Supporting Information

Deciphering the Cellular Targets of Bioactive Compounds using a Chloroalkane Capture Tag

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Supporting Information is divided into two parts (excluding Supporting References): Supporting Methods and Supporting Figures. The later is listed according to the main text and the main figures to which they are related.

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SUPPORTING METHODS

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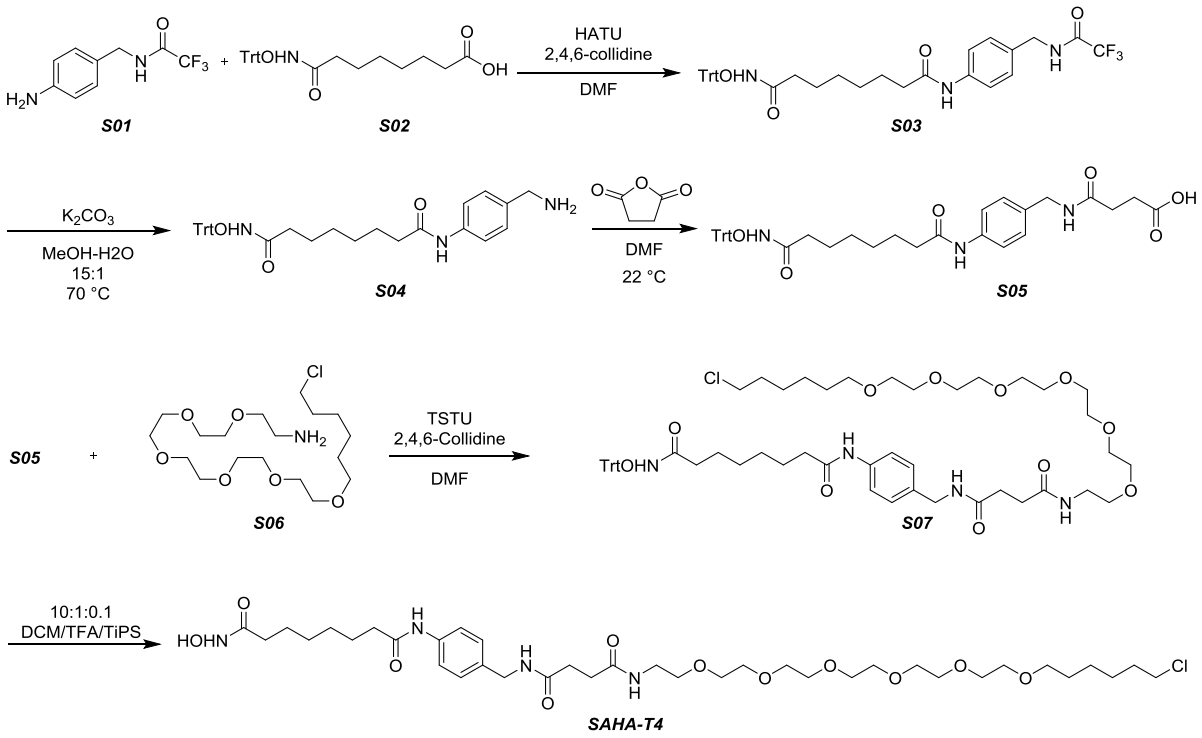
SAHA-T4



S03: To a solution of N-(4-aminobenzyl)-trifluoroacetamide (**S01**)[1] (456 mg, 2.01 mmol) in DMF (15 mL) was added solution of 8-oxo-8-((trityloxy)amino) octanoic acid (**S02**)[2] (902 mg, 209 mmol), HATU (993 mg, 2.61 mmol), 2,4,6-collidine (0.83 mL, 6.27 mmol) in DMF (10 mL). The clear yellow solution was stirred at 22 °C for 17 h, at which point LCMS analysis indicated full consumption of starting material. The reaction mixture was concentrated *in vacuo*, and purified by silica gel chromatography (0→100% EtOAc/Heptane) to provide 1.18 g (89% yield) of anilide **S03** as white solids. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 10.13 (s, 1H), 9.91 (t, *J* = 5.8, 1H), 9.82 (s, 1H), 7.53 (d, *J* = 8.5, 2H), 7.30 (m, 15 H), 7.17 (d, *J* = 8.5, 2H), 4.30 (d, *J* = 6.0, 2H), 2.21 (t, *J* = 7.4, 2H), 1.75 (t, *J* = 7.2, 2H), 1.46 (dq, *J* = 7.3, 7.6, 2H), 1.13 (m, 4H), 0.96 (m, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 171.2, 170.3, 156.23 (q, *J* = 36 Hz), 142.5, 138.5, 131.8, 128.9, 127.9, 127.5, 127.4, 119.1, 116.0 (q, *J* = 287 Hz), 91.3, 42.3, 36.3, 32.0, 28.3, 28.1, 24.9, 24.6; HRMS (SI) Calcd for $C_{36}H_{37}F_3N_3O_4^+$ $[M+H]^+$ 632.27, found 632.67.

S04: To a 20 mL microwave vial, equipped with stir bar was added solid K_2CO_3 (1.05 g, 7.60 mmol), water (1 mL) and solution **S03** (320 mg, 0.51 mmol) in MeOH (15 mL). The resulting

suspension was heated in microwave at 70 °C for 60 min. Upon cooling the reaction mixture was filtered and concentrated *in vacuo*, and purified by silica gel chromatography (0→20% MeOH/(DCM, 1% NEt_3) to provide 251 mg (93% yield) of **S04** as yellow solids. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 10.12 (s, 1H), 9.83 (s, 1H), 7.54 (d, *J* = 7.6, 2H), 7.30 (m, 17 H), 6.19 (br. s, 2H), 3.80 (s, 2H), 2.22 (t, *J* = 7.1, 2H), 1.75 (t, *J* = 7.2, 2H), 1.46 (dq, *J* = 7.3, 7.6, 2H), 1.14 (m, 4H), 0.96 (m, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 171.4, 170.3, 142.5, 139.5, 129.4, 128.9, 128.3, 127.5, 127.4, 119.0, 91.7, 41.8, 36.3, 32.0, 28.3, 28.2, 24.9, 24.7; HRMS (SI) Calcd for $C_{34}H_{38}N_3O_3^+$ $[M+H]^+$ 536.29, found 536.32.

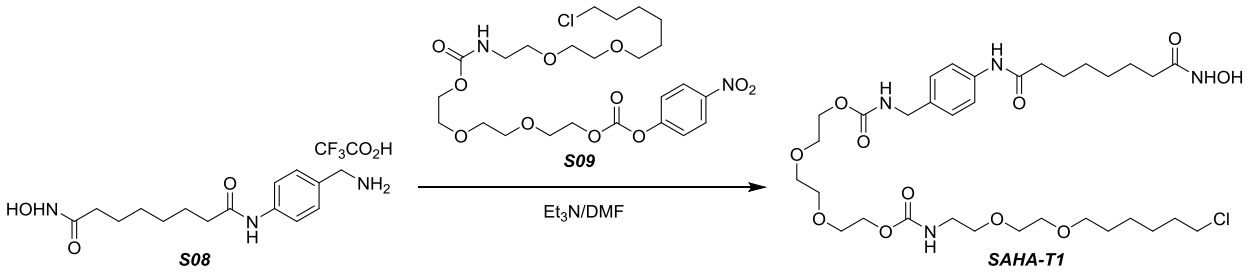
S05: To a solution of **S04** (24.5 mg, 45.7 µmol) in DMF (5 mL) and triethylamine (1 drop) was added succinic anhydride (4.6 mg, 46 µmol). The resulting solution was left stirred for 10 min, at which point LCMS analysis indicated full consumption of starting material. The reaction mixture was concentrated *in vacuo*, and purified by silica gel chromatography (0→20% MeOH/DCM) to provide 13.4 mg (46% yield) of acid **S05** as a clear oil. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 12.24 (br. s, 1H), 10.13 (s, 1H), 9.76 (s, 1H), 8.26 (t, *J* = 5.7, 1H), 7.50 (d, *J* = 8.3, 2H), 7.30 (m, 15 H), 7.14 (d, *J* = 8.3, 2H), 4.17 (d, *J* = 5.9, 2H), 2.45 – 2.31 (m, 4H), 2.21 (t, *J* = 7.3, 2H), 1.75 (t, *J* = 7.0, 2H), 1.46 (dq, *J* = 7.3, 7.6, 2H), 1.14 (m, 4H), 0.98 (m, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 206.7, 173.9, 171.1, 170.9, 170.3, 142.5, 138.0, 134.0, 129.0, 127.5, 118.9, 91.7, 41.7, 36.3, 32.0, 30.7, 30.3, 30.1, 30.0, 29.6, 29.4, 29.2, 28.4, 28.2, 25.0, 24.7; HRMS (SI) Calcd for $C_{38}H_{41}N_3NaO_6^+$ $[M+Na]^+$ 658.29, found 658.29.

S07: To a solution of **S05** (25 mg, 39 µmol) in DMF (5 mL) and 2,4,6-collidine (24 mg, 43 µmol) was added **S06**[3] (16 mg, 39 µmol) followed by TSTU (13 mg, 43 µmol). The resulting solution was left stirred for 18 h, at which point LCMS analysis indicated full consumption of starting material. The reaction mixture was concentrated *in vacuo* and purified by silica gel chromatography (0→30% MeOH/DCM) to provide 28 mg (69% yield) of chloroalkane **S07** as a clear oil. ^{1}H-NMR (300 MHz, CD_3OD) δ 7.51 (d, *J* = 8.5, 2H), 7.45 – 7.9 (m, 5H), 7.35 – 7.26 (m, 10H), 7.23 (d, *J* = 8.5, 2H), 4.32 (s, 2H), 3.65 – 3.59 (m, 16H), 3.58 – 3.55 (m, 3H), 3.55 – 3.45 (m, 7H), 3.39 -3.32 (m, 2H), 3.52 (s, 4H), 2.31 (t, *J* = 7.4, 2H), 1.88 (t, *J* = 7.2, 2H), 1.81 – 1.70 (m, 2H), 1.66 – 1.51 (m, 4H), 1.49 – 1.19 (m, 8H), 1.13 (m, 2H). ^{13}C-NMR (75 MHz, CD_3OD) δ 174.7, 174.5, 174.4, 143.6, 139.0, 135.6, 130.4, 129.0, 128.7, 121.2, 94.4, 72.2, 71.5, 71.2, 71.1, 70.7, 45.7, 43.7, 40.3, 37.9, 33.8, 33.4, 32.3, 30.5, 29.8, 29.7, 27.7, 26.7, 26.5, 26.2; HRMS (SI) Calcd for $C_{56}H_{78}ClN_4O_{11}^+$ $[M+H]^+$ 1017.54, found 1017.82.

SAHA-T4: **S07** (9.2 mg, 9.0 µmol) was treated with 5 mL of a cleavage cocktail (DCM/TFA/TiPS 10:1:0.1) at 22 °C. The resulting solution was left stirring for 30 min, at which point LCMS analysis indicated full consumption of starting material. The reaction mixture was concentrated *in vacuo*, purified by preparative HPLC chromatography (3→95% MeCN/H_2O-0.1% TFA) and lyophilized to provide 3.3 mg (47% yield) of chloroalkane **SAHA-T4** as a white fluffy solid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 10.29 (s, 1H), 9.78 (s, 1H), 8.61 (s, 1H), 8.23 (t, *J* = 5.8, 1H), 7.85 (t, *J* = 5.2, 1H), 7.49 (d, *J* = 8.1, 2H), 7.12 (d, *J* = 8.1, 2H), 4.16 (d, *J* = 5.6, 2H), 3.60 (t, *J* = 6.6,

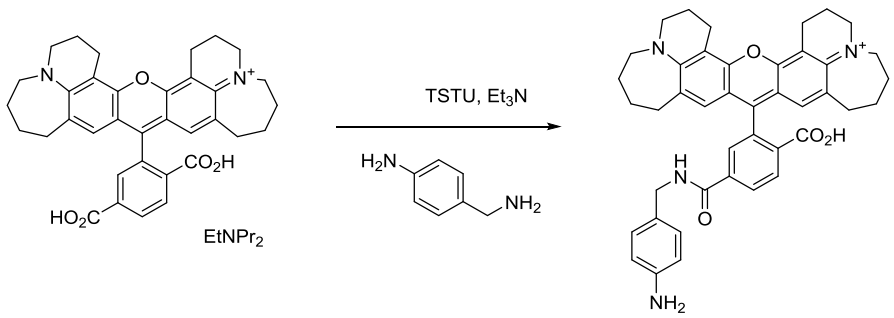
2H), 3.50 - 3.40 (m, 20H), 3.36 (q, *J* = 6.3, 4H), 3.16 (q, *J* = 5.7, 2H), 2.32 (s, 4H), 2.25 (t, *J* = 7.3, 2H), 1.91 (t, *J* = 7.2, 2H), 1.68 (dq, *J* = 7.3, 7.6, 2H), 1.60 – 1.40 (m, 6H), 1.39 – 1.22 (m, 8H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 172.2, 172.0, 171.9, 169.9, 138.2, 134.5, 127.9, 119.5, 70.5, 70.1, 70.0, 69.9, 69.8, 69.3, 45.8, 42.0, 36.66, 32.6, 32.31, 31.2, 31.1, 29.3, 28.7, 28.7, 26.4, 25.4, 25.4, 25.2; HRMS (SI) Calcd for $C_{37}H_{64}ClN_4O_{11}^+$ $[M+H]^+$ 775.43, found 776.06.

