromatography.

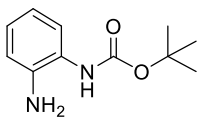
BOC deprotection:

The BOC-protected compound (1 eq) was dissolved in CH_2Cl_2, and TFA (35% of total volume) was added. The reaction (monitored via TLC) was complete after 45 minutes. After the solvent was removed under reduced pressure, the residue was purified via flash chromatography.

Hydroxylamine synthesis:

To a solution of methylcarboxylester (1 eq) with hydroxylamine hydrochloride (15 eq) in dry MeOH, sodium methoxide (25 % (w/v) in methanol, 25 eq) was added slowly at -78°C. The resulting mixture was stirred at -20 °C for 1 h before it was warmed up to RT. After 2 more hours, the reaction (monitored via TLC) was complete. The solvent was removed under reduced pressure, and the remaining residue was partitioned between EtOAc and water. The aqueous phase was extracted three times with EtOAc and the combined organic fractions were washed with brine and dried over $MgSO_4$. The residue was purified via flash chromatography.

***tert*-butyl (2-aminophenyl)carbamate (9)**

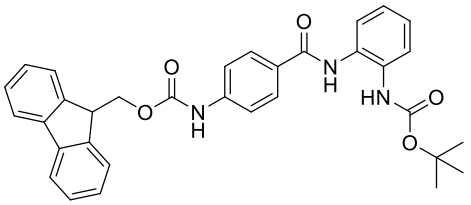


A solution of di-*tert*-butyl dicarbonate (10.1 g, 46.2 mmol, 1 eq) in CH_2Cl_2 (25 mL) was added dropwise at 0°C to a solution of *o*-phenylenediamine **8** (5 g, 46.2 mmol, 1 eq) in CH_2Cl_2 (20 mL). The reaction mixture was stirred overnight at RT. The solvent was removed under reduced pressure. The residue was purified by flash chromatography with hexane/EtOAc (70:30), yielding compound **9** as a white solid (1.48 g, 77 %).

^{1}H-NMR (250 MHz, DMSO-d_6): δ = 8.26 (s, 1H), 7.22 – 7.12 (m, 1H), 6.83 (td, J = 7.9, 1.5 Hz, 1H), 6.67 (dd, J = 8.0, 1.5 Hz, 1H), 6.52 (td, J = 7.8, 1.5 Hz, 1H), 4.80 (s, 2H), 1.45 (s, 9H) ppm.

MS (ESI-): m/z 231.12 ([M-H], 100)

(9H-fluoren-9-yl)methyl (4-((2-((*tert*-butoxycarbonyl)amino)phenyl)carbamoyl)phenyl)carbamate (11)



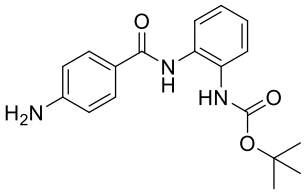
The reaction was performed by following the general procedure for the HATU coupling reaction with 4-(Fmoc-amino)benzoic acid **10** (400 mg, 1.1 mmol) and **9** (231.8 mg, 1.1 mmol). Compound **11** was obtained (370 mg, 60.5 %) as a colorless solid.

R_f(hexane/EtOAc = 60:40) = 0.43.

^{1}H-NMR (250 MHz, DMSO-d_6): δ = 10.05 (s, 1H), 9.73 (s, 1H), 8.66 (s, 1H), 7.90 (t, $^3J_{HH}$ = 7.8 Hz, 4H), 7.77 (d, $^3J_{HH}$ = 7.3 Hz, 2H), 7.54 (ddd, $^3J_{HH}$ = 9.9, $^3J_{HH}$ = 7.5, $^3J_{HH}$ = 5.6 Hz, 4H), 7.48 – 7.32 (m, 4H), 7.17 (ddd, $^3J_{HH}$ = 7.7 Hz, 5.4 Hz, $^4J_{HH}$ = 1.9 Hz, 2H), 4.54 (d, $^3J_{HH}$ = 6.5 Hz, 2H), 4.34 (t, $^3J_{HH}$ = 6.4 Hz, 1H), 1.45 (s, 9H) ppm.

MS (ESI-): m/z 572.13 ([M-H], 100).

***tert*-butyl (2-(4-aminobenzamido)phenyl)carbamate (12)**



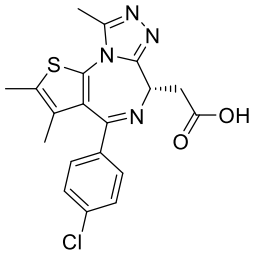
Reaction performed by following the general procedure for FMOC deprotection with **11** (370 mg, 0.67 mmol). Compound **12** was obtained (150 mg, 68.1 %) as a colorless solid.

Rf(hexane/EtOAc = 40:60) = 0.54.

^{1}H-NMR (250 MHz, DMSO-d_6): δ = 9.59 (s, 1H), 8.67 (s, 1H), 7.76 (d, $^3J_{HH}$ = 8.6 Hz, 2H), 7.59 – 7.44 (m, 2H), 7.20 - 7.07 (m, 2H), 6.80 (d, $^3J_{HH}$ = 7.9 Hz, 2H), 1.45 (s, 9H) ppm.

MS (ESI+): m/z 350.09 ([M+Na), 100)

(S)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl) acetic acid (13)



To a solution of (+)-JQ1 **4** (350 mg, 0.77 mmol, 1 eq) in THF/MeOH/H_2O (3:2:1), LiOH (321 mg, 7.7 mmol, 10 eq) was added. The reaction was stirred for 24 h at RT. After removing the solvents under reduced pressure, the residue was dissolved in water, acidified and extracted with EtOAc. The organic layer was washed with brine and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the residue was purified via flash chromatography with CH_2Cl_2/MeOH (9:1) as mobile phase, yielding compound **13** (286 mg, 93 %) as a colorless solid.

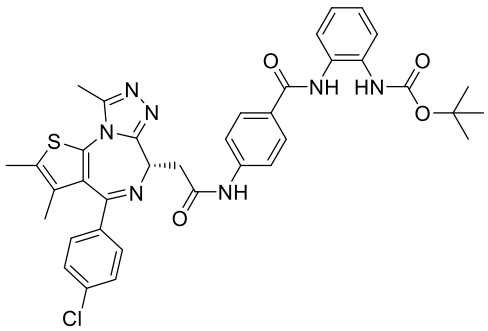
R_f(EtOAc/AcOH = 98:2) = 0.30.

