Supporting information

Identification of mercaptoacetamide-based HDAC6 inhibitors via a lean inhibitor strategy: screening, synthesis, and biological evaluation

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Synthetic procedures, structural and spectral data

General methods

All ^{1}H, ^{13}C, and ^{19}F NMR spectra were recorded with a Bruker Avance III NMR spectrometer, equipped with 1H/BB z-gradient probe (BBO, 5 mm) at 400, 100.6, and 376.5 MHz, respectively. The synthesized compounds were dissolved in d_6-DMSO or $CDCl_3$, and trimethylsilane (TMS) was used as an internal chemical shift standard. All spectra were processed using TOPSPIN 3.6. The chemical shifts were reported as δ-values that were assigned in combination with COSY, HSQC, and HMBC spectra. Mass spectrometric analyses were performed using an Agilent 1100 series MSD SL mass spectrometer with electrospray ionization geometry (ESI) at 4000 V (positive mode) and 3500 V (negative mode), and fragmentation at 70 eV and a mass-selective quadrupole detector. High-resolution electron spray (ES-TOF) mass spectra were obtained with an Agilent Technologies 6210 Series Time of Flight. IR spectra of the synthesized compounds with an S/N-ratio of 30,000:1 were recorded using a Shimadzu IRAFFINITY-1S Fourier Transform Infrared Spectrophotometer (FTIR). The melting points of solid synthesized compounds were determined using a Kofler WME melting bench of Wager and Munz (temperature range 50 to 260°C). Calibration was performed with various standards with a specific melting point. Automated 'reversed phase' chromatography was performed on a Grace Reveleris chromatography system, equipped with a C_{18} column, and a mixture of water and acetonitrile was used as a mobile phase. Eluding compounds were detected by a UV detector (200-400 nm) at two wavelengths of choice (determined after LC-MS analysis of crude product), as well as by an evaporative light scattering detector (ELSD). Microwave reactions were performed in a CEM FocusedTM Microwave Synthesis System, Model Discover, with adaptable power from 0-300W, monitored with the Synergy software v. 1.32. Reagents were added to a 10 mL thick-walled vial and sealed with a PTFE septum. The temperature of the reaction was controlled using an infrared sensor, and the reaction mixture was stirred using a magnetic stirrer plate. The purity of the synthesized compounds was assessed with ^{1}H NMR, confirming a purity $\geq$ 98% unless stated otherwise.

Synthesis of 5-(4-nitrobenzyl)-1,3,4,5-tetrahydrothiopyrano[4,3-*b*]indoles **6a, c, e** and 4-(4-nitrobenzyl)-3,4-dihydro-2*H*-thieno[3,2-*b*]indoles **6b, d**

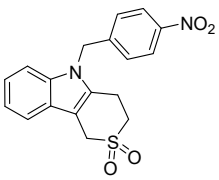
The syntheses of 1,3,4,5-tetrahydrothiopyrano[4,3-*b*]indoles **4a-e** were accomplished using a Fisher-indole reaction, followed by oxidation of the sulfur atom with 3-chloroperbenzoic acid (*m*CPBA) to give sulfones **4a-d** (y = 3) and sulfoxide **4e** (y = 1) using similar procedures as described in previous work. The spectral data of these intermediates were identical to corresponding literature data.[1-3]

As a representative example, the synthesis of 5-(4-nitrobenzyl)-1,3,4,5-tetrahydrothiopyrano[4,3-*b*]indole 2,2-dioxide **6c** is described.

In a flask of 50 mL, 500 mg (2.09 mmol) 8-fluoro-1,3,4,5-tetrahydrothiopyrano[4,3-b]indole 2,2-dioxide **4c** was dissolved in 10 mL dry *N*,*N*-dimethylformamide (DMF) and cooled to 0°C. 92 mg (2.30 mmol; 1.1 equiv.) sodium hydride (60 wt. % in mineral oil) was added and the reaction mixture was stirred for 30 minutes at room temperature and under nitrogen atmosphere. Afterward, 451 mg (2.09 mmol, 1 equiv.) 4-nitrobenzylbromide, dissolved in DMF, was added to the reaction mixture. After stirring the reaction mixture for an additional 30 minutes at room temperature, the reaction was quenched with 30 mL water. 3 x 20 mL ethyl acetate was added to extract the aqueous mixture, and the combined organic fractions were washed with water (2 x 30 mL), a 2 M LiCl solution (2 x 20 mL), and a saturated NaCl solution (2 x 20 mL). The organic phase was dried over magnesium sulfate, filtered, and evaporated. The resulting product was purified using automated column chromatography

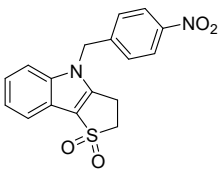
(C_{18}, gradient CH_3CN/H_2O 30/70-100/0). This resulted in 62 mg (0.17 mmol, yield = 8%) 8-fluoro-5-(4-nitrobenzyl)-1,3,4,5-tetrahydrothiopyrano[4,3-*b*]indole 2,2-dioxide **6c**.

5-(4-Nitrobenzyl)-1,3,4,5-tetrahydrothiopyrano[4,3-*b*]indole 2,2-dioxide 6a



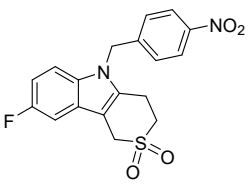
^{1}H NMR (400 MHz, d_6-DMSO): δ 3.21 (2H, t, *J* = 6.2 Hz); 3.51 (2H, t, *J* = 6.2 Hz); 4.51 (2H, s); 5.61 (2H, s); 7.07-7.11 (1H, m); 7.14-7.18 (1H, m); 7.26 (2H, d, *J* = 8.8 Hz); 7.46 (1H, d, *J* = 7.9 Hz); 7,52 (1H, d, *J* = 7.9 Hz); 8.19 (2H, d, *J* = 8.8 Hz). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 22.3, 45.8, 46.7, 48.4, 103.1, 110.3, 118.3, 120.2, 122.6, 124.4, 126.7, 128.0, 131.5, 137.0, 146.3, 147.3. **IR** (cm^{-1}): ν_{NO} = 1520, 1344,$\nu_{S=O}$ = 1317, 1115; ν_{max} = 1520, 1466, 1287, 1163, 1125, 737. **MS**: m/z (%) = 357 ([M + H^+], 50), 374 ([M + NH_4^+], 55). Yellow powder. Yield after automated column chromatography (C_{18}, gradient CH_3CN/H_2O 30/70-100/0) = 16%. T_m = 230°C.