SAHA-T1

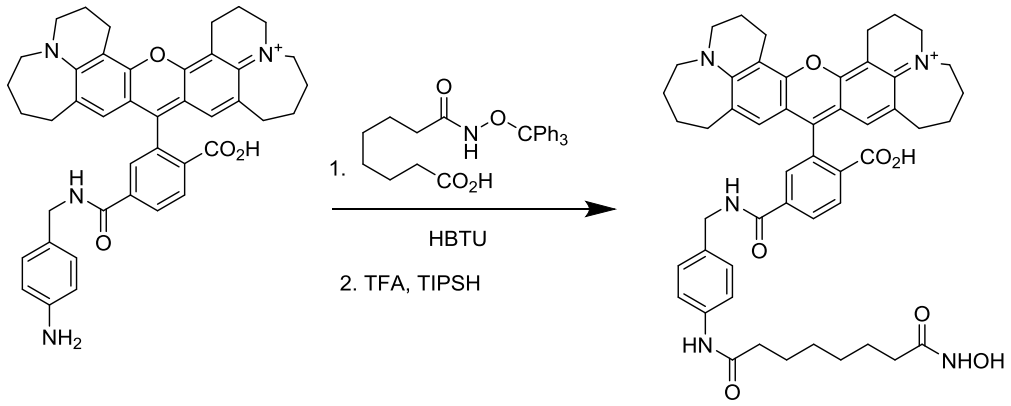


To a solution of hydroxamic acid **S08** [4] (490 mg, 1.20 mmol) in DMF (25 mL) and TEA (330 µL, 2.35 mmol) was added solution of *p*-NO_2-carbamate **S09** [5] (680 mg, 1.20 mmol) in DMF (0.5 mL). The resulting solution was left stirring for 18 h at 22 °C, at which point LCMS analysis indicated full consumption of starting material. The reaction mixture was concentrated *in vacuo*, and purified by silica gel chromatography (0→20% MeOH/DCM) to provide 560 mg (65% yield) of chloroalkane **SAHA-T1** as a white solid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 10.31 (s, 1H), 9.80 (s, 1H), 8.63 (s, 1H), 7.69 (t, *J* = 5.8 Hz, 1H), 7.51 (d, *J* = 8.3 Hz, 2H), 7.15 (m, 3H), 4.13 – 3.99 (m, 5H), 3.56 – 3.42 (m, 16H), 3.42 – 3.33 (m, 4H), 3.11 (q, *J* = 5.8 Hz, 2H), 2.27 (t, *J* = 7.3 Hz, 2H), 1.93 (t, *J* = 7.3 Hz, 2H), 1.70 (p, *J* = 6.7 Hz, 2H), 1.60 – 1.14 (m, 6H), 1.41 – 1.21 (m, 7H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 171.1, 169.1, 156.4, 156.2, 138.0, 134.2, 127.4, 118.9, 70.2, 69.7, 69.5, 69.4, 69.1, 68.9, 63.2, 63.1, 45.3, 43.4, 40.4, 36.3, 32.2, 32.0, 29.0, 28.4, 26.1, 25.0, 24.9; HRMS (SI) Calcd for $C_{33}H_{56}ClN_4O_{11}^+$ $[M+H]^+$ 719.36, found 719.86.

SAHA-NCT

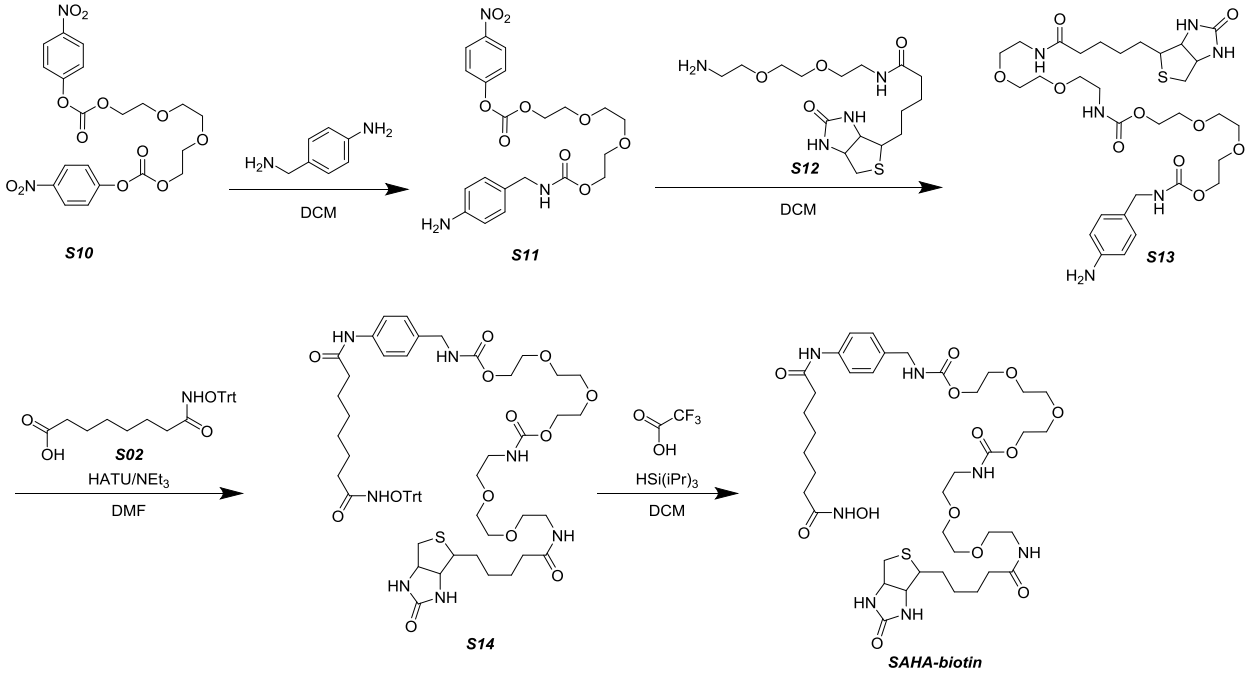


NCT acid (25 mg, 44 µmol) was stirred in 1 mL of DMF and treated with triethylamine (40 µL, 300 µmol) and TSTU (16 mg, 53 µmol). After 40 min, this reaction was added to a solution of 4-aminobenzylamine (54 mg, 442 µmol) in 0.5 mL of DMF and stirred for 1 h. The reaction was then acidified with AcOH, diluted with H2O and MeCN, and subjected to preparative HPLC (25→100% MeCN). The appropriate fractions were concentrated and lyophilized to afford 16 mg of a dark blue solid (54% yield). ^{1}H-NMR (MeOH-d_4): δ 9.34 (br t, <1 H); 8.38 (d, 1 H); 8.20 (dd, 1 H); 7.79 (d, 1 H); 7.52 (d, 2 H); 7.33 (d, 2 H); 6.67 (s, 2 H); 4.62 (s, 2 H); 3.72-3.76 (m, 4 H); 3.54-3.58 (M, 4 H); 3.02-3.08 (m, 4 H); 2.80-2.85 (m, 4 H); 2.65 (br t, < 1 H); 2.04-2.12 (m, 4 H); 1.92-2.01 (m, 4 H); 1.81-1.89 (m, 4 H). MS: Calcd for $C_{42}H_{43}N_4O_4^+$: 667.3; found 667.8. Analytical HPLC: 99.2% @ 600 nm.



NCT aminobenzylamine adduct (16 mg, 24 µmol) was combined with O-trityl suberoylhydroxamic acid (20 mg, 46 µmol) and HBTU (26 mg, 70 µmol) in 1 mL of DMF and stirred overnight. The reaction was subjected to preparative HPLC (40→100% MeCN) and the appropriate fractions (MS Calcd for $C_{69}H_{70}N_5O_7^+$: 1080.5, found 1080.9. Analytical HPLC: 96% @ 600 nm) were combined, concentrated and lyophilized. The resulting dark blue solid (16 mg, 14.8 µmol) was dissolved in 2 mL of DCM and stirred in an ice bath. Triisopropyl silane (0.75 mL) and TFA (0.25 mL) were added and the reaction was monitored by HPLC. After 25 min the reaction was concentrated under reduced pressure. Some excess silane was removed by pipet; the remainder of the reaction was taken up in MeCN/H_2O and the product was isolated by preparative HPLC (25→100% MeCN in 0.1% aqueous TFA). Lyophilization of the appropriate fractions yielded 7.2 mg (58%) of the desired product. ^{1}H-NMR (DMSO-d_6) δ 10.31 (br s, < 1H); 9.82 (s, 1 H); 9.23 (br t, < 1 H); 8.27 (s, 2 H), 7.81 (s, 1 H); 7.52 (d, 2 H); 7.24 (d, 2 H); 6.62 (s, 2 H); 4.38-4.45(m, 2 H); 3.66-3.73 (m, 4 H); 3.49-3.56 (m, 4 H); 2.91-2.99 (m, 4 H); 2.74-2.82 (m, 4 H); 2.26 (t, 2 H); 1.81-2.02 (m, 10 H); 1.70-1.78 (m, 4 H); 1.43-1.58 (m, 4 H); 1.21-1.31 (m, 4 H). HRMS: Calcd for $C_{50}H_{56}N_5O_7^+$: 838.4174; found 838.4172. Analytical HPLC: 94.2% @ 254 nm, 97.0% @ 600 nm.[6, 7]

SAHA-biotin



S11: To a solution of bis-carbamate (**S10**)[5] (50.0 mg, 104 µmol) in dichloromethane (5 mL) was slowly (over 10 min) added solution of 4-(aminomethyl) aniline (3.8 mg, 31 µmol) in dichloromethane (5 mL). Upon completion of the addition the resulting yellow solution was stirred at 22 °C for 12 h. The reaction mixture was purified by silica gel chromatography (0→5% MeOH/DCM) to provide 8.0 mg (55% yield) of carbamate **S11** as a yellow oil. ^{1}H-NMR (300 MHz, $CDCl_3$) 8.26 (d, *J* = 9.1, 2H), 7.36 (d, *J* = 9.1, 2H), 7.05 (d, *J* = 8.3, 2H), 6.64 (d, *J* = 8.3, 2H), 5.06 (s, 1H), 4.51 – 4.35 (m, 2H), 4.27 – 4.20 (m, 4H), 3.87 – 3.74 (m, 2H), 3.74 – 3.53 (m, 6H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 156.3, 155.5, 152.5, 145.4, 145.1, 128.9, 128.7, 125.3, 121.7, 115.5, 70.7, 70.5, 69.7, 68.7, 68.3, 63.9, 44.7; HRMS (SI) Calcd for $C_{21}H_{26}N_3O_9^+$ $[M+H]^+$ 464.17, found 464.35.

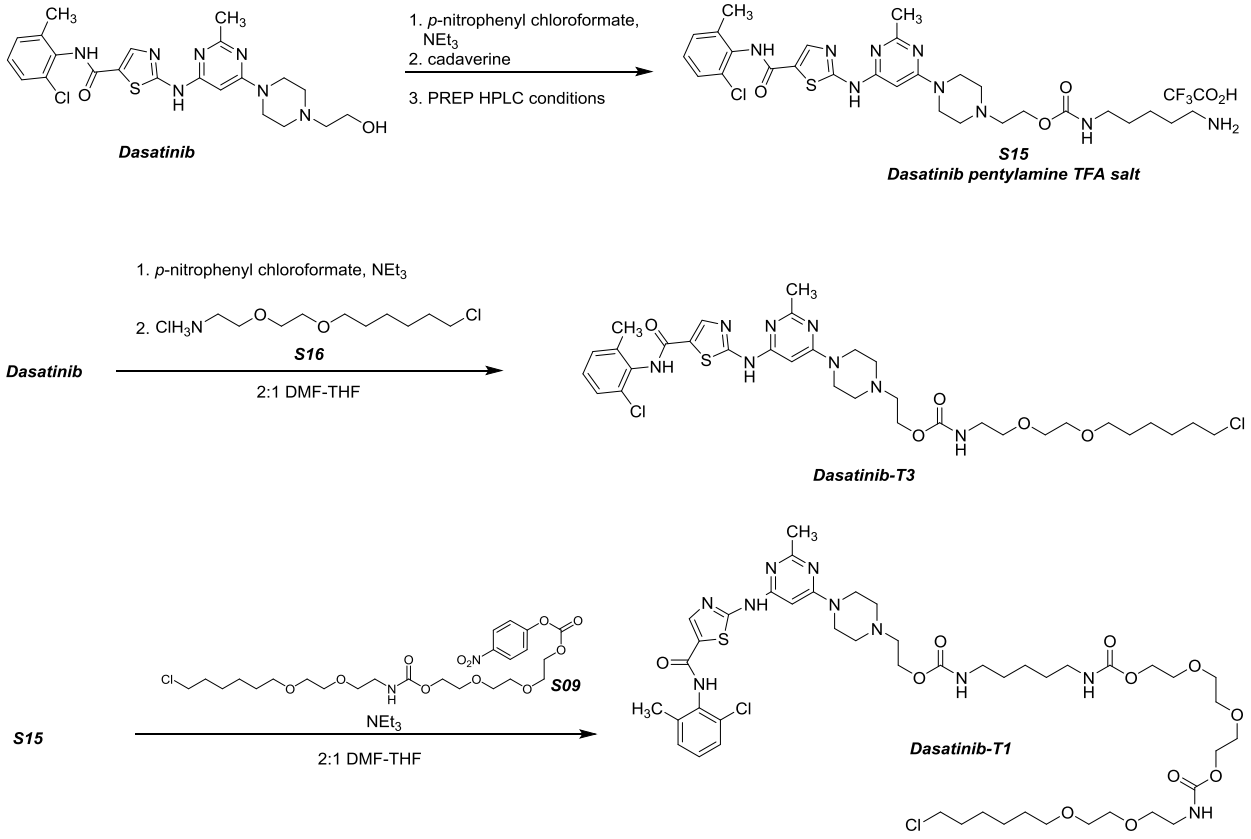
S13: To a solution of **S11** (8.0 mg, 17 µmol) in dichloromethane (5 mL) was added solution of "Thermo EZ-Link Amine-PEG2-Biotin" (**S12**) (7.1 mg, 19 µmol) in DMF (5 mL). The clear yellow reaction mixture was stirred at 22 °C for 20 h, at which point LCMS analysis indicated full consumption of starting material. The reaction mixture was concentrated *in vacuo*, and the residue was dissolved in 5 mL DCM and purified by silica gel chromatography (0→30% MeOH/DCM) to provide 12.0 mg (99% yield) of aniline **S13** as a clear oil. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.81 (t, *J* = 5.5, 1H), 7.52 (t, *J* = 5.9, 1H), 7.19 (t, *J* = 5.5, 1H), 6.89 (d, *J* = 8.2, 2H), 6.48 (d, *J* = 8.2, 2H), 6.40 (s, 1H), 6.34 (s, 1H), 4.91 (s, 2H), 4.39 – 4.22 (dd, , *J* = 7.6, 5.0, 1H), 4.21 – 4.01 (m, 6H), 3.98 (d, *J* = 6.1, 2H), 3.56 (m, 2H), 3.50 (m, 7H), 3.39 (m, 3H), 3.18 (m, 6H), 3.14 – 3.01 (m, 2H), 2.82 (dd, *J* = 5.1, 12.4, 1H), 2.57 (d, *J* = 12.4, 1H), 2.06 (t, *J* = 7.3, 2H), 1.68 –