^{1}H-NMR (250 MHz, DMSO-d_6): δ = 12.22 (s, 1H), 7.54 – 7.40 (m, 4H), 4.45 (t, J = 7.1 Hz, 1H), 3.52 – 3.23 (m, 2H), 2.60 (s, 3H), 2.41 (s, 3H), 1.63 (s, 3H) ppm.

^{13}C-NMR (126 MHz, DMSO-d_6): δ = 172.04, 163.12, 154.85, 149.89, 136.67, 135.27, 132.25, 130.75, 130.16, 129.86, 129.52, 128.52, 128.51, 53.60, 14.06, 12.69, 11.29 ppm.

MS (ESI-): m/z 399.01 ([M-H), 100)

***tert*-butyl (S)-(2-(4-(2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)acetamido)benzamido)phenyl)carbamate (14)**



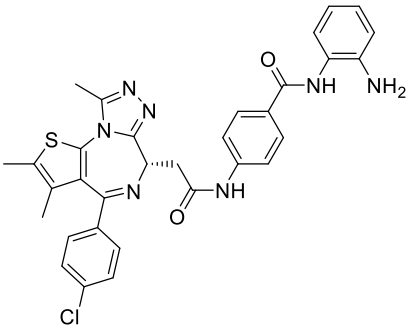
Reaction performed by following the general procedure for the HATU coupling reaction with **12** (124.9 mg, 0.31 mmol) and **13** (102 mg, 0.31 mmol). Compound **14** was obtained (85 mg, 38.4%) as a light yellow solid.

R_f(CH_2Cl_2/MeOH = 90:10) = 0.6.

^{1}H-NMR (250 MHz, DMSO-d_6): δ = 10.62 (s, 1H), 9.76 (s, 1H), 8.67 (s, 1H), 7.94 (d, $^3J_{HH}$ = 8.8 Hz, 2H), 7.79 (d, $^3J_{HH}$ = 8.8 Hz, 2H), 7.58 - 7.35 (m, 6H), 7.24 – 7.08 (m, 2H), 4.63 (t, $^3J_{HH}$ = 7.1 Hz, 1H), 3.57 (d, $^3J_{HH}$ = 7.1 Hz, 2H), 2.60 (d, $^3J_{HH}$ = 4.6 Hz, 3H), 2.43 (s, 3H), 1.64 (s, 3H), 1.45 (s, 9H) ppm.

MS (ESI-): m/z 732.30 ([M-H], 100).

(S)-N-(2-aminophenyl)-4-(2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)acetamido)benzamide (1) – TW9



Reaction performed by following the general procedure for BOC deprotection with **14** (85 mg, 0.12 mmol). Compound **1** (TW9) was obtained (47 mg, 64.3 %) as a light yellow solid.

$R_f(CH_2Cl_2/MeOH = 9.1) = 0.42$.

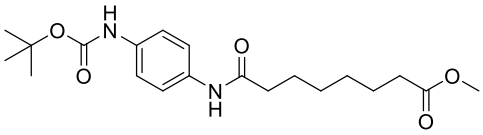
^{1}H-NMR (500 MHz, DMSO-d_6): δ = 10.59 (s, 1H), 9.58 (s, 1H), 7.97 (d, $^3J_{HH}$ = 8.6 Hz, 2H), 7.76 (d, $^3J_{HH}$ = 8.7 Hz, 2H), 7.49 (d, $^3J_{HH}$ = 8.8 Hz, 2H), 7.43 (d, $^3J_{HH}$ = 8.5 Hz, 2H), 7.16 (d, $^3J_{HH}$ = 7.4 Hz, 1H), 6.98 – 6.95 (m, 1H), 6.78 (dd, $^3J_{HH}$ = 8.0 Hz, $^4J_{HH}$ = 1.2 Hz, 1H), 6.62 - 6.58 (m, 1H), 4.89 (s, 2H), 4.62 (t, $^3J_{HH}$ = 7.1 Hz, 1H), 3.56 (d, $^3J_{HH}$ = 7.1 Hz, 2H), 2.61 (s, 3H), 2.43 (s, 3H), 1.64 (s, 3H) ppm.

^{13}C-NMR (126 MHz, DMSO-d_6): δ = 169.13 (1C, CO), 164.72 (1C, CO), 163.31 (1C, CO), 155.01 (1C), 149.98 (1C), 143.17 (2C), 141.96 (1C), 136.74 (1C), 135.29 (1C, C_{Ar}), 130.79 (1C, C_{Ar}), 130.16 (2C, C_{Ar}), 129.90 (2C, C_{Ar}), 129.56 (1C, C_{Ar}), 128.77 (2C, C_{Ar}), 128.54 (2C, C_{Ar}), 126.69 (2C, C_{Ar}), 123.39 (1C, C_{Ar}), 123.48 (1C, C_{Ar}), 118.18 (2C, C_{Ar}), 116.29 (1C), 116.15 (1C), 53.69 (1C), 14.10 (1C, CH_3), 12.72 (1C, CH_3), 11.34 (1C, CH_3) ppm.

MS (ESI-): m/z 608.26 ([M-H], 100).

HRMS (MALDI) m/z calculated 610.17865 for $C_{32}H_{29}ClN_7O_2$, found 610.17855. HPLC: purity > 95%

Methyl 8-((4-((tert-butoxycarbonyl)amino)phenyl)amino)-8-oxooctanoate (17)

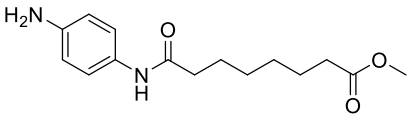


Reaction performed by following the general procedure for the HATU coupling reaction with tert-butyl (4-aminophenyl)carbamate **15** (500 mg, 2.4 mmol) and 8-methoxy-8-ococtanoic acid **16** (0.4 mL, 2.4 mmol). Compound **17** was obtained (800 mg, 88 %) as a colorless solid.