4-(4-Nitrobenzyl)-3,4-dihydro-2*H*-thieno[3,2-*b*]indole-1,1-dioxide 6b



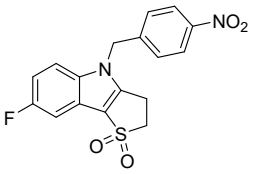
^{1}H NMR (400 MHz, d_6-DMSO): δ 3.45 (2H, t, *J* = 6.5 Hz); 3.95 (2H, t, *J* = 6.5 Hz); 5.64 (2H, s); 7.24-7.32 (2H, m); 7.44 (2H, d, *J* = 8.7 Hz); 7.56 (1H, d, *J* = 8.2 Hz); 7.62 (1H, d, *J* = 7.4 Hz); 8.22 (2H, d, *J* = 8.7 Hz). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 20.9, 46.7, 57.4, 112.2, 116.5, 118.8, 119.4, 122.6, 124.1, 124.5, 128.6, 140.9, 144.7, 147.5, 148.6. **IR** (cm^{-1}): ν_{NO} = 1520, 1346; $\nu_{S=O}$ = 1283, 1109; ν_{max} = 1476, 1449, 1128, 746, 733, 542. **MS**: m/z (%) = 343 ([M + 1]$^+$, 14), 360 ([M + NH_4^+], 100), 365 ([M + Na^+], 5). Beige powder. Yield after automated column chromatography (C_{18}, gradient CH_3CN/H_2O 30/70-100/0) = 70%. T_m = 244°C.

8-Fluoro-5-(4-nitrobenzyl)-1,3,4,5-tetrahydrothiopyrano[4,3-*b*]indole 2,2-dioxide 6c



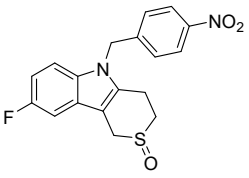
^{1}H NMR (400 MHz, d_6-DMSO): δ 3.20 (2H, t, *J* = 6.3 Hz); 3.51 (2H, t, *J* = 6.3 Hz); 4.49 (2H, s); 5.62 (2H, s); 7.01 (1H, t x d, *J* = 9.1, 2.5 Hz); 7.24 (2H, d, *J* = 8.6 Hz); 7.34 (1H, d x d, *J* = 9.7, 2.5 Hz); 7.48 (1H, d x d, *J* = 9.1, 4.1 Hz); 8.19 (2H, d, *J* = 8.6 Hz). **^{13}C NMR** (100,6 MHz, d_6-DMSO): δ 22.4, 46.0, 46.6, 48.3, 103.4 (d, *J* = 4.4 Hz); 103.5 (d, *J* = 24.1 Hz); 110.5 (d, *J* = 26.1 Hz); 111.5 (d, *J* = 9.7 Hz); 124.4, 127.0 (d, *J* = 10.6 Hz); 128.0, 133.5, 133.7, 146.1, 147.3, 157.8 (d, *J* = 233.0 Hz). **^{19}F NMR** (376.5 MHz, d_6-DMSO): δ (-123.87)-(-123.81) (m). **IR** (cm^{-1}): ν_{NO} = 1514, 1341; $\nu_{S=O}$ = 1315, 1115; ν_{max} = 1285, 1163, 1142, 1126, 806, 432. **MS**: m/z (%) = 375 ([M + 1]$^+$), 10), 392 ([M + NH_4^+], 14), 397 ([M + Na^+], 14)). Beige powder. Yield after automated column chromatography (C_{18}, gradient CH_3CN/H_2O 30/70-100/0) = 8%. T_m = 262°C.

7-Fluoro-4-(4-nitrobenzyl)-3,4-dihydro-2*H*-thieno[3,2-*b*]indole-1,1-dioxide 6d



^{1}H NMR (400 MHz, d_6-DMSO): δ 3.45 (2H, t, *J* = 6,5 Hz); 3,95 (2H, t, *J* = 6.5 Hz); 5,65 (1H, s); 7,17 (1H, d x d x d, *J* = 9.2, 9.2, 2.5 Hz); 7.41-7.45 (1H, m); 7.44 (2H, d, *J* = 8.7 Hz); 7.59 (1H, d x d, *J* = 9.2, 4.3 Hz); 8.22 (2H, d, *J* = 8.7 Hz). **^{13}C NMR** (100,6 MHz, d_6-DMSO): δ 21.0, 46.9, 57.4, 104.4 (d, *J* = 25.5 Hz); 112,2 (d, *J* = 25.8 Hz); 113.7 (d, *J* = 9.8 Hz); 116.5 (d, *J* = 4.4 Hz); 119.6 (d, *J* = 11.5 Hz); 124.5, 128.7, 137.5, 144.5, 147.5, 150.2, 158.8 (d, *J* = 236.8 Hz,). **^{19}F NMR** (376.5 MHz, d_6-DMSO): δ (-120.44)-(-120.38) (m). **IR** (cm^{-1}): ν_{NO} = 1516, 1340; $\nu_{S=O}$ = 1290, 1108; ν_{max} = 1476, 1127, 852, 806, 563, 522. **MS**: m/z (%) = 361 ($[M + 1]^+$, 6), 378 ($[M + NH_4^+]$, 100). White powder. Yield after automated column chromatography (C_{18}, gradient CH_3CN/H_2O 30/70-100/0) = 40%. T_m = 229°C.