1.37 (m, 4H), 1.35 – 1.22 (m, 2H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 172.6, 163.2, 156.71, 156.66, 147.9, 128.5, 127.2, 114.1, 70.1, 70.0, 69.9, 69.6, 69.5, 69.4, 69.3, 63.58, 63.52, 61.5, 59.6, 55.9, 55.3, 49.0, 44.0, 40.80, 38.9, 35.5, 28.6, 28.5, 25.7; HRMS (SI) Calcd for $C_{31}H_{51}N_6O_{10}S^+$ $[M+H]^+$ 699.34, found 699.48

S14: To a solution of **S13** (12.0 mg, 17 µmol) in DMF (2 mL) was added solution of 8-oxo-8-((trityloxy)amino)octanoic acid **S02**[2] (7.4 mg, 17 µmol), HATU (8.0 mg, 21 µmol), NEt_3 (8.7 mg, 86 µmol) in DMF (1 mL). The clear yellow reaction mixture was stirred at 22 °C for 17 h, at which point LCMS analysis indicated full consumption of starting material. The reaction mixture was concentrated *in vacuo*, and the residue was dissolved in 5 mL DCM and purified by silica gel chromatography (0→30% MeOH/DCM) to provide 11.5 mg (60% yield) of anilide **S14** as a clear oil. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 10.15 (s, 1H), 9.79 (s, 1H), 7.81 (t, *J* = 5.5, 1H), 7.69 (t, *J* = 6.0, 1H), 7.51 (d, *J* = 8.4, 3H), 7.32 (m, 15H), 7.15 (m, 3H), 6.40 (s, 1H), 6.34 (s, 1H), 4.28 (dd, *J* = 5.1, 7.4, 1H), 4.36 – 4.00 (m, 7H), 3.60 – 3.46 (m, 12H), 3.42 – 3.35 (m, 4H), 3.22 – 3.04 (m, 5H), 2.81 (dq, *J* = 14.0, 5.0, 1H), 2.58 (d, *J* = 12.6, 1H), 2.23 (t, *J* = 7.3, 2H), 2.06 (t, *J* = 7.3, 2H), 1.77 (m, 2H), 1.67 – 1.38 (m, 6H), 1.38 – 1.07 (m, 6H), 1.06 - 0.92 (m, 2H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 172.2, 171.2, 170.4, 162.8, 156.4, 156.3, 142.5, 138.1, 134.3, 129.0, 127.5, 127.4, 119.0, 91.8, 69.7, 69.54, 69.51, 69.2, 69.1, 68.91, 68.89, 63.3, 63.2, 61.1, 59.2, 55.5, 38.5, 35.1, 28.4, 28.2, 28.1, 25.3, 25.0; HRMS (SI) Calcd for $C_{58}H_{78}N_7O_{13}S^+$ $[M+H]^+$ 1112.54, found 1112.61.

SAHA-biotin: To a solution of **S14** (11.5 mg, 10 µmol) in DCM (1 mL) was added triisopropylsilane (81.9 mg, 517 µmol) followed by TFA (25 µL). The reaction mixture was stirred at 22 °C for 20 min, at which point TLC analysis indicated full consumption of starting material. The reaction mixture was concentrated *in vacuo*, and the residue was purified by preparative RP HPLC (3→95% MeCN/H_2O w/ 0.1% TFA over 45 min) to provide 9 mg (100% yield) of hydroxamic acid **SAHA-biotin** as a white solid after lyophilization. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 10.31 (s, 1H), 9.80 (s, 1H), 8.63 (s, 1H), 7.81 (t, *J* = 5.6, 2H), 7.69 (t, *J* = 5.9, 1H), 7.51 (d, *J* = 8.5, 2H), 7.20 (t, *J* = 5.9, 1H), 7.15 (d, *J* = 8.5, 2H), 6.51 (s, 1H), 6.40 (s, 1H), 6.34 (s, 1H), 4.36 – 4.22 (m, 1H), 4.17 – 3.93 (m, 7H), 3.60 - 3.52 (m, 4H), 3.52 – 3.47 (m, 8H), 3.42 – 3.34 (m, 4H), 3.21 – 3.01 (m, 4H), 2.82 (dd, *J* = 5.1, 12.4, 1H), 2.73 (s, 1H), 2.57 (m, 2H), 2.27 (m, 2H), 2.06 (t, *J* = 7.4, 2H), 1.93 (t, *J* = 7.3, 2H), 1.49 (m, 8H), 1.28 (m, 6H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 172.1, 171.2, 169.0, 162.7, 156.4, 156.2, 138.0, 134.2, 127.4, 118.9, 69.7, 69.49, 69.46, 69.2, 69.1, 68.9, 63.2, 63.1, 38.4, 36.3, 35.1, 32.2, 28.4, 28.2, 28.0, 25.2, 25.0; HRMS (SI) Calcd for $C_{39}H_{64}N_7O_{13}S^+$ $[M+H]^+$ 870.43, found 870.48.

Dasatinib conjugates



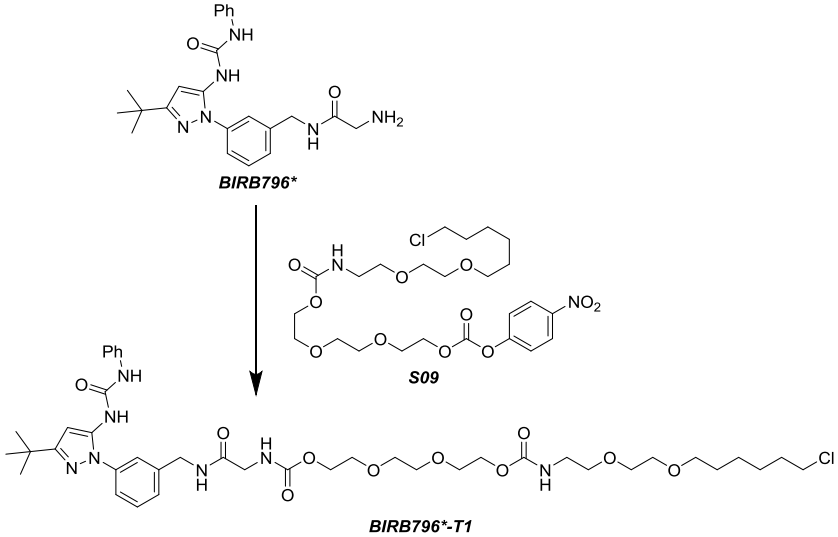
S15 (Dasatinib pentylamine TFA salt): **Dasatinib** (50 mg, 102 µmol) was combined with p-nitrophenyl chloroformate (28 mg, 139 µmol, 1.36 equiv) and 30 µL TEA in 1.8 mL of 2:1 DMF:THF. The reaction mixture was stirred overnight, and then cadaverine was added (209 mg, 2 mmol, 20 equiv). After stirring for 2 h, the reaction mixture was neutralized with AcOH and the desired product was isolated by preparative HPLC using a gradient of 20→60% MeCN in 0.1% aqueous TFA. The appropriate fractions were concentrated and lyophilized to afford the desired product (**S15**) as the TFA salt (69 mg, 92%). ^{1}H-NMR (D_2O): 8.08 (s); 7.30-7.36 (m, 1 H); 7.21-7.25 (m, 2 H); 6.30 (s, 1 H); 4.33 (br m; 4 H); 3.45 (br m, 8 H); 3.03 (t, 2 H); 2.87 (t, 2 H); 2.53 (s, 3 H); 2.18 (s, 3 H); 1.51-1.61 (m, 2 H); 1.38-1.48 (m, 2 H); 1.22-1.32 (m, 2 H). ^{13}C-NMR (DMF-d7) 166.06, 163.12, 160.11, 159.63, 159.15, 157.98, 156.01, 141.10, 139.37, 134.27, 132.94, 129.28, 128.32, 127.31, 126.98, 83.74, 58.64, 55.83, 51.69, 41.22, 40.61, 39.84, 27.25, 25.20, 23.61, 18.22. ^{19}F-NMR (DMF-d7): -75.80 ppm. MS: Calcd for $C_{28}H_{389}ClN_9O_3S^+$: 616.3; found 616.6.

Dasatinib-T3: Dasatinib (20.8 mg, 42.6 µmol) was combined with p-nitrophenyl chloroformate (13.3 mg, 66 µmol, 1.55 equiv) and 30 µL TEA in 1.8 mL of 2:1 DMF:THF. The reaction mixture was stirred overnight, and then 2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-

amine hydrochloride salt (33.3 mg, 3 equiv.) was added. The reaction mixture was stirred for 90 min, then diluted with MeCN and H_2O and acidified with AcOH. Preparative HPLC (20→60% MeCN in 0.1% aqueous TFA) afforded **Dasatinib-T3** as a colorless residue (27 mg, 86% yield.) ^{1}H-NMR(MeCN-d3): 8.67 (s, 1 H); 8.13 (s, 1 H); 7.36-7.41 (m, 1 H); 7.25-7.31 (m, 2 H); 7.12 (s, 1 H); 6.11 (br s, 1 H); 4.39 (br m, 2H); 4.09 (br s, 2 H); 3.37-3.63(m, 16 H); 3.24-3.32 (m, 2 H); 2.55 (s, 3 H); 2.31 (s, 3 H); 1.72-1.81 (m, 2 H); 1.50-1.60 (m, 2 H); 1.30-1.47 (m. 4 H). MS: Calcd for $C_{33}H_{47}Cl_2N_8O_5S^+$:737.2762; found 737.2771. HPLC: 96.3% at 254 nm.

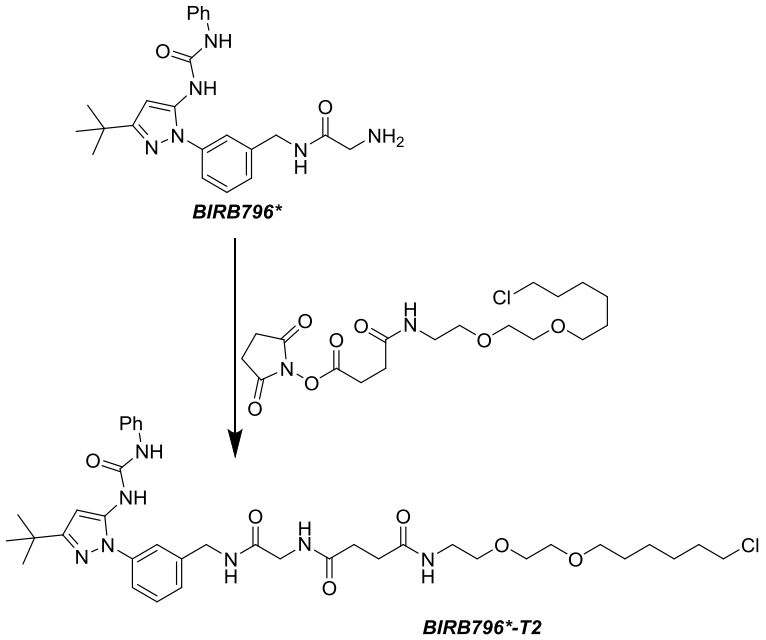
Dasatinib-T1: To a stirred solution of dasatinib pentylamine trifluoroacetate salt (**S15**) (43 mg, 59 µmol) and triethylamine (12 mg, 118 µmol) in DMF was added a DMF solution of **S09** [5] (37 mg, 65 µmol). The reaction mixture was stirred for 75 min at RT, then neutralized with TFA and subjected to preparative HPLC with an elution gradient of 25→100% MeCN in 0.1% aqueous TFA. Concentration and lyophilization afforded **Dasatinib-T1** as a film (39 mg). ^{1}H-NMR (MeCN-d_3) 8.61 (s, 1 H); 8.10 (s, 1 H); 7.36 (dd, 1 H): 7.21-7.28 (m, 2 H); 6.72 (br s, 1 H); 6.12 (br t, 1 H); 5.73 (br s, 1 H); 5.66 (br t, 1 H); 4.31-4.37 (br m, 2 H); 4.06-4.15 (br m, 4 H); 4.01 (br s, 2 H); 3.31-3.63 (m, 28 H); 3.22 (q, 2 H); 3.03-3.11 (m, 4 H); 2.49 (s, 3 H); 2.29 (s, 3 H); 1.69-1.79 (m, 2 H); 1.25-1.57 (m, 12 H). Calcd for $C_{46}H_{71}Cl_2N_{10}O_{11}S^+$: 1041.4396; found 1041.4407. Analytical HPLC: 98.6% @ 254 nm.