^{1}H-NMR (250 MHz, DMSO-d_6): δ = 9.70 (s, 1H), 9.19 (s, 1H), 7.44 (d, J = 9.0 Hz, 2H), 7.33 (d, J = 9.0 Hz, 2H), 3.57 (s, 3H), 2.27 (dt, J = 11.3, 7.4 Hz, 4H), 1.63 – 1.49 (m, 4H), 1.46 (s, 9H), 1.32 – 1.23 (m, 4H) ppm.

MS (ESI-): m/z 377.14 ([M-H], 100).

Methyl 8-((4-aminophenyl)amino)-8-oxooctanoate (18)



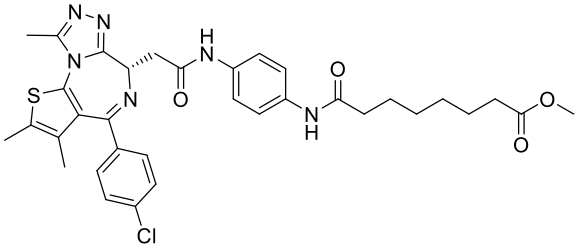
Reaction performed by following the general procedure for FMOC deprotection with **17** (508 mg, 1.34 mmol). Compound **18** was obtained (194 mg, 51.9 %) as a colorless solid.

$R_f(CH_2Cl_2/MeOH = 95:5) = 0.24$.

^{1}H-NMR (250 MHz, DMSO-d_6): δ = 9.86 (s, 1H), 7.55 (d, J = 8.8 Hz, 2H), 7.05 (d, J = 8.7 Hz, 2H), 3.57 (s, 3H), 2.33 – 2.23 (m, 4H), 1.54 (d, J = 7.1 Hz, 4H), 1.28 (q, J = 5.4, 3.8 Hz, 4H) ppm.

MS (ESI-): m/z 277.09 ([M-H], 75)

Methyl (S)-8-((4-(2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)acetamido)phenyl)amino)-8-oxooctanoate (19)



Reaction performed by following the general procedure for the HATU coupling reaction with **13** (120 mg, 0.30 mmol) and **18** (140 mg, 0.50 mmol). Compound **19** was obtained (85 mg, 45 %) as a light purple solid.

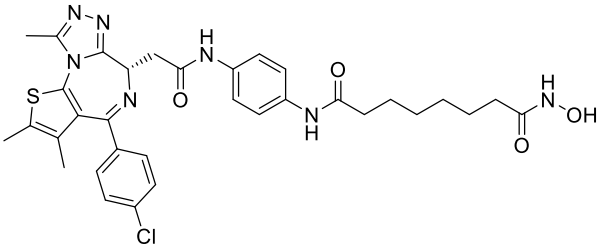
$R_f(CH_2Cl_2/MeOH = 95:5) = 0.15$.

^{1}H-NMR (500 MHz, DMSO-d_6): δ = 10.23 (s, 1H), 9.79 (s, 1H), 7.54 – 7.46 (m, 6H), 7.42 (d, J = 8.5 Hz, 2H), 4.59 (t, J = 7.1 Hz, 1H), 3.58 (s, 3H), 3.48 (d, J = 6.1 Hz, 2H), 2.60 (s, 3H), 2.42 (s, 3H), 2.31 – 2.25 (m, 4H), 1.63 (s, 3H), 1.58 – 1.50 (m, 4H), 1.32 – 1.26 (m, 4H) ppm.

^{13}C-NMR (126 MHz, DMSO-d_6): δ = 173.82, 171.35, 168.73, 163.67, 162.78, 155.53, 150.37, 137.20, 135.72, 135.24, 134.92, 132.77, 131.22, 130.61, 130.34, 130.04, 128.99, 119.94, 119.89, 54.25, 51.65, 36.71, 33.71, 28.80, 28.70, 25.45, 24.80, 14.55, 13.17, 11.79 ppm.

MS (ESI+): m/z 683.30 ([M+Na], 100)

(S)-N[1]-(4-(2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)acetamido)phenyl)-N[8]-hydroxyoctanediamide (2) – TW12



Reaction performed by following the general procedure for hydroxylamine synthesis with **19** (120 mg, 0.18 mmol). Compound **2** (TW12) was obtained (31 mg, 26 %) as a light purple solid.

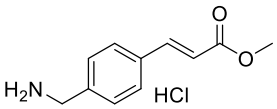
$R_f(CH_2Cl_2/MeOH = 9:1) = 0.24$.

^{1}H-NMR (500 MHz, DMSO-d_6): δ = 10.33 (s, 1H, NH), 10.24 (s, 1H), 9.80 (s, 1H), 8.65 (s, br, 1H), 7.55 – 7.46 (m, 6H), 7.42 (d, J = 8.6 Hz, 2H), 4.59 (t, J = 7.1 Hz, 1H), 3.47 (d, J = 7.1 Hz, 2H), 2.60 (s, 3H), 2.42 (s, 3H), 2.26 (t, J = 7.4 Hz, 2H), 1.93 (t, J = 7.4 Hz, 2H), 1.63 (s, 3H), 1.56 (p, *J* = 7.4 Hz, 2H), 1.48 (p, J = 7.3 Hz, 2H), 1.31 – 1.22 (m, 4H) ppm.

^{13}C-NMR (126 MHz, DMSO-d_6): δ = 170.90, 169.09, 168.26, 163.22, 155.07, 149.93, 136.73, 135.26, 134.78, 134.45, 132.31, 130.77, 130.15, 129.88, 129.57, 128.53, 119.48, 119.42, 54.25, 38.50, 36.20, 32.25, 28.45, 28.42, 25.08, 25.05, 14.09, 12.72, 11.33 ppm.