8-Fluoro-5-(4-nitrobenzyl)-1,3,4,5-tetrahydrothiopyrano[4,3-*b*]indole 2-oxide 6e



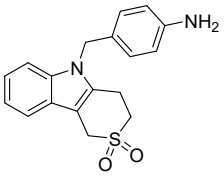
^{1}H NMR (400 MHz, d_6-DMSO): δ 2.98-3.14 and 3.31-3.38 (4H, 2 x m); 4,03 (1H, d, *J* = 15.4 Hz); 4.17 (1H, d, *J* = 15.4 Hz); 5.60 (2H, s); 6.96 (1H, d x d x d, *J* = 9.2, 9.2, 2.6 Hz); 7.25 (2H, d, *J* = 8.8 Hz); 7.34 (1H, d x d, *J* = 9,5, 2,6 Hz); 7.44 (1H, d x d, *J* = 9.2, 4.2 Hz); 8.18 (2H, d, *J* = 8.8 Hz). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 16.5, 43.7, 44.6, 45.9, 100.3 (d, *J* = 4.7 Hz); 103.4 (d, *J* = 23.9 Hz); 110.0 (d, *J* = 26.0 Hz); 111.2 (d, *J* = 9.8 Hz); 124.4, 128.0, 128.1 (d, *J* = 10.2 Hz); 133.3, 135.2 146.3, 147.3, 157.8 (d, *J* = 232.6 Hz). **^{19}F NMR** (376.5 MHz, d_6-DMSO): δ -124.21 (t x d, *J* = 9.5, 9.2, 4.2 Hz). **IR** (cm^{-1}): ν_{NO} = 1518, 1342; $\nu_{S=O}$ = 1040; ν_{max} = 1479, 1462, 1308, 1142, 849, 797, 754. **MS**: m/z (%) = 359 ($[M + H^+]$, 100). Beige powder. Yield after automated column chromatography (C_{18}, gradient CH_3CN/H_2O 30/70-100/0) = 24%. T_m = 234°C.

Synthesis of 5-(4-aminobenzyl)-1,3,4,5-tetrahydrothiopyrano[4,3-*b*]indoles 7a, c, e and 4-(4-aminobenzyl)-3,4-dihydro-2*H*-thieno[3,2-*b*]indoles 7b, d

The synthesis of 5-(4-aminobenzyl)-8-fluoro-1,3,4,5-tetrahydrothiopyrano[4,3-b]indole 2,2-dioxide **7c** is described as an example for the synthesis of compounds **7a-e.**

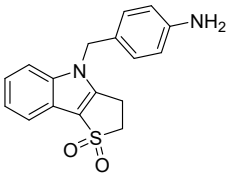
143 mg (0.38 mmol) 8-fluoro-5-(4-nitrobenzyl)-1,3,4,5-tetrahydrothiopyrano[4,3-b]indole 2,2-dioxide **6c** was dissolved in 10 mL acetone in a flask of 25 mL. 690 mg (3.06 mmol; 8 equiv.) tin(II) chloride dihydrate ($SnCl_2.2H_2O$) was dissolved in 989 µL (11.95 mmol; 31.25 equiv.) HCl (≥ 37%) and added dropwise to the solution of compound **6c** in acetone. The reaction was stirred for two hours at 50°C, after which NaOH was added (1M, 18 mL) until pH= 8-10. The solvent was removed in vacuo and the resulting residue was dissolved in 30 mL ethyl acetate. 30 mL water was added and the aqueous phase was extracted with ethyl acetate (2 x 20 mL). The combined organic fractions were washed with water (2 x 20 mL) and brine (20 mL), dried over $MgSO_4$, filtered, and evaporated. This furnished 66 mg (0.19 mmol; yield = 50%) 5-(4-aminobenzyl)-8-fluoro-1,3,4,5-tetrahydrothiopyrano[4,3-b]indole 2,2-dioxide **7c.**

5-(4-aminobenzyl)-1,3,4,5-tetrahydrothiopyrano[4,3-b]indole 2,2-dioxide 7a



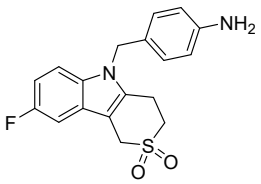
H NMR (400 MHz, d_6-DMSO): δ 3.25 (2H, t, *J* = 6.1 Hz); 3.50 (2H, t, *J* = 6.1 Hz); 4.47 (2H, s); 5.02 (2H, s); 5.19 (2H, s); 6.47 (2H, d, *J* = 8.4 Hz); 6.80 (2H, d, *J* = 8.4 Hz); 7.02-7.06 (1H, m); 7.12-7.16 (1H, m); 7.45-7.51 (2H, m). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 22.5, 46.2, 46.8, 48.5, 102.2, 110.5, 114.3, 118.0, 119.6, 122.1, 125.0, 126.5, 128.1, 131.5, 137.0, 148.4. **IR** (cm^{-1}): ν_{NH} = 3383; $\nu_{S=O}$ = 1282, 1163; ν_{max} = 1618, 1519, 1312, 1283, 1113, 745, 513, 430. **MS**: m/z (%) = 327 ([M + H^+], 11). Beige powder. Yield = 98%. T_m = 238°C.