BIRB796* conjugates



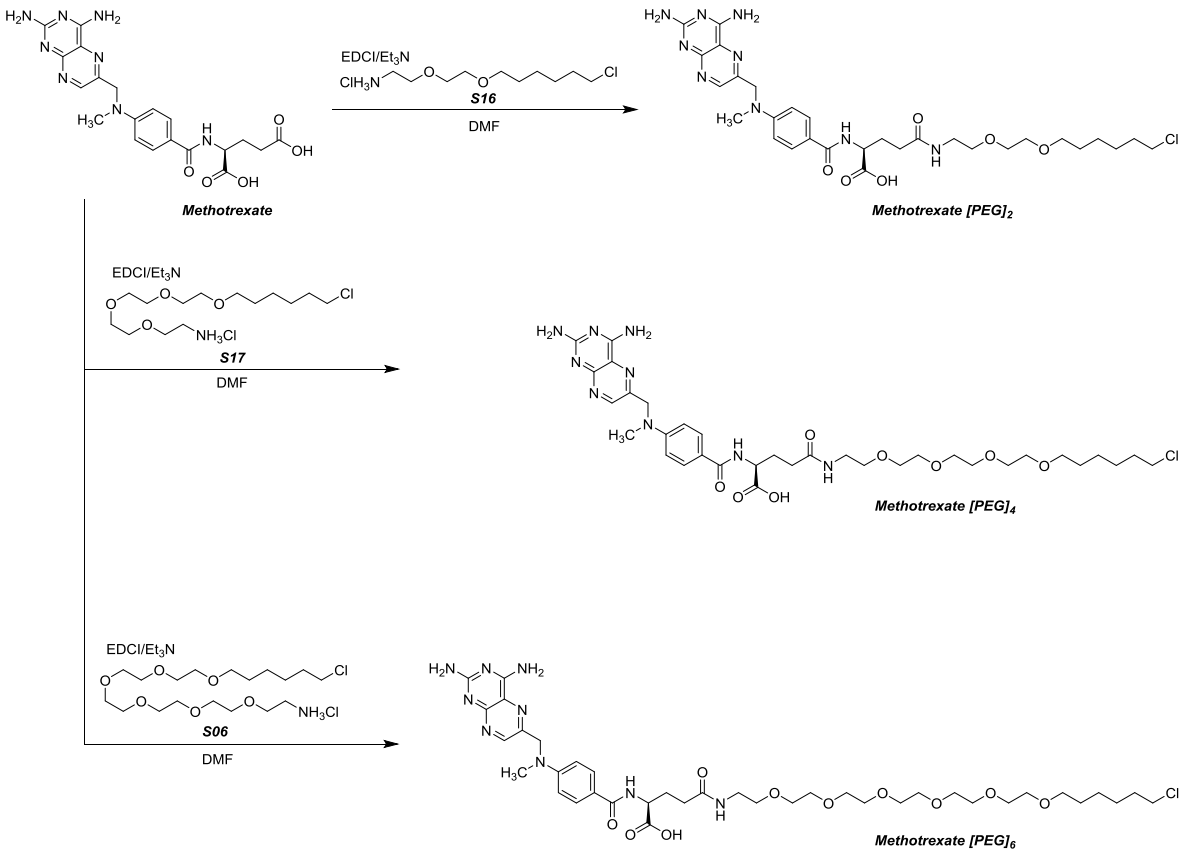
BIRB796*-T1: To a solution of 1-(1-(4-((2-aminoacetamido)methyl)phenyl)-3-tert-butyl-1H-pyrazol-5-yl)-3-phenylurea[8] (**BIRB796***, 20 mg, 0.04 mmol) in DMF (2.5 mL) and diisopropylethylamine (330 µL, 2.35 mmol) was added solution of *p*-NO_2-carbamate **S09**[5] (25 mg, 0.05 mmol) in DMF (3 mL). The resulting solution was stirred for 3 h at 22 °C, at which point LCMS analysis indicated >90% consumption of starting material. The reaction mixture was

quenched with 1% TFA in ACN/H_2O and purified by reverse phase preparative HPLC (5→100% ACN/H_2O) to provide 8 mg (25% yield) of chloroalkane **BIRB796*-T1** as a clear gum: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.79 (s, 1H), 8.50 (s, 1H), 7.99 (s, 1H), 7.40 (d, *J* = 7.8 Hz, 4H), 7.34-7.23 (m, 5H), 7.03 (t, *J* = 7.2 Hz, 3H), 6.77 (s, 1H), 5.56 (s, 1H), 4.31-3.98 (m, 12H), 3.58 – 3.28 (m, 6H), 1.75 (p, *J* = 6.8 Hz, 2H), 1.60 – 1.14 (m, 12H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 171.3, 161.5, 157.2, 156.8, 151.0, 141.1, 139.7, 138.3, 134.2, 130.3, 128.8, 125.2, 123.4, 119.2, 94.2, 70.4, 70.2, 69.9, 69.8, 69.5, 69.2, 64.5, 64.0, 45.0, 44.5, 43.1, 40.8, 32.5, 31.9, 29.8, 29.3, 29.0, 26.6, 25.4, 22.7, 14.1; HRMS (SI) Calcd for $C_{41}H_{60}ClN_7O_{10}^+$ $[M+]^+$ 846.42, found 846.47.



BIRB796*-T2: To a solution of 1-(1-(4-((2-aminoacetamido)methyl)phenyl)-3-tert-butyl-1H-pyrazol-5-yl)-3-phenylurea[8] (**BIRB796***,15 mg, 0.028 mmol) in DMF (2.5 mL) and diisopropylethylamine (500 µL, 3 mmol) was added solution of HaloTag Succinimidyl Ester (O2) Ligand (Promega, 12 mg, 0.03 mmol) in DMF (0.5 mL). The resulting solution was stirred for 3 h at 22 °C, at which point LCMS analysis indicated consumption of starting material. The reaction mixture was quenched with 1% TFA in ACN/H_2O and purified by reverse phase preparative HPLC (5→100% ACN/H_2O) to provide 12 mg (59% yield) of chloroalkane **BIRB796*-T2** as a white solid: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.48 (s, 1H), 8.20 (s, 1H), 7.98 (s, 1H), 7.40-7.36 (m, 3H), 7.31-7.12 (m, 6H), 6.94 (t, *J* = 7.3 Hz, 1H), 6.48 (s, 1H), 6.33 (s, 1H), 4.27 (d, *J* = 5.2 Hz, 2H), 3.74 (d, *J* = 5.4 Hz, 2H), 3.46 – 3.31 (m, 8H) , 3.10 (d, *J* = 5.8 Hz, 2H), 2.47 (br s, 2H), 2.34 (br s, 2H), 1.73-1.64 (m, 2H), 1.56-1.47 (m, 2H), 1.42 – 1.32 (m, 6H), 1.29 (s, 9H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 172.8, 171.6, 169.7, 161.3, 150.8, 138.6, 137.4, 137.3, 136.2, 128.9, 127.9, 126.2, 123.3, 122.5, 122.2, 118.2, 94.1, 70.3, 69.2, 68.9, 68.2, 44.0, 42.3, 42.2, 38.2, 31.4, 30.2, 30.0, 29.2, 28.4, 25.6, 24.3; HRMS (SI) Calcd for $C_{37}H_{53}ClN_7O_6^+$ $[M+H]^+$ 726.31, found 726.44.

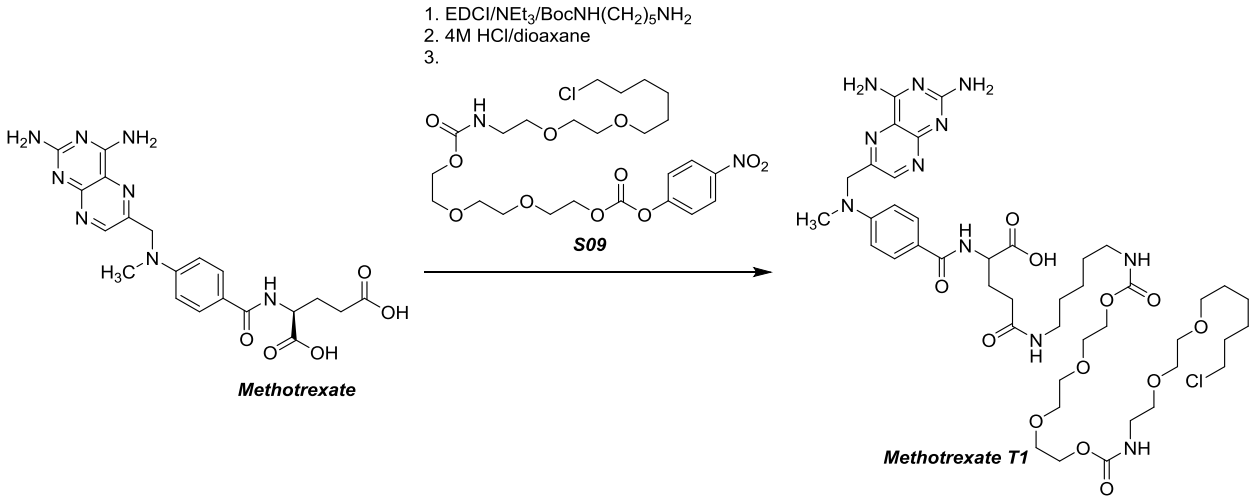
Methotrexate conjugates



Methotrexate [PEG]$_2$: **Methotrexate** (50 mg, 110 µmol) was dissolved in 3 mL of DMF and treated with EDC (63.3 mg, 330 µmol, 3 equiv), 2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-amine hydrochloride salt (**S16**)[5] (21.5 mg, 82.5 µmol, 0.75 equiv) and triethylamine (77 µL, 5 equiv). After 3 h, the reaction mixture was diluted with HCl (1 M) and the desired product was isolated by preparative HPLC eluting with a gradient up to 50% MeCN in 0.1% formic acid. After concentration and lyophilization 16.9 mg of **Methotrexate [PEG]$_2$** was obtained as a yellow solid. ^{1}H-NMR (MeOH-*d$_4$*) 8.59 (s, 1 H); 7.77 (d, 2 H); 6.87 (d, 2 H); 4.48-4.54 (m, 1 H); 3.45-3.59 (m, 10 H); 3.40 (t, 2 H); 3.26 (s, 3 H); 2.43 (t, 2 H); 2.01-2.20 (m, 2 H); 1.68-1.78 (m, 2 H); 1.48-1.57 (m, 2 H); 1.29-1.45 (m, 4 H). HRMS: Calcd for: $C_{34}H_{50}ClN_9O_8^+$: 660.3019, found 660.3027. Analytical HPLC: 98.2% @ 254 nm.

Methotrexate [PEG]$_4$: Synthesized with **S17** [3] using conditions for **Methotrexate [PEG]$_2$**. ^{1}H-NMR (MeOH-d$_4$) 8.48 (s, 1 H); 7.66 (d, 2 H); 6.78 (d, 2 H); 4.81 (s, 2 H); 4.38-4.48 (m, 1 H); 3.40-3.55 (m, 14 H); 3.36 (t, 2 H); 3.26 (t, 2 H); 3.17 (s, 3 H); 2.33 (t, 2 H), 1.92-2.13 (m, 2 H); 1.58-1.68 (m, 2 H); 1.41-1.50 (m, 2 H); 1.20-1.37 (m, 4 H). HRMS: Calcd for: $C_{30}H_{43}ClN_9O_6^+$: 748.3543, found 748.3554. Analytical HPLC: 97.2% @ 254 nm.

Methotrexate [PEG]$_6$: Synthesized with **S06**[3] using conditions for **Methotrexate [PEG]$_2$**. ^{1}H-NMR (MeOH-d$_4$) 8.59 (s, 1 H); 7.77 (d, 2 H); 6.87 (d, 2 H); 4.89 (s, 2 H); 4.47-4.57 (m, 1 H); 3.52-3.63 (m, 22 H); 3.45 (t, 2 H); 3.35-3.40 (m, 2 H); 3.26 (s, 3 H); 2.43 (t, 2 H), 1.98-2.22 (m, 2 H); 1.51-1.61 (m, 2 H); 1.41-1.50 (m, 2 H); 1.29-1.49 (m, 4 H). Calcd for $C_{38}H_{59}ClN_9O_{10}^+$: 836.4068, found 836.4073. Analytical HPLC: 100% @ 254 nm.