MS (ESI+): m/z 662.41 ([M+H], 100). HRMS (MALDI) m/z calculated 662.23108 for $C_{33}H_{37}ClN_7O_4S$, found 662.23109. HPLC: purity > 95%

Methyl (E)-3-(4-(aminomethyl)phenyl)acrylate hydrochloride (21)

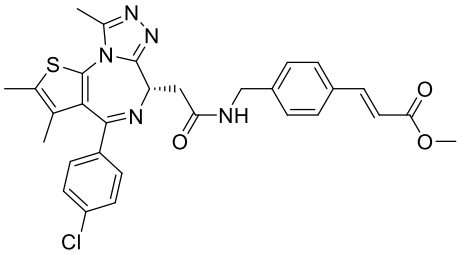


To a solution of methyl (E)-3-(4-(bromomethyl)phenyl)acrylate **20** (1 g, 3.92 mmol, 1eq) in DMF (12 mL), NaN_3 (305.8 mg, 4.7 mmol, 1.2 eq) was added. The reaction was stirred at 80 °C for 2 hours. After the mixture was cooled down, brine was added, and the reaction was extracted with Et_2O/hexane (1:1). The combined organic layers were washed with water, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was dissolved in THF (18 mL) and H_2O (3 mL), then PPh_3 (1.13 g, 4.3 mmol, 1.1 eq) was added. The reaction was stirred overnight at RT. The solvents were removed under reduced pressure. Salt **21** was obtained (0.74 mg 87%) after precipitating in CH_2Cl_2 and HCl (1.0 M in diethyl ether) as a colorless solid.

^{1}H-NMR (250 MHz, DMSO-d_6): δ = 8.58 (s, 3H), 7.76 (d, J = 8.0 Hz, 2H), 7.67 (d, J = 16.1 Hz, 1H), 7.55 (d, J = 8.1 Hz, 2H), 6.69 (d, J = 16.1 Hz, 1H), 4.03 (s, 2H), 3.73 (s, 3H) ppm.

MS (ESI+): m/z 192.11 ([M-Cl], 10).

Methyl (*S*,*E*)-3-(4-((2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4] diazepin-6-yl)acetamido)methyl)phenyl)acrylate (22)



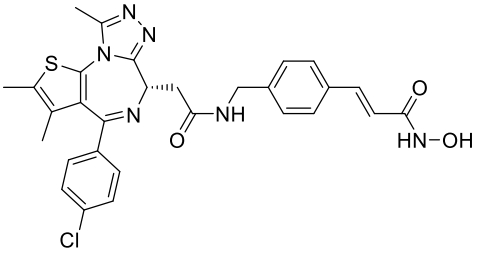
Reaction performed by following the general procedure for the HATU coupling reaction with **13** (100 mg, 0.25 mmol) and **21** (73.8 mg, 0.32 mmol). Compound **22** was obtained (105 mg, 73 %) as a colorless solid.

$R_f(CH_2Cl_2/MeOH = 95:5) = 0.27$.

^{1}H-NMR (250 MHz, DMSO-d_6): δ = 8.77 (t, J = 6.0 Hz, 1H), 7.73 - 7.62 (m, 3H), 7.49 – 7.41 (m, 2H), 7.41 – 7.30 (m, 4H), 6.62 (d, J = 16.1 Hz, 1H), 4.59 – 4.25 (m, 3H), 3.73 (s, 3H), 3.42 – 3.20 (u, 2H, masked by H_2O signal), 2.60 (s, 3H), 2.41 (s, 3H), 1.61 (s, 3H) ppm.

MS (ESI-): m/z 572.24 ([M-H], 70).

(*S*,*E*)-3-(4-((2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3*a*][1,4]diazepin-6-yl)acetamido)methyl)phenyl)-N-hydroxyacrylamide (3) – TW22



Reaction performed by following the general procedure for hydroxylamine synthesis with **22** (105 mg, 0.18 mmol). Compound **3** (TW22) was obtained (31 mg, 29 %) as a colorless solid.

^{1}H-NMR (500 MHz, DMSO-d_6): δ = 10.74 (s, 1H), 9.04 (s, 1H), 8.78 (t, J = 6.0 Hz, 1H), 7.52 (d, J = 8.1 Hz, 2H), 7.49 – 7.42 (m, 3H), 7.40 – 7.31 (m, 4H), 6.45 (d, J = 15.8 Hz, 1H), 4.54 (dd, J = 8.4, 5.9 Hz, 1H), 4.36 (dd, J = 27.3, 5.9 Hz, 2H), 3.37 – 3.31 (u, 2H, masked by H_2O signal), 2.60 (s, 3H), 2.41 (s, 3H), 1.61 (s, 3H) ppm.

^{13}C-NMR (126 MHz, DMSO-d_6): δ = 169.71, 163.14, 162.81, 155.08, 149.88, 141.19, 138.13, 136.74, 135.26, 133.40, 132.30, 130.73, 130.14, 129.85, 129.58, 128.47, 127.78, 127.46, 118.63, 53.95, 41.91, 37.64, 14.09, 12.72, 11.35 ppm.

MS (ESI+): m/z 574.92 ([M+H], 100).

HRMS (MALDI) m/z calculated 575.16266 for $C_{29}H_{28}ClN_6O_3S$, found 575.16165.

HPLC: purity > 95%.

Supplementary Table S1. X-ray data collection and refinement statistics of BRD4(1)-inhibitor structures.

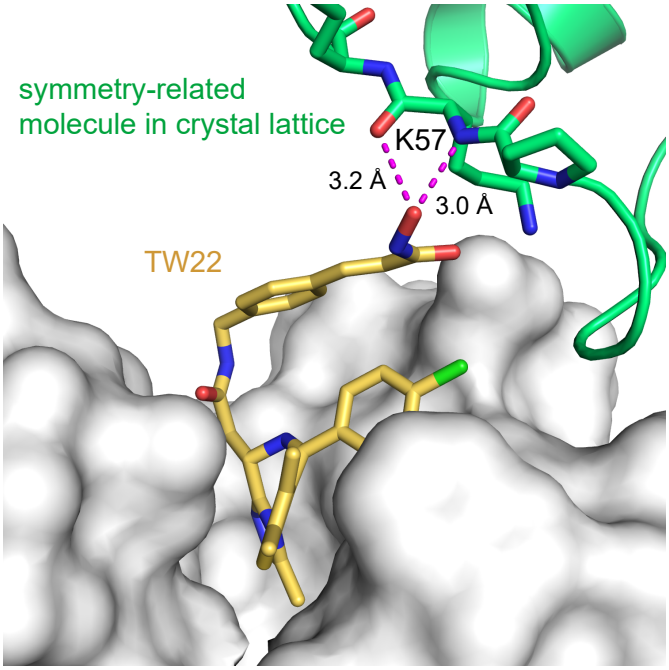
Compound	**TW9 (1)**	**TW12 (2)**	**TW22 (3)**
Data Collection			
Space Group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
a, b, c (Å)	37.25, 44.34, 78.26	37.17,44.2, 78.58	37.03, 44.43, 78.77
Molecules/AU	1	1	1
Resolution (Å)[a]	78.3 -1.05 (1.07-1.05)	44.3 -1.07 (1.09-1.07)	78.8 -1.25 (1.27-1.25)
Unique reflections	61,370	57,949	36,807
Completeness (%)[a]	100 (100)	99.8 (99.0)	99.9 (99.6)
Multiplicity[a]	11.6 (11.8)	10.6 (10.9)	7.5 (7.1)
R_{merge} (%)[a]	8.4 (69.2)	7.9 (74.2)	7.1 (48.8)
CC(1/2)	0.998 (0.896)	0.998 (0.889)	0.998 (0.884)
Mean $I/\sigma(I)$[a]	14.3 (2.8)	14.3 (3.0)	13.1 (2.8)
Refinement			
R_{work}, (%)[b]	16.4	15.7	15.9
R_{free}, (%)[b]	17.6	17.4	19.3
No. of atoms			
Protein[c]	1067	1087	1086
Water	147	145	168
Ligands	35	46	52
RMSD bonds (Å)	0.005	0.005	0.005
RMSD angles (°)	0.82	0.83	0.80
Mean *B* (Å^2)	14.2	13.9	16.2
Ramachandran favored (%)[d]	98.5	98.5	98.5
Ramachandran outliers (%)[d]	0	0	0
PDB ID	6YQN	6YQO	6YQP