4-(4-Aminobenzyl)-3,4-dihydro-2*H*-thieno[3,2-*b*]indole 1,1-dioxide 7b



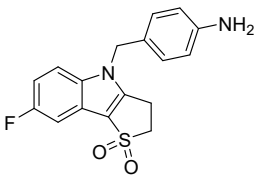
^{1}H NMR (400 MHz, d_6-DMSO): δ 3.45 (2H, t, *J* = 6.5 Hz); 3.93 (2H, t, *J* = 6.5 Hz); 5.22 (2H, s); 6.53 (2H, d, *J* = 8.5 Hz); 6.99 (2H, d, *J* = 8.5 Hz); 7.22 (1H, d x d, *J* = 7.5, 7.5 Hz); 7.29 (1H, d x d, *J* = 8.0, 7.5 Hz); 7.57 (1H, d, *J* = 7.5 Hz); 7.64 (1H, d, *J* = 8.0 Hz). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 21.1, 47.3, 57.4, 112.5, 114.5, 115.6, 118.6, 119.2, 122.2, 123.6, 123.8, 129.0, 140.9, 148.3, 148.5. **IR** (cm^{-1}): ν_{NH} = 3352; $\nu_{S=O}$ = 1279, 1105; ν_{max} = 1518, 1447, 1128, 735, 604, 546, 257. **MS**: m/z (%) = 313 ([M + H^+], 32), 330 ([M + NH_4^+], 31). Brown powder. Yield = 95%. T_m = 212°C.

5-(4-Aminobenzyl)-8-fluoro-1,3,4,5-tetrahydrothiopyrano[4,3-*b*]indole 2,2-dioxide 7c



^{1}H NMR (400 MHz, d_6-DMSO): δ 3.25 (2H, t, *J* = 6.2 Hz); 3.50 (2H, t, *J* = 6.2 Hz); 4.44 (2H, s); 5.03 (2H, s); 5.19 (2H, s); 6.47 (2H, d, *J* = 8.4 Hz); 6.79 (2H, d, *J* = 8.4 Hz); 6.97 (1H, d x d x d, *J* = 9.5, 9.1, 2.5 Hz); 7.27 (1H, d x d, *J* = 9.5, 2.5 Hz); 7,51 (1H, d x d, *J* = 9.1, 4.5 Hz). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 22.6, 46.4, 46.7, 48.4, 102.4 (d, *J* = 4.2 Hz); 103.2 (d, *J* = 24.2 Hz); 110.0 (d, *J* = 26.0 Hz); 111.6 (d, *J* = 9.2 Hz); 114.7, 125.3, 126.8 (d, *J* = 10.1 Hz); 128.1, 133.5, 133.6, 149.0, 157.1 (d, *J* = 231.2 Hz). **^{19}F NMR** (376.5 MHz, d_6-DMSO): δ -124.47 (d x d x d, *J* = 9.5, 9.1, 4.5 Hz). **IR** (cm^{-1}): ν_{NH} = 3377; $\nu_{S=O}$ = 1285, 1115; ν_{max} = 1518, 1481, 1317, 1165, 1146, 729, 440. **MS**: m/z (%) = 367 ([M + Na^+], 100). Orange oil. Yield = 50%.

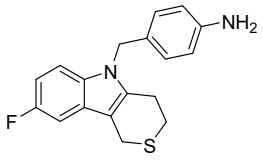
4-(4-Aminobenzyl)-7-fluoro-3,4-dihydro-2*H*-thieno[3,2-*b*]indole 1,1-dioxide 7d



^{1}H NMR (400 MHz, d_6-DMSO): δ 3.45 (2H, t, *J* = 6.6 Hz); 3.93 (2H, t, *J* = 6.6 Hz); 5.12 (2H, s); 5.22 (2H, s); 6.51 (2H, d, *J* = 8.4 Hz, 2 x CH_{arom}); 6.98 (2H, d, *J* = 8.4 Hz); 7.15 (1H, d x d x d, *J* = 9.2, 9.2, 2.5 Hz); 7.35 (1H, d x d, *J* = 9.2, 2.5 Hz, CH_{arom}); 7.66 (1H, d x d, *J* = 9,2, 4,7 Hz). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 21.2, 47.6, 57.3, 104.0 (d, *J* = 25.1 Hz); 111.7 (d, *J* = 26.0 Hz); 113.9 (d, *J* = 9.9 Hz); 114.4, 115.6 (d, *J* = 4.4 Hz,); 119.4 (d, *J* = 11.1 Hz); 123.4, 129.0, 137.5, 148.8, 149.8, 158.5 (d, *J* = 236.1 Hz). **^{19}F NMR** (376.5

MHz, d_6-DMSO): δ -120.96 (d x d x d, *J* = 9.2, 9.2, 4.7 Hz,). **IR** (cm^{-1}): ν_{NH} = 3352; $\nu_{S=O}$ = 1279, 1105; ν_{max} = 1518, 1481, 1184, 1142, 1125, 569, 523. **MS** (70 e): m/z (%) = 331 ([M + H^+], 35), 348 ([M + NH_4^+], 100). Orange powder. Yield = 94%. T_m = 186°C.

4-[(8-Fluoro-3,4-dihydrothiopyrano[4,3-*b*]indol-5(1*H*)-yl)methyl]aniline 7e



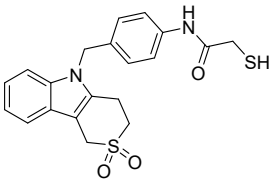
^{1}H NMR (400 MHz, d_6-DMSO): δ 2.90-2.99 (4H, m); 3.77 (2H, s); 4.99 (2H, s, NH_2); 5.14 (2H, s); 6.46 (2H, d, *J* = 8.4 Hz); 6.75 (2H, d, *J* = 8.4 Hz); 6.90 (1H, d x d x d, *J* = 9.5, 9.2, 2.6 Hz); 7.22 (1H, d x d, *J* = 9.5, 2.6 Hz); 7.44 (1H, d x d, *J* = 9.2, 4.4 Hz). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 22.2, 23.9, 25.1, 45.5, 102.4 (d, *J* = 23.3 Hz); 106.1 (d, *J* = 4.4 Hz); 108.5 (d, *J* = 25.7 Hz); 110.5 (d, *J* = 9.8 Hz); 113.8, 124.7, 126.3 (d, *J* = 10.3 Hz); 127.5, 131.9, 136.9, 147.8, 156.9 (d, *J* = 231.6 Hz). **^{19}F NMR** (376.5 MHz, d_6-DMSO): δ -125.29 (d x d x d, *J* = 9.5, 9.2, 4.4 Hz). **IR** (cm^{-1}): ν_{NH} = 3364; ν_{max} = 1479, 1362, 1179, 1146, 1134, 791, 496, 484, 432. **MS**: m/z (%) = 313 ([M + H^+], 100), 335 ([M + NH_4^+], 27). Orange oil. Yield = 82%.