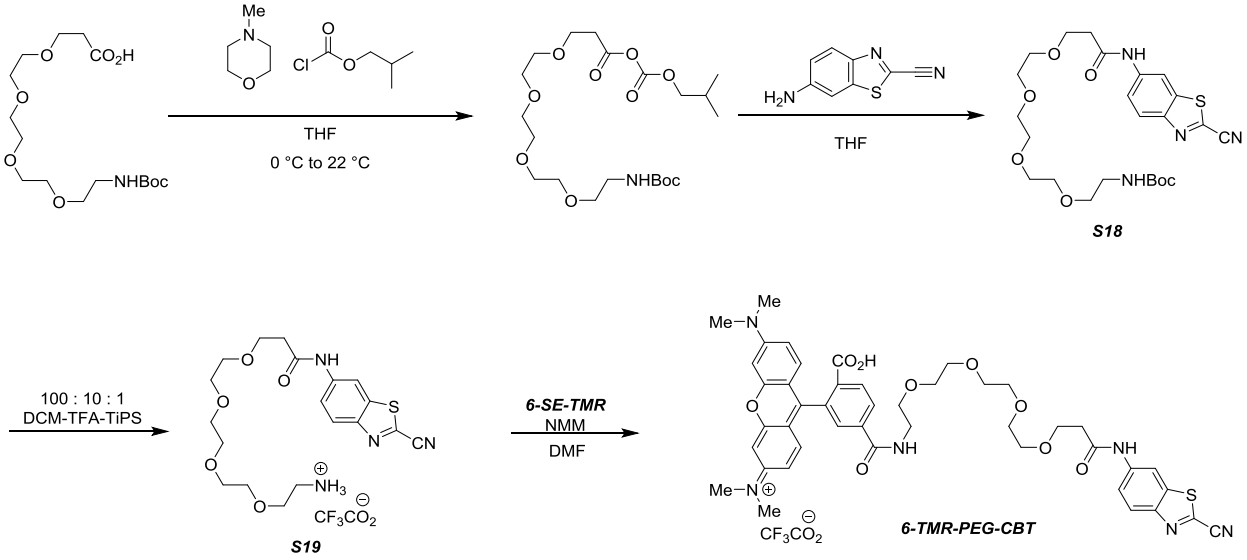
Methotrexate T1: To a mixture of methotrexate hydrate (50 mg, 110 µmol), EDCI (63 mg, 330 µmol) and triethylamine (77 µL, 550 µmol) in 2 mL of DMF, N-Boc cadaverine (22 mg, 110 µmol) was added. The reaction mixture was stirred for 90 min and quenched with 2 mL of 1 N HCl, diluted with water and subjected to preparative HPLC (20→50% MeCN in 0.1% aqueous formic acid). The appropriate fractions were concentrated and lyophilized to yield methotrexate N-Boc-cadaverine, which was used without further characterization.

Methotrexate N-Boc-cadaverine adduct (24 mg, 38 µmol) was treated with 4 M HC1 15 in dioxane (0.5 mL) at 22 C. Upon completion of the reaction mixture, the solvents were removed under reduced pressure, and the resulting residue was stirred with diethyl ether to form a yellow precipitate which was isolated by centrifugation. The hydrochloride salt was used without further characterization.

N-(5-aminopentyl) methotrexate amide (10 mg, 17.4 µmol) was combined with 10 µL of triethylamine and 14.7 mg (26.1 µmol) of **S09**[5] in 1 mL of DMF. After 1 h, the reaction mixture was diluted with brine and extracted with a mixture of DCM and EtOAc. The combined organics were evaporated under reduced pressure, taken up in MeCN and H2O, and subjected to preparative HPLC eluting with 25→50% MeCN in 0.1% formic acid. Concentration and lyophilization of the appropriate fractions yielded 6.2 mg (36.9%) of **Methotrexate T1** as a yellowish solid. ^{1}H-NMR (MeOH-d$_4$) 8.60 (br s, 1 H); 7.76 (d, 2 H); 6.86 (d, 2 H); 4.47-4.55 (m, 1 H); 4.12-4.16 (m, 4 H); 3.45-3.67 (m, 22 H); 3.26 (s, 3 H); 3.02-3.11 (m, 4 H); 2.18-2.36 (m, 2 H)

1.70-1.80 (m, 2 H); 1.54-1.62 (m, 2 H); 1.36-1.51 (m, 10 H); 1.23-1.35 (m, 2 H). HRMS: Calcd for $C_{43}H_{67}ClN_{11}O_{12}^{+}$: 964.4653, found 964.4657. Analytical HPLC: 98% @ 254 nm.

6-TMR-PEG-CBT



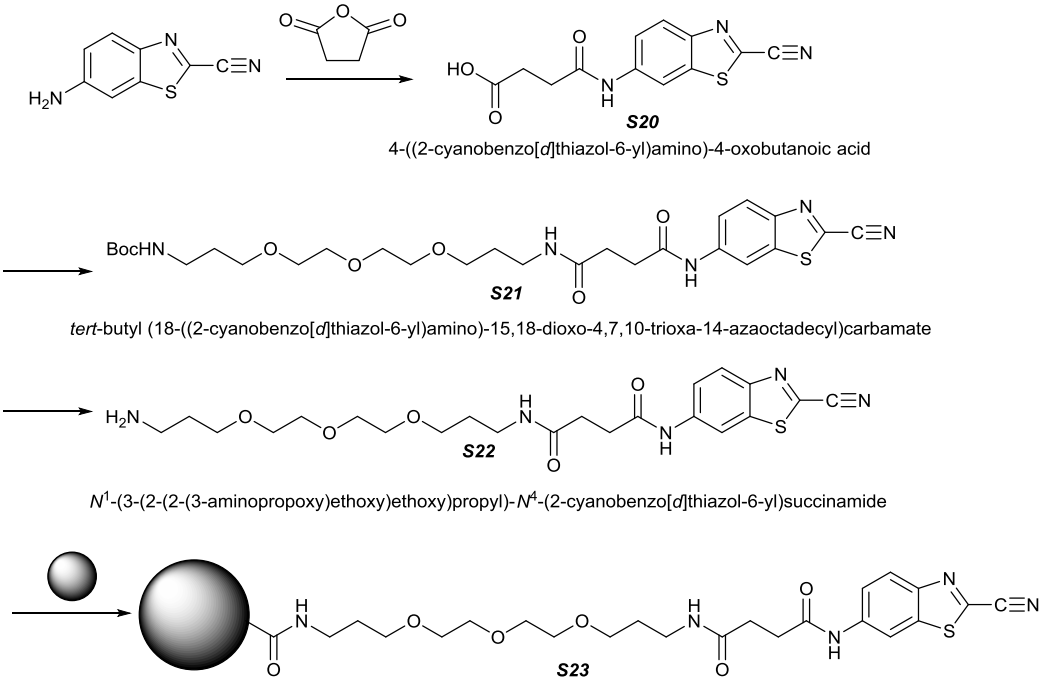
S18: To a stirred, ice-cold solution of Boc-NH-$(PEG)_4$-$CH_2CH_2CO_2H$ (78.0 mg, 214 µmol) and NMM (24 µL, 218 µmol) in THF (10 mL) was slowly added solution of isobutyl chloroformate (28 µL, 215 µmol) in THF (2 mL) under N_2. The reaction mixture was stirred at 0 °C for 10 min, and for 20 min at 22 °C at which point the murky solution was transferred to a 20 mL microwave vial equipped with a stir bar under N_2. 6-Amino-cyanobenzothiazole (38 mg, 217µmol) was added and the resulting suspension was heated in the microwave at 110 °C for 2 h. The reaction mixture was concentrated *in vacuo*, and the residue was purified by silica gel chromatography (0→50% MeOH/DCM) to provide 82.0 mg (74% yield) of anilide **S18** as a yellow oil. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 10.45 (s, 1H), 8.76 (d, *J* = 2.0 Hz, 1H), 8.18 (d, *J* = 9.0 Hz, 1H), 7.74 (dd, *J* = 9.0, 2.1 Hz, 1H), 6.72 (t, *J* = 6.4 Hz, 1H), 3.73 (t, *J* = 6.2 Hz, 2H), 3.49 (m, 12H), 3.34 (t, *J* = 6.1 Hz, 3H), 3.04 (q, *J* = 6.0 Hz, 2H), 2.63 (t, *J* = 6.2 Hz, 2H), 1.36 (s, 9H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 170.0, 155.5, 147.5, 139.7, 136.7, 134.8, 124.85, 120.6, 113.6, 111.0, 77.6, 69.7, 69.4, 69.1, 66.5, 37.3, 28.20; HRMS (SI) Calcd for $C_{24}H_{35}N_4O_7S^+$ $[M+H]^+$ 523.22, found 523.64.

6-TMR-PEG-CBT: To a solution of **S18** (80.0 mg, 153 µmol) in DCM (20 mL), was added triisopropylsilane (0.2 mL) followed by TFA (2 mL). The resulting solution was left stirring at 22 °C for 2 h at which point HPLC analysis indicated consumption of the starting material and the reaction mixture was concentrated to yellow oil, which was used in the next step without further purification. To a solution of crude **S19** (27 mg, 50 µmol) and NMM (100 µL, 910 µmol), was added 6-SE-TMR (27 mg, 51 µmol) and the resulting deep purple solution was stirred at

22 °C for 17 h at which point HPLC analysis indicated consumption of the starting material. The reaction mixture was concentrated *in vacuo*, and the residue was purified by preparative HPLC (C18) chromatography (3→75% MeCN/H_2O-0.1% TFA) and lyophilized to provide 13.0 mg (31% yield) of **6-TMR-PEG-CBT** as a fluffy deep purple solid. ^{1}H-NMR (300 MHz, CD_2Cl_2) δ 9.98 (s, 1H), 8.37 (m, 2H), 8.13 (d, *J* = 7.9 Hz, 1H), 8.01 (s, 1H), 7.87 (d, *J* = 8.8 Hz, 1H), 7.77 (m, 2H), 7.04 (d, *J* = 8.9 Hz, 2H), 6.73 (d, *J* = 9.0 Hz, 2H), 6.53 (s, 2H), 3.78 (t, *J* = 5.8 Hz, 2H), 3.71 – 3.50 (m, 16H), 3.23 (s, 12H), 2.69 (t, *J* = 5.8 Hz, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 171.1, 166.3, 160.6, 157.5, 157.2, 148.2, 139.9, 137.9, 136.4, 134.7, 134.4, 133.7, 132.0, 131.5, 129.5, 128.7, 124.6, 121.6, 114.3, 113.9, 113.6, 111.5, 105.2, 96.3, 70.6, 70.52, 70.47, 70.4, 70.1, 70.0, 69.5, 69.5, 67.1, 40.9, 40.2, 37.6; HRMS (SI) Calcd for $C_{44}H_{47}N_6O_9S^+$ $[M+H]^+$ 835.31, found 835.82.

Synthesis of paramagnetic HaloTag particles

Chemical synthesis of the paramagnetic cyanobenzothiazole (CBT) particles



4-((2-cyanobenzo[d]thiazol-6-yl)amino)-4-oxobutanoic acid (S20): 6-aminobenzo[d]thiazole-2-carbonitrile (2.0 g, 11.4 mmol), succinic anhydride (1.3 g, 13 mmol) and THF (15 mL) were placed in a 25 mL vessel and heated in a microwave synthesizer for 90 min at 110 °C. Upon cooling, the reaction mixture was triturated with Et_2O, filtered, dried, and evaporated to give 3.1 g (99%) of acid **S20** as a light yellow solid. ^{1}H-NMR (d_6-DMSO, 300 MHz):

δ 12.15 (s, 1H), 10.45 (s, 1H), 8.71 (s, 1H), 8.16 (d, 1H, *J* = 8.2 Hz), 7.70 (d, 1H, *J*= 8.2 Hz), 2.62 (m, 2H), 2.55 (m, 2H). ESI-MS: Calc. $C_{12}H_{10}N_3O_3S^+$: m/z 276.3; found m/z 276.

tert-butyl(18-((2-cyanobenzo[d]thiazol-6-yl)amino)-15,18-dioxo-4,7,10-trioxa-14-azaoctadecyl)carbamate (S21). Acid **S20** (4.93g, 17.9 mmol), tert-butyl (3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)carbamate (7.40 g, 23.1 mmol) and DCM:DMF (10:1, 100 mL) were stirred together in a 250 mL round bottomed flask at room temperature. EDIC (4.0 g, 20.9 mmol) was added and the reaction mixture was stirred for 20 h. The solvent was evaporated and purified by normal phase chromatography with DCM/MeOH as solvent to give 6.62 g (64%) of Boc-protected amine **S21** a white solid. ^{1}H-NMR (d_3-ACN, 300 MHz): δ 9.21 (s, NH), 8.62 (d, 1H, *J* = 2.0 Hz), 8.09 (d, 1H, *J* = 8.4 Hz), 7.63 (d, 1H, *J* = 8.4 Hz), 6.65 (bs, NH), 5.40 (bs, NH), 3.5 (m, 12 H), 3.28 (m, 2H), 3.06 (m, 2H), 2.65 (m, 2H), 2.51 (m, 2H), 2.70 (m, 4H), 1.40 (s, 9H). ESI-MS: Calc. $C_{27}H_{40}N_5O_7S^+$: m/z 578.7; found m/z 578.4.