[a]Values in parentheses are for the highest resolution shell.
[b]R_{work} and $R_{free} = \sum||F_{obs}| - |F_{calc}||/\sum|F_{obs}|$, where R_{free} was calculated with 5 % of the reflections chosen at random and not used in the refinement.
[c]Number includes alternative conformations.
[d]Ramachandran statistics were calculated using MolProbity (1).

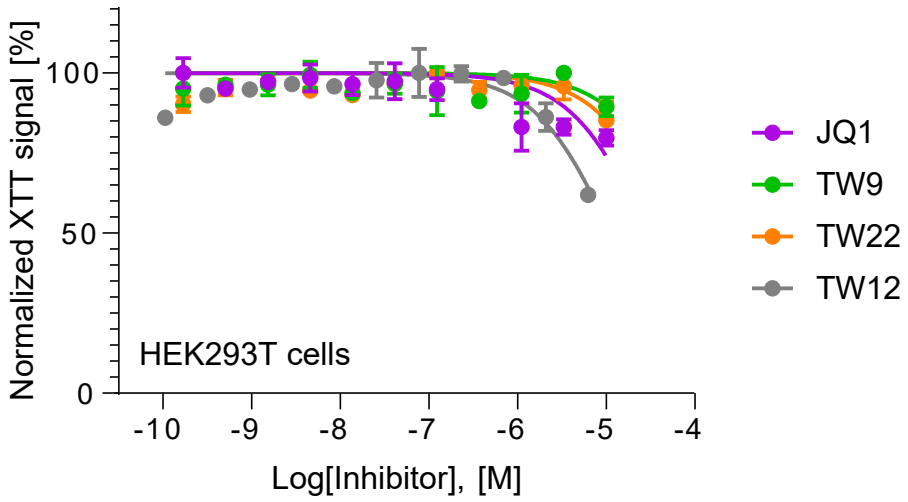
Supplementary Table S2. Primer sequences.

Gene	Forward sequence	Reverse sequence	Ref.
p57	AGATCAGCGCCTGAGAAGTCGT	CTCGGGGCTCTTTGGGCTCT	(2)
KRT10	AGGGGGCAGTTTCGGAGGTG	AAGTAGGAAGCCAGGCGGTCATT	(3)
KRT14	CCAGTTCTCCTCTGGATCGCAG	GATCTTCCAGTGGGATCTGTGTCCA	(4)
TGM1	ACATGAAGTACGACACGCCT	TTGGAGCTGATGGCCTTTGT	
FOSL1	GGCCTCTGACCTACCCTCA	CTTCCTCCGGGCTGATCT	(5)
Myc	CAGCTGCTTAGACGCTGGATT	GTAGAAATACGGCTGCACCGA	(6)
Hexim1	CTAGGGAACTGGGAGCTTGG	AAGGGTTAAATCCCCTGCCG	