Synthesis of *N*-{4-[(3,4-dihydrothiopyrano[4,3-b]indol-5(1H)-yl)methyl]phenyl}-2-mercaptoacetamides 10a, c, e and *N*-{4-[(1,1-dioxido-2,3-dihydro-4H-thieno[3,2-*b*]indol-4-yl)methyl]phenyl}-2-mercaptoacetamide 10b, d.

The synthesis procedure for *N*-{4-[(8-fluoro-2,2-dioxido-3,4-dihydrothiopyrano[4,3-*b*]indol-5(1*H*)-yl)methyl]phenyl}-2-mercaptoacetamide **10c** described underneath can be considered as a general method to obtain compounds **10a-d**, except for compounds **10b** and **10d**, for which the purification was more difficult and differed from the general procedure. Crystallization from different solvents was not succesful, therefore, automated column chromatography was used to obtain pure **10d**. No full conversion to compound **10b** could be attained, and maximal purity after several purification steps was reached at 95%. As such, *N*-{4-[(1,1-dioxido-2,3-dihydro-4H-thieno[3,2-*b*]indol-4-yl)methyl]phenyl}-2-mercaptoacetamide **10b** was obtained in 95% purity, in a mixture with 5% starting product 4-(4-aminobenzyl)-3,4-dihydro-2*H*-thieno[3,2-*b*]indole 1,1-dioxide **7b**.

In a 10 mL glass tube, 66 mg (0.19 mmol) 5-(4-aminobenzyl)-8-fluoro-1,3,4,5-tetrahydrothiopyrano[4,3-b]indole 2,2-dioxide **7c** was dissolved in 214 µL (3.07 mmol; 16 equiv.) mercaptoacetic acid. The reaction mixture was stirred for 20 minutes at 80°C under microwave conditions. Afterward, 10mL ethyl acetate and 10 mL water was added. The aqueous phase was extracted with ethyl acetate (2 x 10 mL). The combined organic fractions were washed with saturated sodium bicarbonate solution (10 mL), water (10 mL) and brine (10mL) and dried over $MgSO_4$. After filtration and evaporation, the resulting residue was crystallized from acetonitril. This resulted in 8 mg (0.02 mmol, yield = 10%) *N*-{4-[(8-fluoro-2,2-dioxido-3,4-dihydrothiopyrano[4,3-*b*]indol-5(1*H*)-yl)methyl]phenyl}-2-mercaptoacetamide **10c**.

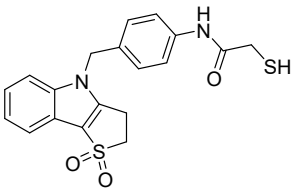
***N*-{4-[(2,2-dioxido-3,4-dihydrothiopyrano[4,3-*b*]indol-5(1*H*)-yl)methyl]phenyl}-2-mercaptoacetamide 10a**



^{1}H NMR (400 MHz, d_6-DMSO): δ 2.90-2.93 (1H, m); 3.23-3.27 (4H, m); 3.49-3.51 (2H, m); 4.48 (2H,s); 5.36 (2H, s); 7.01-7.08 (3H, m); 7.12-7.16 (1H, m); 7.47-7.51 (4H, m), 10.08 (1H, s). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 22.0, 28.2, 45.5, 46.3, 48.0, 102.1, 109.9, 117.7, 119.3, 119.4, 121.8, 126.1, 127.0, 131.0, 132.8, 136.5, 138.1, 168.5. **IR** (cm^{-1}): ν_{NH} = 3337; $\nu_{C=O}$ =1674, $\nu_{S=O}$ = 1315, 1115; ν_{max} = 1516, 1412, 1283, 1163, 737, 515. **MS**: m/z (%) = 418 ([M + NH_4^+], 100). **HRMS** (ESI): calculated for $C_{20}H_{20}N_2O_3S_2^+$: 401.0988 [M+H^+], found: 401.0973. Beige powder. Yield after crystallisation from acetonitril = 13%. T_m = 179°C.

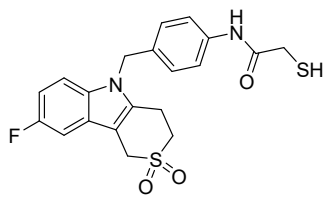
***N*-{4-[(1,1-dioxido-2,3-dihydro-4H-thieno[3,2-*b*]indol-4-yl)methyl]phenyl}-2-mercaptoacetamide 10b**

Analysis of spectral data was performed in presence of 5% 4-(4-aminobenzyl)-2,3-dihydrothieno[3,2-*b*]indole-1,1-dioxide **7b** (starting product).