N^1-(3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)-N^4-(2-cyanobenzo[*d*]thiazol-6-yl)succinamide hydrochloride (S22). Boc-protected amine **S21** (6.62 g, 11.5 mmol) was stirred in a 500 mL round bottomed flask with DCM (200 mL) and triisopropylsilane (1 mL). A 4.0 M solution of HCl in dioxane (30 mL, 120 mmol) was added and stirred at room temperature for 3h. The solvent was evaporated to give 6.4 g (98%) of amine **S22** a yellow hygroscopic solid. ^{1}H-NMR (d_6-DMSO, 300 MHz): δ 10.62 (s, 1H), 8.74 (d, 1H, *J* = 2.0 Hz), 8.15 (d, 1H, *J* = 8.4 Hz), 7.77 (d, 1H, *J* = 8.4 Hz), 3.4 (m, 16 H), 3.28 (m, 2H), 3.08 (m, 2H), 2.80(m, 2H), 2.61 (m, 2H), 2.41 (m, 2H), 1.80, (m, 2H), 1.60 (m, 2H). ESI-MS: Calc. $C_{22}H_{32}N_5O_5S^+$: m/z 478.59; found m/z 478.2.

Immobilized cyanobenzothiazole-magnetic cellulose (S23). Carboxymethyl magnetic cellulose (7.24g, 30–50 μm, Iontosorb MG CM) was taken up in a 250 mL round bottomed flask with amine **S22** (800 mg, 1.53 mmol) in DMF (100 mL). EDIC (387 mg, 2.01 mmol) was added and the reaction mixture was stirred for 20h at room temperature. The particles were filtered on a frit and rinsed first with DMF (200 mL) then 25% EtOH (300 mL) and stored as a 50% suspension at 4 ^{o}C.

Conjugation of HaloTag onto the paramagnetic CBT particles

His-HaloTag fusion protein separated by a linker containing a modified TEV protease recognition site (EPTTEDLYFQCDN) was expressed in *Escherichia coli* KRX according to Ohana *et al.*[9] The fusion was affinity purified on a Ni Sepharose 6 FF resin (GE Healthcare) and then cleaved with ProTEV protease (Promega) at 200 units/mg of fusion protein in the presence of 2 mM TCEP to generate a HaloTag protein with an N-terminal cysteine. The cysHaloTag protein was further purified on a Q Sepharose FF resin (GE Healthcare) and conjugated to the paramagnetic CBT particles over night at 4 °C in the presence of 2 mM TCEP at 20 mg protein/mL settled resin. Unbound HaloTag was determined by absorbance at 280 nm and was used to determine the amount (mg) of HaloTag immobilized/mL settled particles.

DNA constructs

NLuc N and C terminal fusions were generated by cloning the protein coding regions into pFN31 *NLuc* CMV-neo Flexi Vector and pFC32 *NLuc* CMV-neo Flexi Vector (Promega), respectively, using the Flexi Vector Cloning System.[10] HDAC1, HDAC2, HDAC3, HDAC6, HDAC8, and HDAC10 were obtained from Kazusa DNA Research Institute. ADO and CPPED1 were obtained from Origene.

HaloTag N and C terminal fusions were generated by cloning the protein coding regions into pFN21 HaloTag CMV Flexi Vector and pFC14 HaloTag CMV Flexi Vector (Promega), respectively, using the Flexi Vector Cloning System. For the generation of purified and labeled HDAC6, the HDAC6 protein coding region was cloned into pFN21 HaloTag CMV Flexi Vector. The linker sequence separating the two fusion partners was replaced with a linker encoding EPTTEDLYFQCDN.

Table 1: List of the human coding regions used in this study

Gene	Description	Accession #
HDAC1	Histone Deacetylase 1	AB463736
HDAC2	Histone Deacetylase 2	NM_001527.3
HDAC3	Histone Deacetylase 3	AB463738
HDAC6	Histone Deacetylase 6	AB385398
HDAC8	Histone Deacetylase 8	AB527476
HDAC10	Histone Deacetylase 10	AB463069
ADO	Human 2-aminoethanethiol dioxygenase	NM_032804.5
CPPED1	Human calcineurin-like phosphoesterase domain	NM_018340.1

Determining the specific activity of immobilized HaloTag

In triplicate experiments, 2 nmol of HaloTag TMR ligand (Promega) were incubated with 2.5 µL settled HaloTag paramagnetic particles for 30 min. Following binding, the unbound fractions as well as three washes of the particles were collected. The fluorescence intensities of these fractions were measured on a Safire plate reader (Tecan) together with the fluorescence intensity of a calibration curve comprised of serial dilution of the HaloTag TMR ligand. The linear calibration curve was used to calculate the amount of HaloTag TMR ligand that was bound to the particles. Because the binding of the chloroalkane ligand to HaloTag is at a 1:1 molar ratio, the amount of bound HaloTag TMR ligand is equivalent to the amount of active HaloTag. The percent of HaloTag that retained activity after immobilization was determined by dividing the amount of active HaloTag by the total amount of immobilized HaloTag.

Cell culture and transfections

HEK293T cells (ATCC-CRL-3216) were maintained in DMEM medium (Sigma) supplemented with 10% FBS (Hyclone) at 37 °C at 5% CO_2. K-562 cells (ATCC-CCL-243) and THP-1 cells (ATCC-TIB-202) were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS at 37 °C at 5% CO_2. HEK293T were transfected with DNA constructs at 0.8 µg mL^{-1} using PEI as transfection reagent as previously described.[11] For transfection of DNA constructs encoding NLuc fusions, in order to reduce expression levels the DNA was diluted 1:100 with a promoterless carrier DNA plasmid (pCI-neo; Promega) to generate a final total DNA concentration of 0.8 µg mL^{-1}. K-562 cells were transfected with DNA constructs at 0.8 µg mL^{-1} using Fugene-HD (Promega) as recommended by the manufacturer.

Phenotypic assays

BIRB796 model: THP-1 cells were plated in 96-well plates at 100,000 cells/well, treated with serial dilutions of BIRB796* and BIRB796*-chloroalkane for 2 h, and then stimulated with LPS (Sigma) at final concentration of 250 ng mL^{-1} for 24 h. Supernatants were analyzed for human TNFα secretion by ELISA (R&D systems). **Dasatinib model**: K-562 cells were transfected with a pGL4.52 [*luc2P*/STAT5] vector expressing a Luc2:STAT5 reporter gene (Promega) and plated in 96-well plates at 20,000 cells/well. 24 h post transfection, cells were treated with serial dilutions of dasatinib (LC Laboratories) and dasatinib-chloroalkane for 24 h and then tested for inhibition of STAT5 activation using the Bright-Glo luciferase assay (Promega) and for apoptosis induction using the Caspase-Glo 3/7 assay (Promega). **SAHA model**: K-562 cells were plated in 96-well plates at a 10,000 cells/well and treated with serial dilutions of SAHA (Selleckchem) and SAHA-chloroalkane for 2 h or 48 h. The cell treated for 2 h were tested for inhibition of HDAC activity using a non-lytic HDAC-Glo I/II assay (Promega). The cells treated for 48 h were tested for apoptosis induction using the Caspase-Glo 3/7 assay (Promega).

Optimization of enrichment protocols using NLuc fusion proteins

Optimization of chloroalkane tagged compound concentration

Target engagement inside cells: HEK293T cells were transfected in a 96-well plate with DNA constructs encoding NLuc:HDAC6 or HDAC1:NLuc (diluted 1:100 with carrier plasmid pCI-neo). The enrichment experiments were done in six replicates where 24 h post transfection, cells were treated with serial dilution of SAHA-chloroalkane (0–40 µM). Following 2.5 h incubation, medium was removed, cells were washed with PBS and then lysed with 30 µL/well detergent lysis buffer for 10 min. Cell lysates were diluted 1:2 with pull-down buffer and transferred to a 96-well plate containing 0.5 µL/well settled paramagnetic HaloTag particles. Following 15 min of binding, the unbound fraction was removed, particles were washed three times, 3 min each, and the captured NLuc fusions were released by 15 min of competitive

elution with 400 µM SAHA. The released NLuc fusions were detected by NanoGlo reagent (Promega) on a GENios Pro plate reader (Tecan). The specific enrichment was determined by the increase in bioluminescence over control.

Target engagement in lysate: HEK293T cells were transfected in 10 cm dishes with DNA constructs encoding NLuc:HDAC6 or HDAC1:NLuc (diluted 1:100 with carrier plasmid pCI-neo). 24 h post-transfection, cells were collected, harvested and lysed with 3 mL detergent lysis buffer for 10 min. The lysates were then diluted 1:2 with pull-down buffer, and dispensed to a 96-well plate at 90 µL/well. The enrichment experiments were done in six replicates where lysates were treated with serial dilution of SAHA-chloroalkane (0–10 µM). Following 2.5 h of binding, the lysates were transferred to a 96-well plate containing 0.5 µL/well settled paramagnetic HaloTag particles and processed as described above.

Optimization of competitive elution

Experiments were done as described above except 20 µM or 1 µM SAHA-chloroalkane were used for target engagement inside cells or in lysates, respectively. The captured NLuc fusions were released by 15 min of competitive elution with 5–450 µM SAHA and the specific enrichment was determined by the increase in bioluminescence over control as described above.

Western blot analysis

A fraction of the eluted proteins (10%) was analyzed by SDS-PAGE and electro-transferred onto a PVDF membrane (Life Technologies). The membrane was blocked for 1 h with 5% BSA (Promega) in TBS buffer and probed overnight at 4 °C with the primary antibody in TBS supplemented with 0.1% Tween-20 (TBST). After three washes in TBST, the membrane was incubated with the appropriate secondary HRP conjugated antibody (Jackson laboratories) in TBST for 1 h, washed 5 times with TBST, and 1 time with TBS. The immuno-stained proteins were detected using enhanced chemiluminescent (ECL) reagent (Promega) and detected on the LAS400 imager (GE Healthcare).

Antibody source: anti-HDAC1 (Abcam), anti-HDAC2 (Abcam), anti-HDAC10 (Abcam), anti HDAC3 (Thermo-Scientific), anti HDAC6 (Millipore) and anti HDAC8 (Rockland/Promega).

LC-MS/MS analysis

Eluted proteins from the enrichment experiments were analyzed and processed by MS Bioworks, LLC (Ann Arbor, Michigan). 25% of each elution was separated by SDS-PAGE gel which was subsequently Coomassie stained and cut into 10 equally sized fragments. Each gel fragment was processed using Progest, Protein Digestion Station (Digilab). Briefly, the gel

fragments were reduced, alkylated and in-gel digested with trypsin (Promega) for 4 h. Gel digests were analyzed directly by nano LC/MS/MS with a NanoAcquity HPLC system (Waters) interfaced to a LTQ-Orbitrap Velos Pro (Thermo Scientific). The mass spectrometer was operated in data-dependent mode, with MS performed in the Orbitrap at 60,000 FWHM resolution and MS/MS performed in the LTQ. The fifteen most abundant ions were selected for MS/MS. Data were searched with Mascot (Matrix Science) against a SwissProt Human database allowing for common protein modifications. Mascot DAT files were filtered by Scaffold (Proteome software) using a 1% protein and peptides false discovery rates and requiring at least two unique peptides per protein. Normalized spectral abundance factors (NSAF) were determined as described before.[12] Interacting proteins were determined based on the following criteria: protein had at least five spectral counts in the test sample and non in the control or detected with a least 4-fold more spectral counts in the test sample over the control.

Protein purifications

ADO and HDAC6 HaloTag fusions were expressed in HEK293T cells and purified using the HaloTag mammalian protein purification system[11] (Promega). The linker in the HaloTag:HDAC6 fusion protein separating HaloTag and HDAC6 contained a modified TEV protease recognition site (EPTTEDLYFQCDN). For the generation of a labeled HDAC6, the TEV proteolytic release was carried out in the presence of 0.1 mM TCEP and 4-fold molar excess of TMR-cyanobenzothiazole (6-TMR-PEG-CBT). This step generated an HDAC6 with an N-terminal cysteine that served as single point of conjugation with 6-TMR-PEG-CBT. The purified labeled HDAC6 was dialyzed to remove the unconjugated 6-TMR-PEG-CBT.

ADO activity assay

ADO catalyzed cysteamine dioxygenation was analyzed through the detection of sulfhydryl groups using Ellman's reagent (Sigma). Briefly, 1.5 µM purified ADO was pre-incubated with 0–30 µM $FeSO_4$ for 15 min at 37 °C prior to the addition of 8 mM cysteamine (Sigma) and then incubated for up to 60 min. Reactions were done in triplicate, in 100 mM Tris buffer pH 8.5 with constant mixing (900 rpm). For the detection of sulfhydryl groups, 10 µL of the reaction as well as 10 µL of a calibration curve comprised of serial dilution of cysteamine were diluted into 90 µL of 0.5 mM Ellman's reagent and incubated for 15 min at room temperature. Absorbance at 412 nm was measured on a Safire plate reader (Tecan) and the remaining cysteamine concentrations were determined from the linear calibration curve.