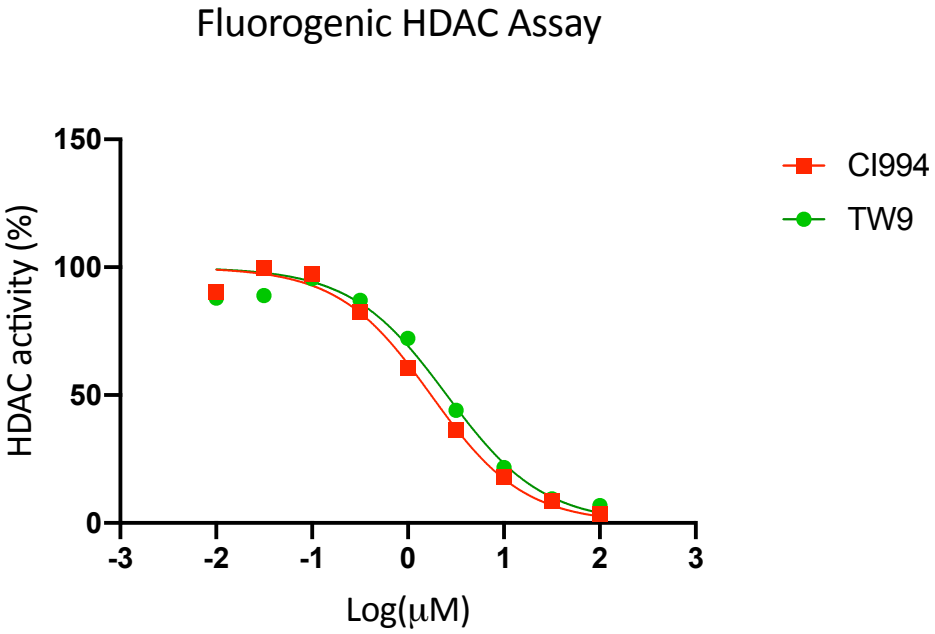
Supplementary Figures



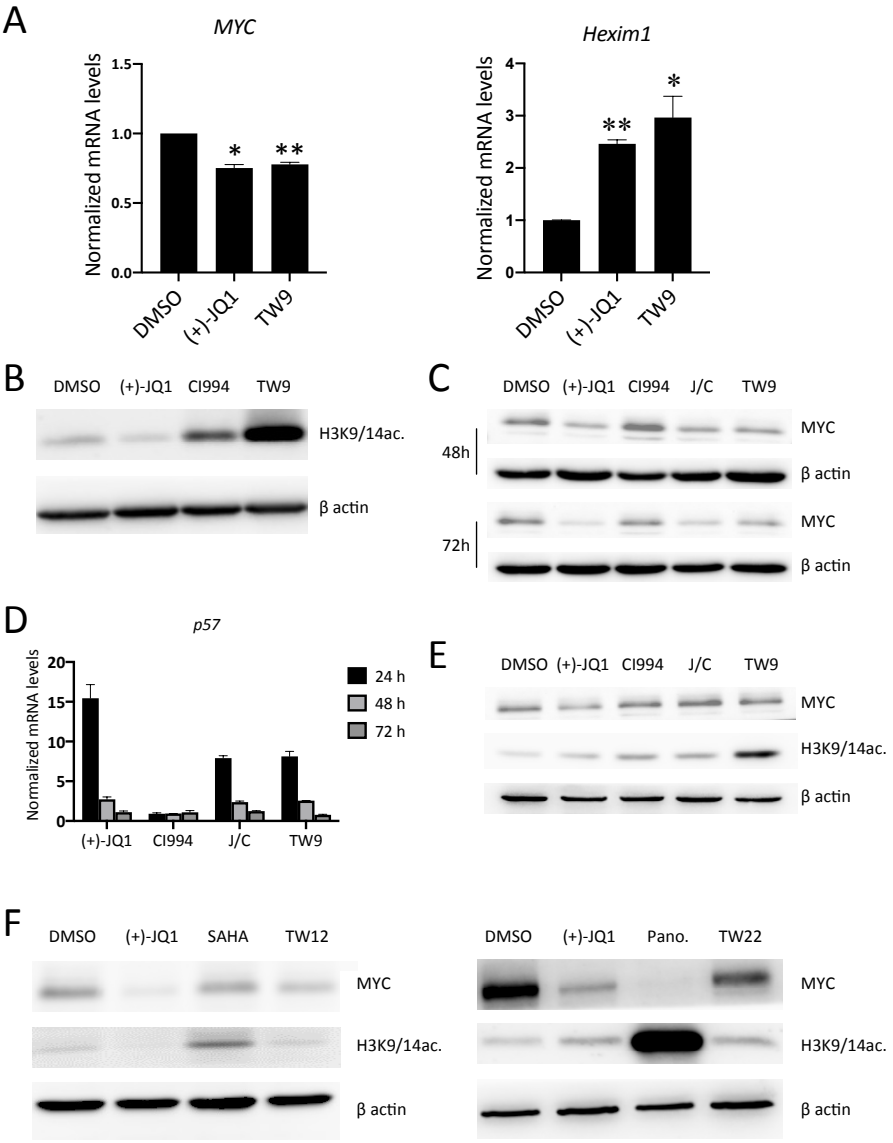
Supplementary Fig. S1.
Crystal structure of TW22 (3) in complex with BRD4(1). The bromodomain is shown as a grey surface representation and bound TW22 (3) as a yellow stick model. The HDACi moiety is stabilized in the crystal lattice via interaction with a symmetry-related molecule shown in green. Hydrogen bonds between the inhibitor and the symmetry-related molecule are highlighted as magenta broken lines.



Supplementary Fig. S2.
Viability of HEK293T cells after treatment with different doses of the indicated inhibitors for 24 h.



Supplementary Fig. S3.
Cell-free fluorogenic HDAC2-activity assay in the presence of CI994 or TW9 at the indicated concentrations.



Supplementary Fig. S4.

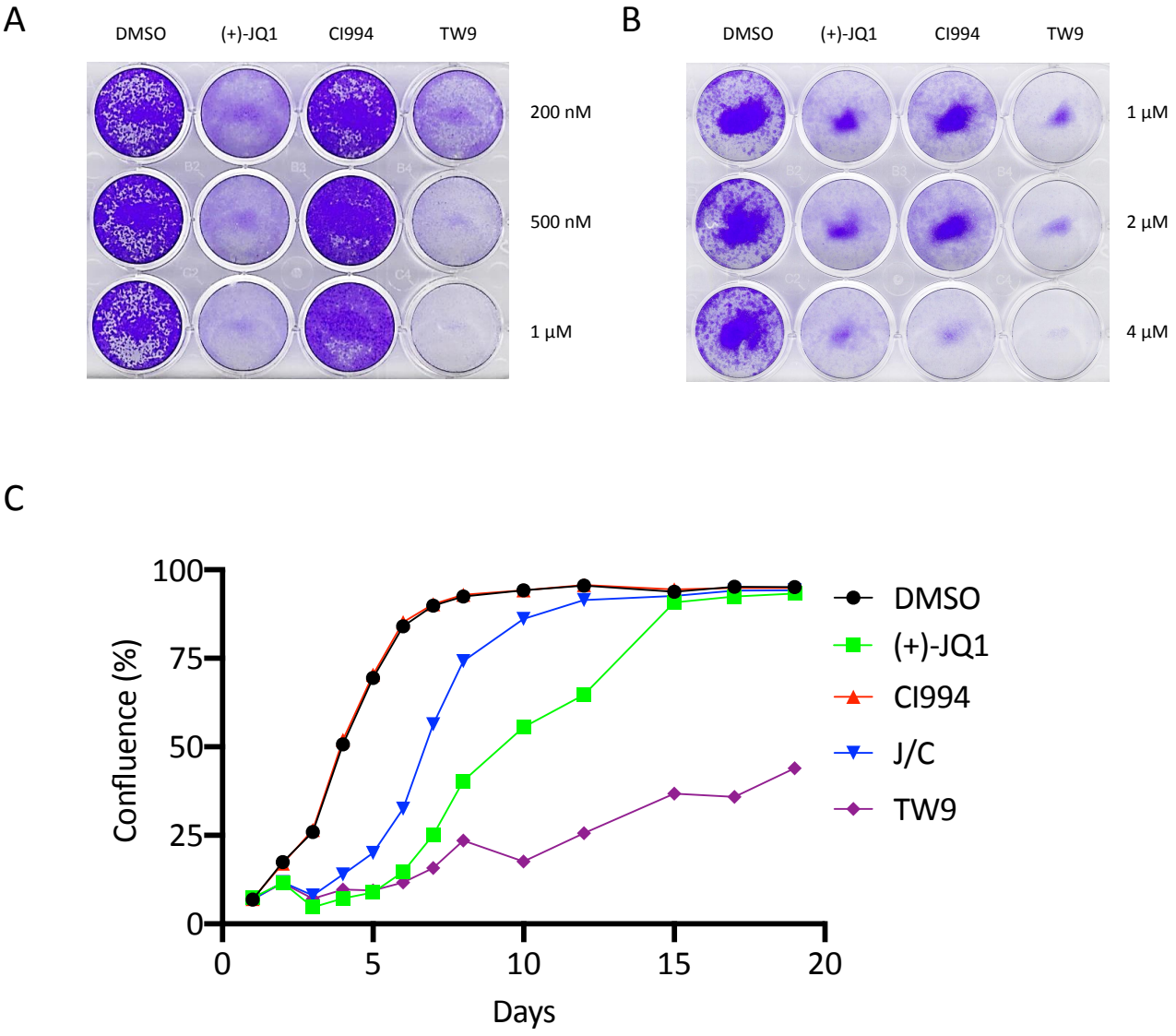
(A) Quantitative RT-PCR analysis for BETi-responsive genes (*MYC and HEXIM1*) upon treatment of 500 nM (+)-JQ1 or TW9 for 6 h in PaTu 8988t cells. Mean ± SEM from three independent experiments, ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$; n.s., not significant.