^{1}H NMR (400 MHz, d_6-DMSO): δ 2.92 (1H, t, *J* = 8.1 Hz); 3.27 (2H, d, *J* = 8.1 Hz); 3.46 (2H, t, *J* = 6.0 Hz); 3.94 (2H, t, *J* = 6.0 Hz); 5.39 (2H, s); 7.20-7.30 (4H, m); 7.53-7.60 (4H, m); 10.12 (1H, s). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 21.0, 28.7, 47.0, 57.4, 112.4, 116.0, 118.7, 119.3, 119.8, 120.0, 122.4, 123.8, 128.4, 131.8, 140.9, 148.4, 169.1. **IR** (cm^{-1}): ν_{NH} = 3332; $\nu_{C=O}$ = 1678; $\nu_{S=O}$ = 1126, 1101; ν_{max} = 1537, 1269, 1240, 743, 544, 457. **HRMS** (ESI): calculated for $C_{19}H_{18}N_2O_3S_2^+$: 387.0832 [M+H]$^+$, found: 387.0829. **MS**: m/z (%) = 387 ([M + H^+], 10), 404 ([M + NH_4^+], 100), 409 ([M + Na^+], 18). White powder. Yield = 57%. Purity = 95%. T_m = 240°C.

***N*-{4-[(8-fluoro-2,2-dioxido-3,4-dihydrothiopyrano[4,3-*b*]indol-5(1*H*)-yl)methyl]phenyl}-2-mercaptoacetamide 10c**



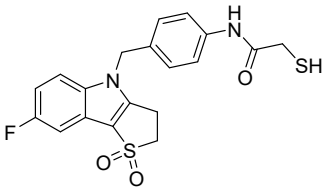
^{1}H NMR (400 MHz, d_6-DMSO): δ 2.91 (1H, t, *J* = 8.1 Hz); 3.23 (2H, t, *J* = 6.2 Hz); 3.26 (2H, d, *J* = 8.1 Hz); 3.50 (2H, t, *J* = 6.2 Hz); 4.46 (2H, s); 5.37 (2H, s); 6.98 (1H, d x d x d, *J* = 9.4, 9.4, 2.5 Hz); 7.02 (2H, d, *J* = 8.6 Hz); 7.30 (1H, d x d, *J* = 9.6, 2.5 Hz); 7.48-7.51 (1H, m); 7.50 (2H, d, *J* = 8.6 Hz); 10.08 (1H, s, NH). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 22.6, 28.7, 46.2, 46.6, 48.4, 102.8 (d, *J* = 4.5 Hz); 103.3 (d, *J* = 24.1 Hz); 110.2 (d, *J* = 25.8 Hz); 111.6 (d, *J* = 9.6 Hz); 119.9, 126.8 (d, *J* = 10.4 Hz); 127.5, 133.0, 133.5, 133.7, 138.7, 157.6 (d, *J* = 232.6 Hz); 169.0. **^{19}F NMR** (376.5 MHz, d_6-DMSO): δ -124.24 (d x d x d, *J* = 9.6, 9.4, 4.1 Hz). **IR** (cm^{-1}): $\nu_{C=O}$ = 1667; $\nu_{S=O}$ = 1125, 1115; ν_{max} = 1542, 1480, 1414, 1312,

1288, 1176, 1145. **MS**: m/z (%) = 419 ([M + H^+], 10), 436 ([M + NH_4^+], 100), 441 ([M + Na^+], 17). Beige powder. Yield after crystallisation from acetonitril = 10%. T_m = 221 °C.

Signals originating from the dimer of *N*-{4-[(8-fluoro-2,2-dioxido-3,4-dihydrothiopyrano[4,3-*b*]indol-5(1*H*)-yl)methyl]phenyl}-2-mercaptoacetamide **10c:**

^{1}H NMR (400 MHz, d_6-DMSO): δ 3.68 (4H, s); 5.38 (4H, s); 10.19 (2H, s). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 43.5; 167.2.

***N*-{4-[(7-fluoro-1,1-dioxido-2,3-dihydro-4*H*-thieno[3,2-*b*]indol-4-yl)methyl]phenyl}-2-mercaptoacetamide 10d**

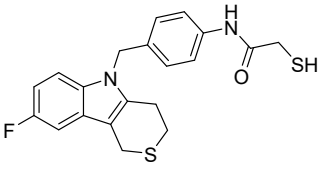


^{1}H NMR (400 MHz, d_6-DMSO): δ 2.93 (1H, s); 3.27 (2H, s); 3.46 (2H, t, *J* = 6.4 Hz); 3.94 (2H, t, *J* = 6.4 Hz); 5.39 (2H, s); 7.16 (1H, d x d x d, *J* = 9.2, 9.2, 2.5 Hz); 7.22 (2H, d, *J* = 8.5 Hz); 7.38 (1H, d x d, *J* = 9.1, 2.5 Hz); 7.55 (2H, d, *J* = 8.5 Hz); 7.62 (1H, d x d, *J* = 9.2, 4.4 Hz); 10.12 (1H, s). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 21.1, 28.7, 47.2, 57.3, 104.2 (d, *J* = 24.9 Hz); 111.9 (d, *J* = 25.6 Hz); 113.8 (d, *J* = 10.9 Hz); 116.0 (d, *J* = 4.4 Hz); 119.4 (d, *J* = 10.6 Hz); 119.8, 128.4, 131.6, 137.8, 139.1, 150.0 , 158.6 (d, *J* = 236.3 Hz); 169.1. **^{19}F NMR** (376.5 MHz, d_6-DMSO): δ -120.72 (d x d x d, *J* = 9.2, 9.1, 4.4 Hz). **HRMS** (ESI): calculated for $C_{19}H_{18}FN_2O_3S_2^+$: 405.0737 $[M+H]^+$, found: 405.0720. **MS**: m/z (%) = 422 ([M + NH_4^+], 100). White powder. Yield after automated column chromatography (C_{18}, gradient CH_3CN/H_2O 30/70-100/0) = 17%.

Results originating from the dimer of *N*-{4-[(7-fluoro-1,1-dioxido-2,3-dihydro-4*H*-thieno[3,2-*b*]indol-4-yl)methyl]phenyl}-2-mercaptoacetamide **10d:**

IR (cm^{-1}): $\nu_{C=O}$ = 1607; $\nu_{S=O}$ = 1125, 1107; ν_{max} = 1481, 1277, 1142, 800, 569, 523, 447. T_m = 222°C.