For the analysis of ADO inhibition by SAHA, 1.5 µM purified ADO was pre-incubated with 10 µM $FeSO_4$ for 15 min at 37 °C prior to a 60 min treatment with 0 or 10 µM SAHA. 8 mM cysteamine was then added to the reactions and incubated for an additional 60 min. The detection of sulfhydryl groups and determination of cysteamine concentrations was done as described above. To avoid any interference of buffer, $FeSO_4$ or SAHA with the detection of the

sulfhydryl groups, the reactions and calibration curves were set up in the same buffer and in the presence of identical concentrations of iron and SAHA.

Analysis of capture capacity and kinetics

To determine the capture capacity of the HaloTag and Streptavidin (GE Healthcare) paramagnetic particles, 125 pmol of purified and fluorescently labeled HDAC6 (^{TMR}HDAC6) were incubated with 0–125 pmol of the two SAHA conjugates for 2 h. The SAHA conjugates and the ^{TMR}HDAC6 bound to them were captured onto the appropriate particles (exhibiting capacity for 125 pmol of biotin or chloroalkane). After 30 min of binding, the unbound fractions were removed and the bound ^{TMR}HDAC6 was released from the particles by SDS elution. The eluted proteins were resolved on SDS-PAGE, scanned on a Typhoon 9400 fluorescent imager (GE Healthcare) and bands were quantified using ImageQuant (GE Healthcare). The amount of captured ^{TMR}HDAC6 was determined from an in-gel linear calibration curve generated with serial dilutions of the ^{TMR}HDAC6.

To determine the capture kinetics, 125 pmol of ^{TMR}HDAC6 were incubated with 60 pmol of the two SAHA conjugates for 2 h. The SAHA conjugates and the ^{TMR}HDAC6 bound to them were then captured onto the appropriate particles for up to 60 min. Analysis of the captured ^{TMR}HDAC6 was performed as described above.

SUPPORTING FIGURES

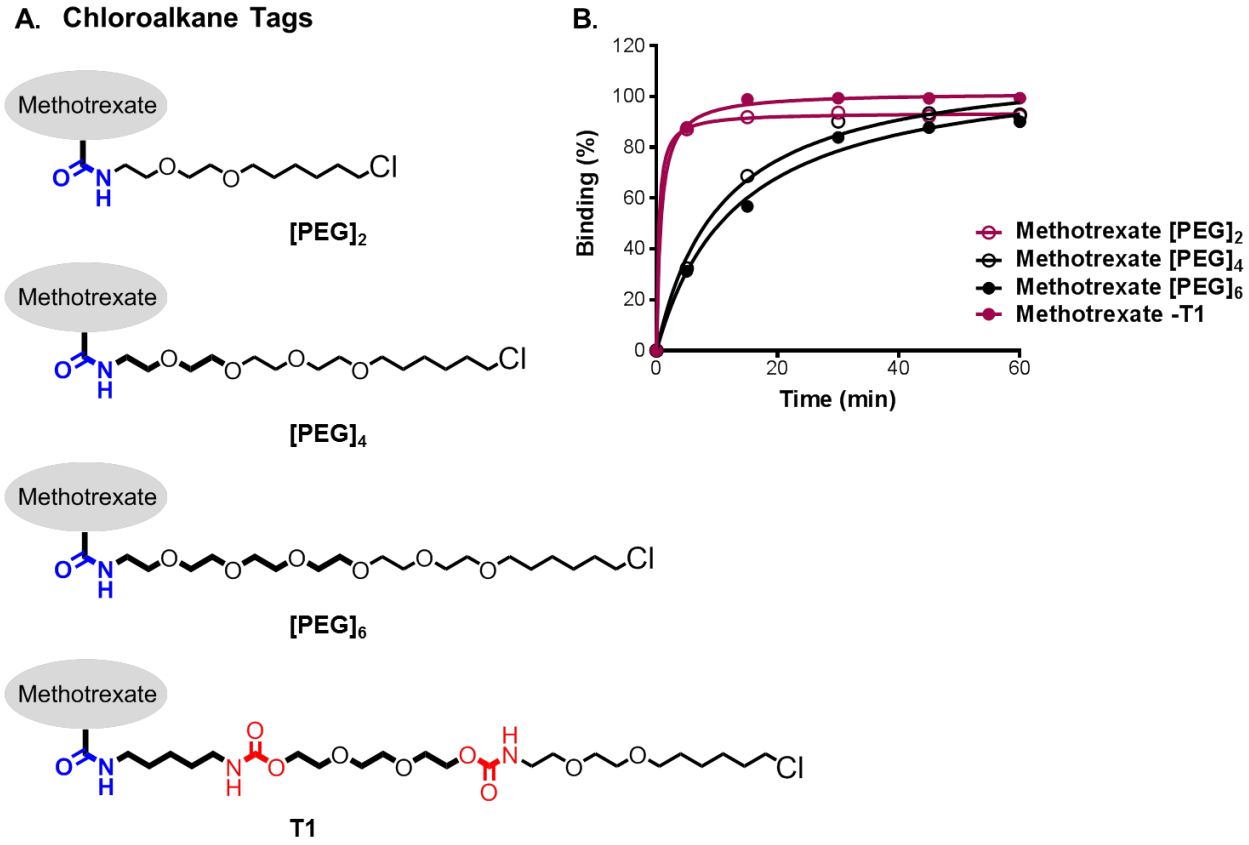


Figure 1. **Influence of spacers on kinetics of binding between the chloroalkane tags and HaloTag.** (A) Chemical structure of chloroalkane tags conjugated to methotrexate. The spacers are shown by thicker bonds, carbamate groups are indicated in red, and amide groups are indicated in blue. (B) Binding kinetics of the methotrexate conjugates to a HaloTag fusion protein in cell lysates. Results indicate that the $[PEG]_4$ and $[PEG]_6$ significantly reduced the binding kinetics to HaloTag while the longer T1 maintained the rapid binding kinetics of the $[PEG]_2$ linker.

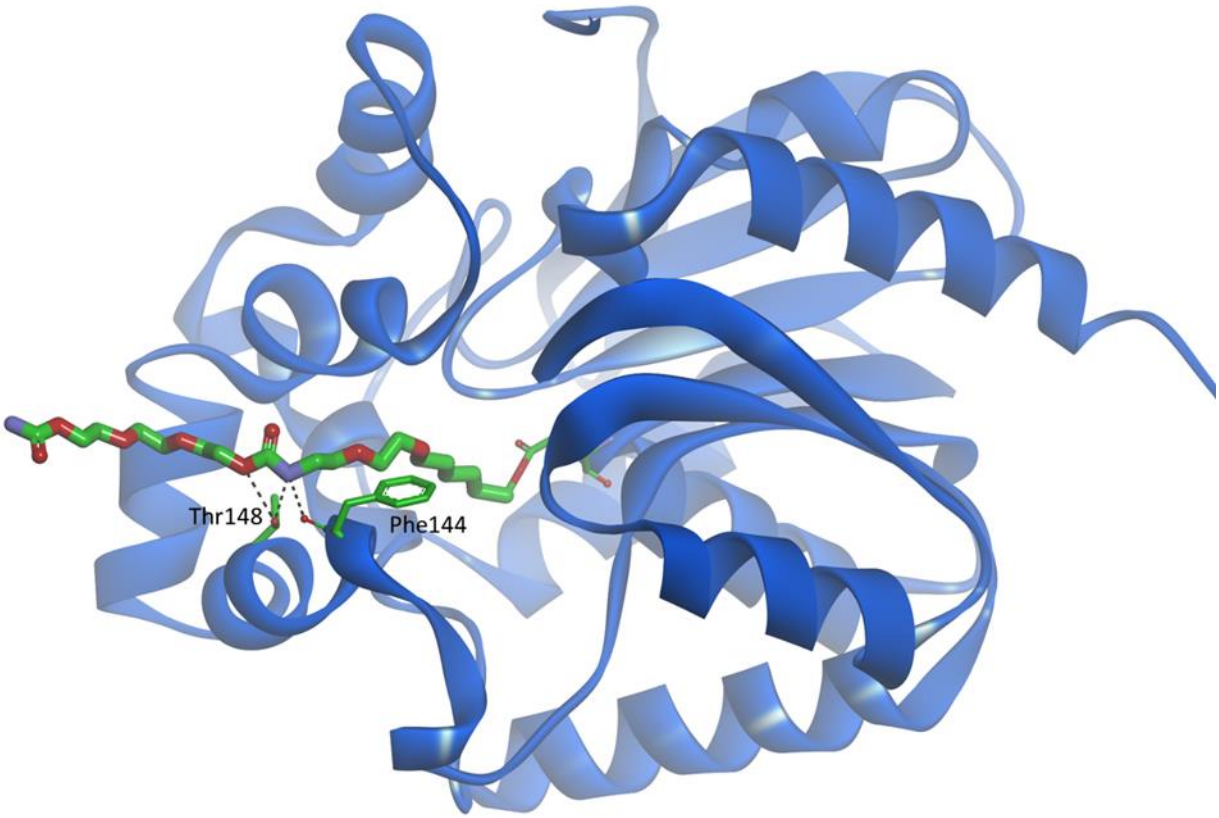


Figure 2. A docking model of T1 with the crystal structure of HaloTag. HaloTag (ribbon) with bound T1 (thick stick) and relevant amino acids (Thr148 and Phe144, thin sticks) that can form hydrogen bonds (dashed lines) with one of the T1 carbamate groups. Atom coloring is green for carbon, red for oxygen, and blue for nitrogen. Hydrogen atoms are hidden for clarity. Analyses and visualization were performed with Discovery Studio 3.5 (Accelrys Software Inc.). T1 was manually docked into HaloTag (PDB code 4KAJ) and subjected to a molecular dynamics simulation, using the Standard Dynamics Cascade protocol with implicit solvent model and default parameters (steps were increased to ensure system equilibration). Analysis of 40 conformers from the production phase indicated that the carbamate can form one or more hydrogen bonds with the Thr148 side chain (35 conformers) and the Phe144 backbone oxygen (6 conformers).

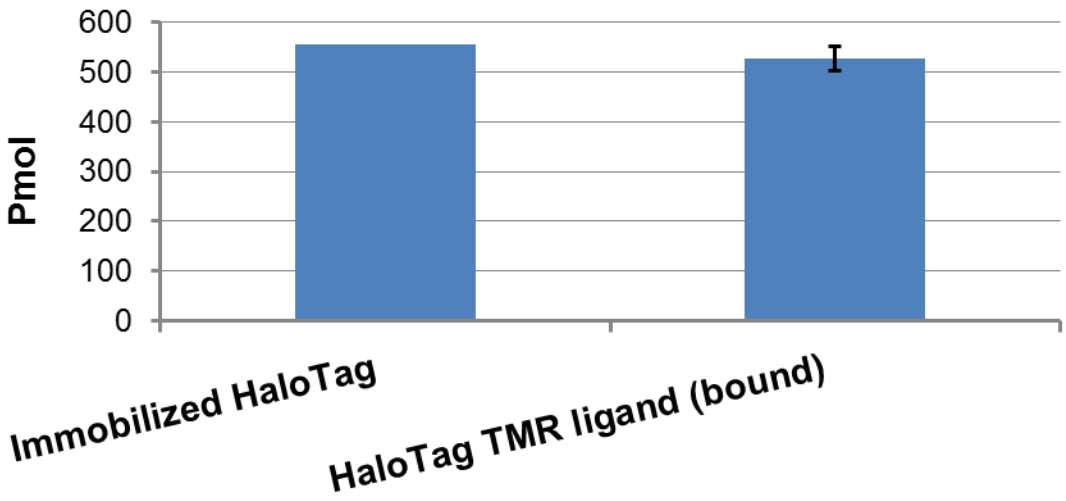


Figure 3. Specific activity of HaloTag immobilized onto paramagnetic particles. Comparison between the amount of immobilized HaloTag and the amount of HaloTag TMR ligand bound to the particles. Because the binding of HaloTag to the ligand is at a 1:1 molar ratio, the pmol of HaloTag TMR ligand bound to the particles equals the pmol of active HaloTag. The percent of HaloTag that retained activity after immobilization was determined by dividing the amount of active HaloTag by the total amount of immobilized HaloTag. These results indicate that by using a homogenously oriented single point of attachment, HaloTag retained 95% of its specific activity after immobilization.