(B) Immunoblot analysis of H3K9/14ac in PaTu 8988t cells treated with 1 μM (+)-JQ1, CI994 or TW9 for 48 h.

(C) and (D), Time-course analysis of MYC protein levels by immunoblot (C) and *p57* mRNA levels by quantitative RT-PCR (D). PaTu 8988t cells were treated with 1 μM indicated inhibitors and harvested at the indicated time points. Mean ± SEM from three independent experiments.

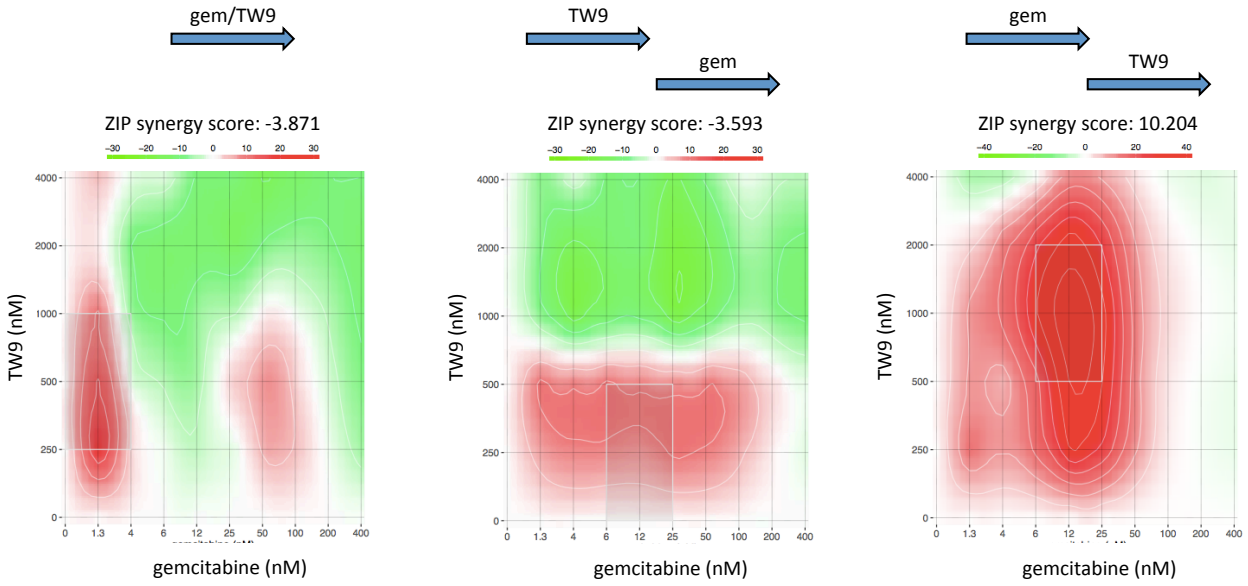
(E) Immunoblot analysis of drug washout experiment in PaTu 8988t cells treated with 1 μM indicated inhibitors (24 h on and 48 h off).

(F) Immunoblot analysis of MYC and H3K9/14ac in PaTu 8988t cells treated with 1 μM indicated inhibitors for 48 h.