***N*-{4-[(8-fluoro-3,4-dihydrothiopyrano[4,3-*b*]indol-5(1*H*)-yl)methyl]phenyl}-2-mercaptoacetamide 10e**

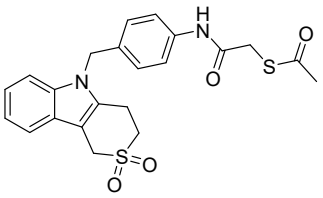


^{1}H NMR (400 MHz, d_6-DMSO): δ 2.87-2.97 (5H, m); 3.25 (2H, d, *J* = 8.1 Hz); 3.78 (2H, s); 5.31 (2H, s); 6.90 (1H, d x d x d, *J* = 9.2, 9.2, 2.4 Hz); 6.98 (2H, d, *J* = 8.4 Hz); 7.24 (1H, d x d, *J* = 9.6, 2.4 Hz); 7.43 (1H, d x d, *J* = 9.2, 4.3 Hz); 7.48 (2H, d, *J* = 8.4 Hz); 10.08 (1H, s). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ 22.6, 24.3, 25.9, 28.7, 45.8, 103.0 (d, *J* = 23.5 Hz); 107.0 (d, *J* = 4.6 Hz); 109.2 (d, *J* = 25.8 Hz); 110.9 (d, *J* = 9.7 Hz); 119.9, 126.9 (d, *J* = 9.8 Hz); 127.4, 132.4, 133.5, 137.5, 138.5; 157.5 (d, *J* = 231.9 Hz), 169.0 (C=O). **^{19}F NMR** (376.5 MHz, d_6-DMSO): δ -125.04 (d x d x d, *J* = 9.6, 9.2, 4.3 Hz). **IR** (cm^{-1}): $\nu_{C=O}$ = 1667; ν_{max} = 1605, 1518, 1479, 1460, 1414, 1180, 1146, 1136, 756. **MS**: m/z (%) = 387 ([M + H^+], 69). Orange powder. Yield after crystallisation from acetonitril = 29%. T_m = 180 °C.

Synthesis of [2-({4-[(2,2-dioxido-3,4-dihydrothiopyrano[4,3-*b*]indol-5(1*H*)-yl)methyl]phenyl}amino)-2-oxoethyl] ethanethioate 9

119 mg (0.365 mmol) aniline x was dissolved in 10 mL THF in a 25 mL flask and cooled to 0°C. 1.5 equivalents of *N,N*-diisopropylethylamine were added to this cooled solution, followed by 1.1 equivalents of 2-chloroacetyl chloride. The reaction mixture was stirred overnight at room temperature. After addition of 10 mL water, THF was evaporated and the remaining aquous solution was extracted with ethyl acetate (2 x 15 mL). The combined organic layers were washed with brine, dried over $MgSO_4$ and filtration and evaporation gave 2-chloro-*N*-{4-[(2,2-dioxido-3,4-dihydrothiopyrano[4,3-b]indol-5(1H)-yl)methyl]phenyl}acetamide **8** in 84% yield. Compound 8 was dissolved in DMF, 1.2 equivalents of potassium thioacetate were added and the reaction was stirred at room temperature for four hours. Water was added to the resulting solution, which was then extracted with ethyl acetate. The combined organic layers were washed with water, brine, dried with $MgSO_4$, filtered and concentrated *in vacuo*, which furnished prodrug **9** in 82% yield.

[2-({4-[(2,2-dioxido-3,4-dihydrothiopyrano[4,3-*b*]indol-5(1*H*)-yl)methyl]phenyl}amino)-2-oxoethyl] ethanethioate 9



^{1}H NMR (400 MHz, d_6-DMSO): δ 2.37 (3H, s); 3.23 (2H, d, *J* = 5.8 Hz); 3.50 (2H, t, *J* = 5.8 Hz); 3.79 (2H, s); 4.49 (2H, s); 5.37 (2H, s); 7.01-7.08 (3H, m); 7.13-7.16 (1H, m); 7.47-7.49 (4H, m); 10.23 (1H, s). **^{13}C NMR** (100.6 MHz, d_6-DMSO): δ. 22.4, 30.6, 34.2, 45.9, 46.8, 48.5, 102.5, 110.4, 118.1, 119.8, 122.3, 126.6, 127.5, 131.5, 133.3, 137.0, 138.5, 166.1, 195.0 **IR** (cm^{-1}): $\nu_{C=O}$ = 1692; $\nu_{S=O}$ = 1319, 1115; ν_{max} = 2926, 1535, 1514, 1466, 1287, 1125, 627. **MS**: m/z (%) = 460 ($[M + NH_4]^+$, 100). **HRMS** (ESI): calculated for $C_{22}H_{22}N_2O_4S_2^+$: 443.1094 $[M+H]^+$, found: 443.1086. Beige/grey powder. Yield = 82%. T_m = 104°C.

Docking studies (performed by the Centre for Synthetic Biology)

The molecular modelling program YASARA and the YASARA/WHATIF twinset was used for all manipulations.[4, 5] Figures were created with PyMOL 2.5 (Schrödinger LLC. The PyMOL Molecular Graphics System, Version 2.5). The crystal structure of catalytic domain 2 from *Homo sapiens* histone deacetylase 6 (accession code 5EDU) was used as template for docking.[6] HDAC inhibitor structures were created with YASARA Structure and subsequently minimised with the AMBER14 force field.[7] The grid box for docking had a dimension of 25 x 25 x 25 ångström and comprised the entire catalytic cavity, including the zinc ion and the outer surface of the active site entrance. Docking was performed with AutoDock VINA[8] using default parameters and ligands were allowed to rotate freely during the simulation. The first conformer from the cluster that had its zinc-binding group in the vicinity of the zinc ion was selected as the binding mode for further analysis. Ligplot diagrams were made with LigPlot⁺v1.4.