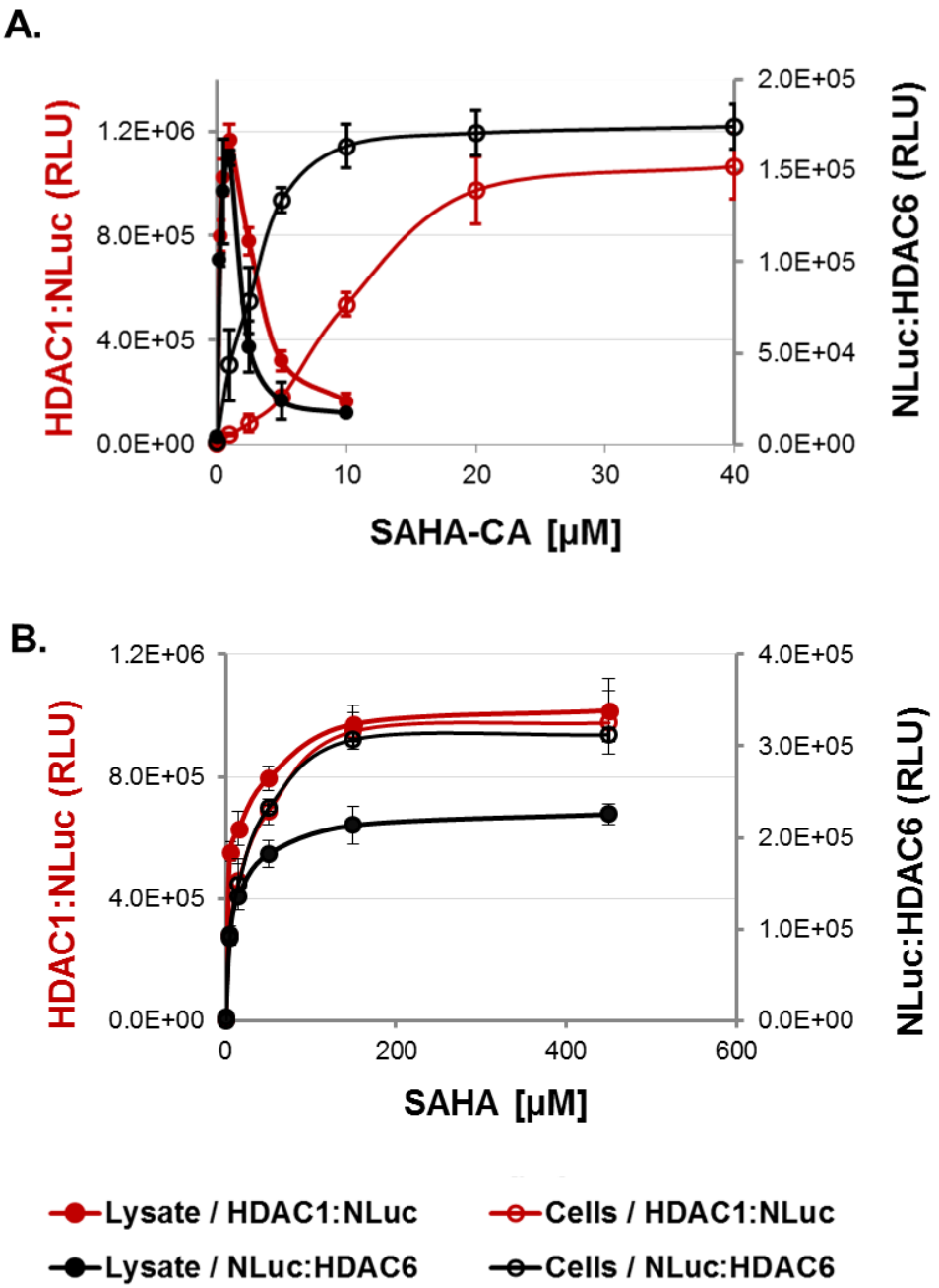


Figure 4. Optimization of target enrichment methods using NLuc fusion proteins. (A) Optimization of SAHA-chloroalkane concentration for target engagement in lysates or inside cells (n=3). Results indicate optimal enrichment of the two fusions using 1 µM or 20 µM SAHA-chloroalkane for target engagement in lysate (●) or inside cells (○), respectively. (B) Optimization of competitive elution (n=3). Both methods indicate that optimal elution is achieved using ≥150 µM SAHA.

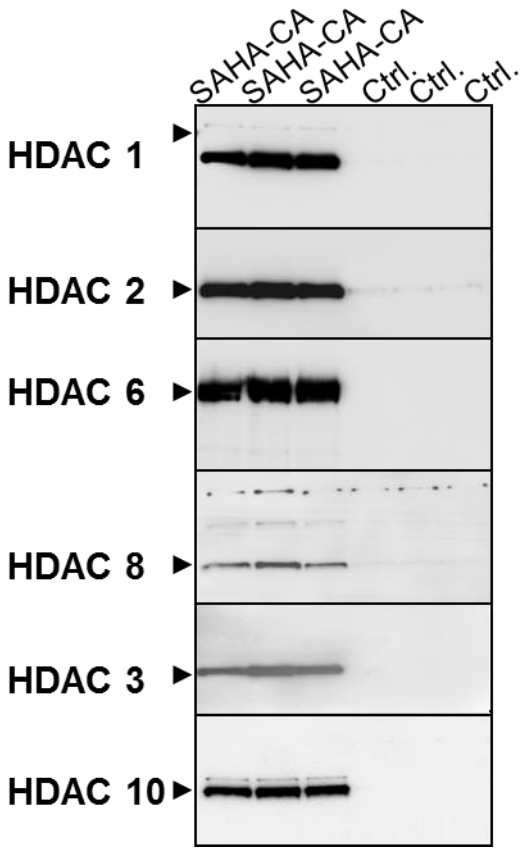


Figure 5. Enrichment of SAHA's HDAC targets from K-562 cells analyzed by Western. Results indicate specific isolation of all the HDACs expected to interact with SAHA (i.e., HDAC 1, 2, 3, 6, 8 and 10).

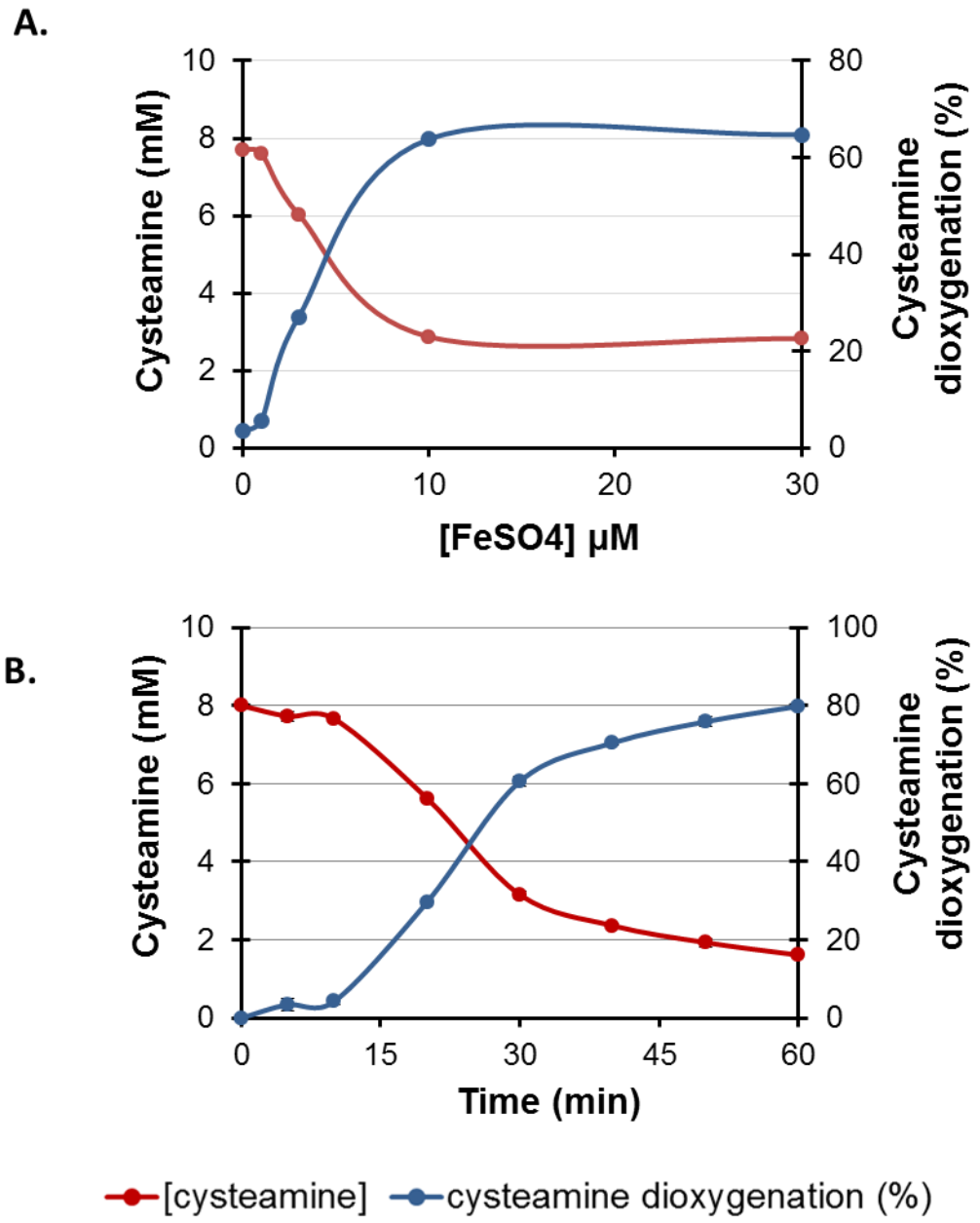


Figure 6. Biochemical analysis of ADO catalyzed cysteamine dioxygenation. The detection of sulfhydryl groups was utilized to determine the remaining cysteamine concentrations and the % of cysteamine dioxygenation. The level of cysteamine dioxygenation was optimized for (A) iron (II) concentration and (B) reaction time. The analysis demonstrates a time dependent dioxygenation that is reliant on iron (II) as a cofactor for ADO activity with an optimal concentration of 10 µM.

	SAHA	SAHA-chloroalkane	SAHA-biotin
	K_i (nM)	K_i (nM)	K_i (nM)
HDAC6	2	9	130
HDAC10	10	9	60
HDAC1	7	40	180
HDAC2	11	60	330
HDAC3	130	140	500
HDAC8	350	70	570

1—10 nM 10—100 nM 100—1000 nM

Figure 7. Influence of chloroalkane and biotin tags on SAHA's interactions with HDACs. A BRET assay was used to compare the ability of SAHA, SAHA-chloroalkane and SAHA-biotin to displace SAHA-NCT bound to each HDAC. Apparent affinities were determined as described in Materials and Methods. Results indicate that the biotin modification displayed a higher degree of interference with SAHA interactions. This was particularly apparent for the affinities of HDAC 1, 2 and 6, which were reduced 26-, 30-, and 65-fold, respectively, by the biotin modifications.

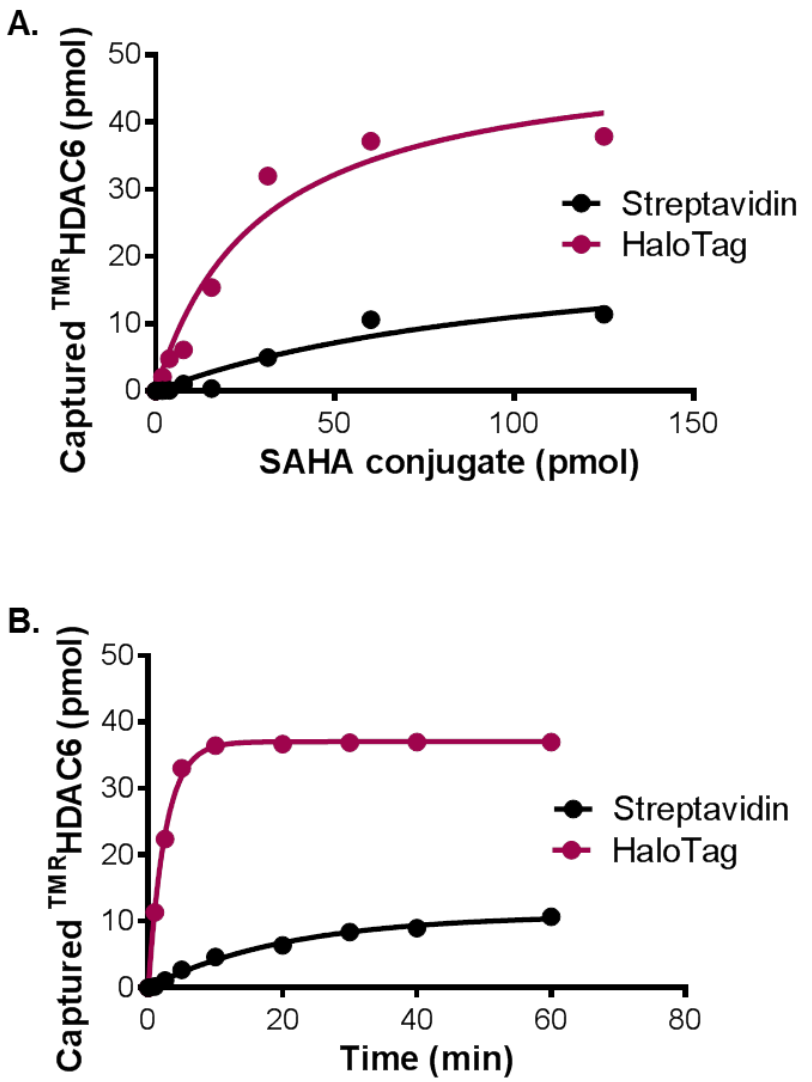


Figure 8. Capture efficiency of purified HDAC6 onto HaloTag and streptavidin particles. (A) Capture capacity and (B) capture kinetics. To determine the capture capacity, ^{TMR}HDAC6 was allowed to bind to serial dilutions of the SAHA conjugates for 2 h before being captured onto the appropriate particles. The unbound fractions were removed and the bound ^{TMR}HDAC6 was released from the particles by SDS denaturation and analyzed by SDS-PAGE. The amount of captured ^{TMR}HDAC6 was determined from an in-gel linear calibration curve. These results indicate that the HaloTag particles had 3.5-fold higher capture capacity. To compare the capture kinetics, following a 2 h incubation the SAHA conjugates and their bound ^{TMR}HDAC6 were captured onto the appropriate particles for up to 60 min. The amount of captured ^{TMR}HDAC6 revealed that capture onto the HaloTag particles was 10-fold faster.

Supporting References

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