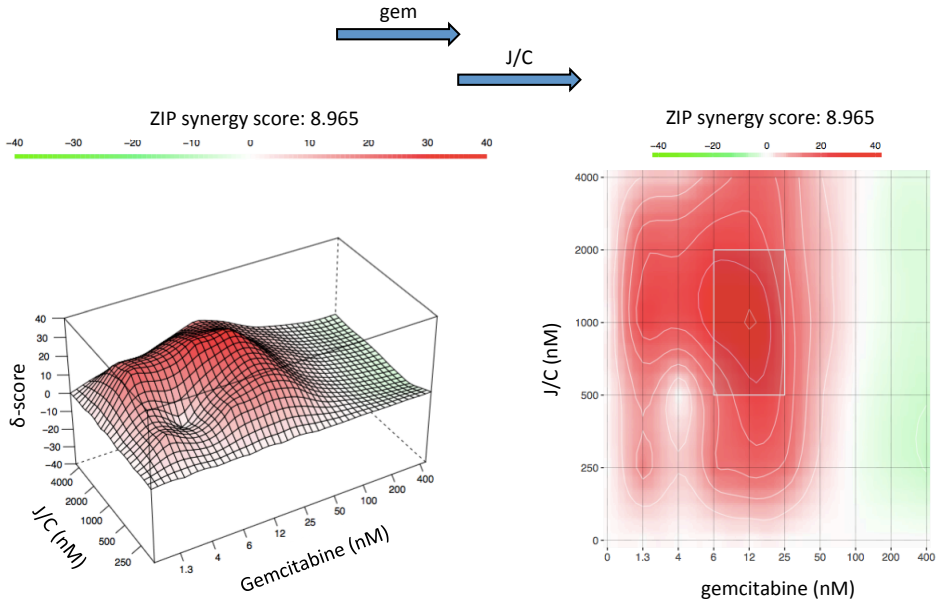


Supplementary Fig. S5.
(A) and (B), colony formation assay of HPAC cells treated with indicated inhibitors for 4 days (A) and DanG cells for 5 days (B).
(C) Cell proliferation assay. MiaPaCa2 cells were seeded in 12-well plates (30,000 cells/ well) and treated with 1 µM indicated inhibitors on day 1, 8 and 15 for 24 h. Cell confluence was measured at the indicated time points by NYONE image cytometry.

A



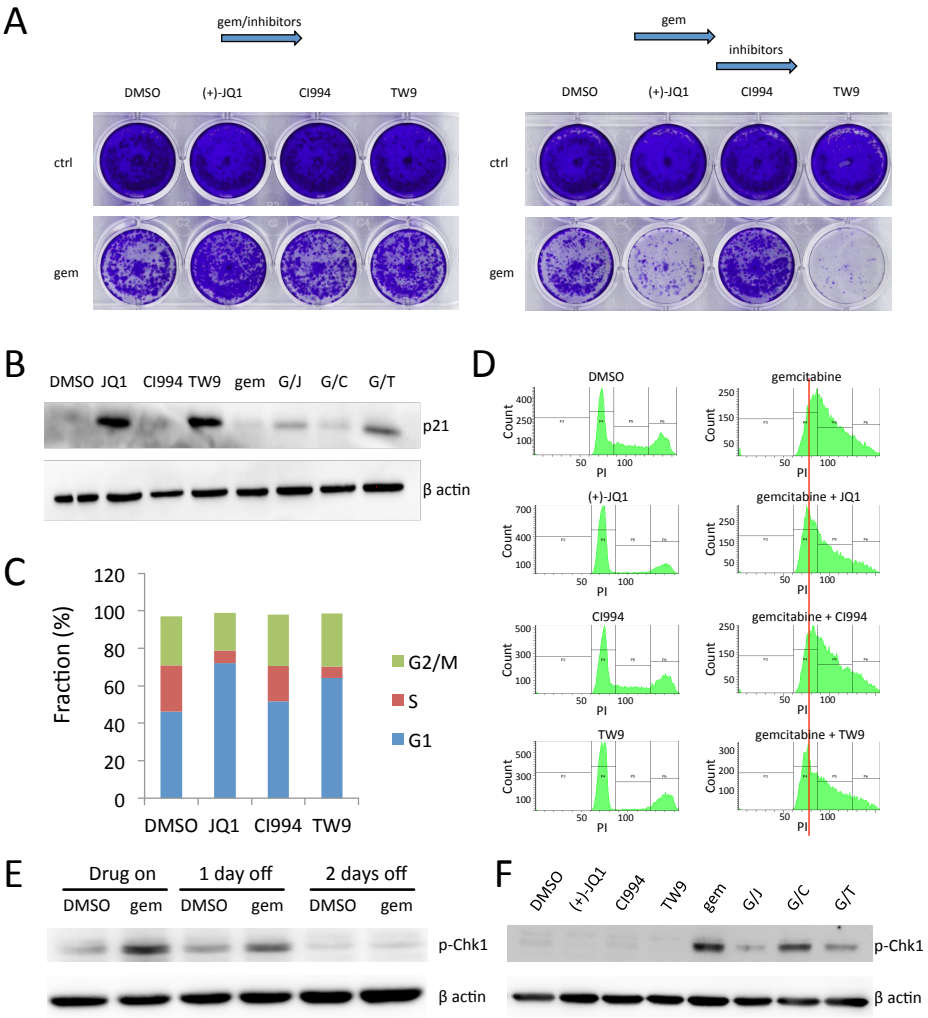
B



Supplementary Fig. S6.

(A) The ZIP synergy scores from Fig. 4A are plotted in 2D graph.

(B) Combination response to JQ1/CI994 (1:1 ratio) and gemcitabine for HPAC cells treated with the following administration schedule: sequential administration of gemcitabine and JQ1/CI994 (each for 24 h) and incubation for another 3 days. CellTiter Glo cell viability assay was performed to measure cell viabilities for all the indicated dose combinations. Synergy effects were evaluated using SynergyFinder (synergyfinder.fimm.fi). The ZIP synergy score is averaged over all the dose combination cells.



Supplementary Fig. S7.

(A) PaTu 8988t cells were seeded in 12-well plates (10,000 cells/well) and treated with 2 μM (+)-JQ1, CI994 and TW9 alone or together with 100 nM gemcitabine for 24 h. Then drugs were removed and cells were cultured for another 8 days for colony formation assay (left panel). Alternatively, PaTu 8988t cells were first treated with 100 nM gemcitabine for 24 h and then 2 μM (+)-JQ1, CI994 and TW9 for another 24 h. Later, drugs were removed and cells were cultured for another 7 days for colony formation assay (right panel).

(B) HPAC cells were treated with 2 μM (+)-JQ1, CI994 and TW9 alone or together with 10 nM gemcitabine for 24 h and then harvested for immunoblot analysis of p21 protein.

(C) HPAC cells were treated with 2 μM (+)-JQ1, CI994 and TW9 for 24 h and then harvested for cell-cycle analysis by flow cytometry.

(D) HPAC cells were treated with 2 μM (+)-JQ1, CI994 and TW9 alone or together with 10 nM gemcitabine for 24 h and then harvested for cell-cycle analysis by flow cytometry.

(E) HPAC cells were treated with 10 nM gemcitabine for 24 h and then gemcitabine was removed. Cells were harvested at indicated time points for immunoblot analysis of phospho-CHK1.

(F) HPAC cells were treated with 2 μM (+)-JQ1 **(4)**, CI994 **(5)** and TW9 **(1)** alone or together with 10 nM gemcitabine for 24 h and then harvested for immunoblot analysis of phospho-CHK1.

Supplementary References

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