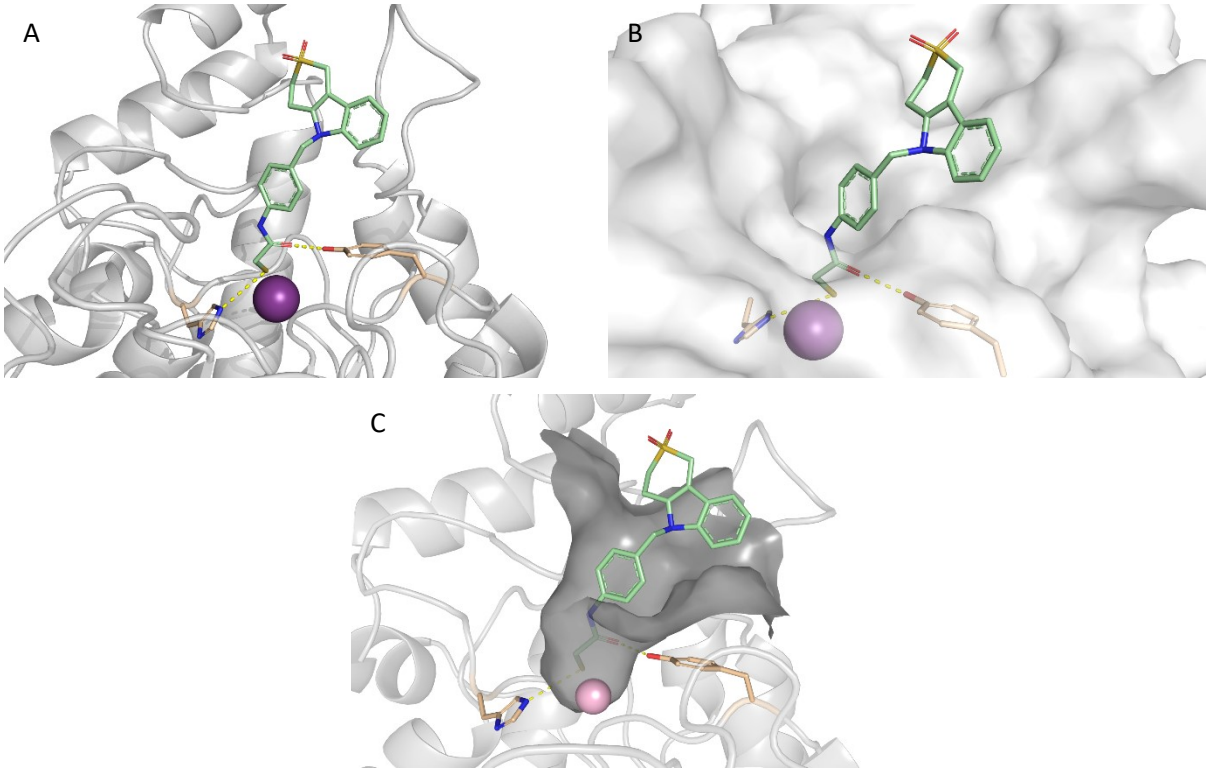


*Figure 1 Docking of compound **10a** in the catalytic domain CD2 of human HDAC6 (PDB 5EDU) (A), with (B) surface view of the active site pocket and (C) surface view of the protein surface and access tunnel. Hydrogen bonds are depicted in yellow dashed lines, zinc ion; purple, carbon; green, nitrogen; blue, sulfur; orange, oxygen; red.*

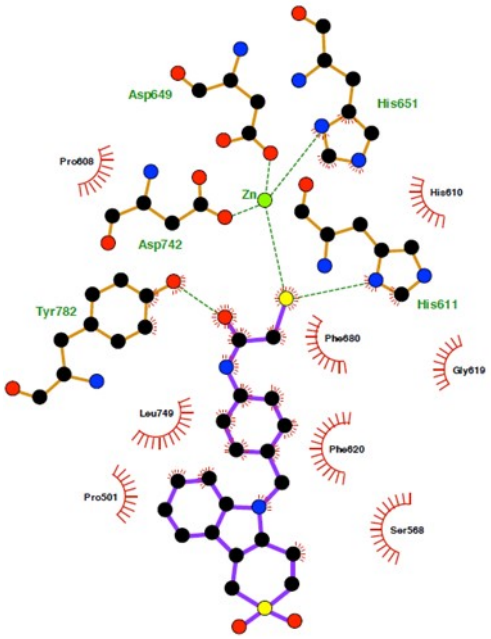


*Figure 2 Docking of compound **10a** in catalytic domain CD2 of human HDAC6 (PDB 5EDU).[6] Results show a monodentate binding of mercaptoacetamide to Zn^{2+} while engaging in additional hydrogen bonds (hydrogens bonds; green dashed lines, oxygen; red, carbon; black, nitrogen; blue, sulfur; yellow).*

Bioassay results

Cell lines and reagents

MM1.S cells were cultured in RPMI1640 GlutaMAX (Gibco, life technologies), supplemented with 10% fetal bovine serum (Tico Europe) and grown in a 5% CO_2 incubator at 37˚C. MM1.S cells were purchased from ATCC. All cell lines were regularly tested for mycoplasma contamination and were negative. Tubastatin A was purchased from Selleckchem, dissolved in DMSO and stored at -20˚C. All test compounds were dissolved in DMSO and stored at -20˚C. The total solvent concentration in all experiments was kept equal in each condition.

Protein lysates and Western Blotting (WB)

MM1.S cells were treated for one hour with control (0.1% DMSO) or treatment conditions (1-10-50 µM **10a**, **9**, **10a** + TCEP (tris(2-carboxyethyl)phosphine) or 1 µM Tubastatin A. Following treatment, MM1.S cells were lysed using Totex lysis buffer (Hepes/KOH, pH = 7.9, 20 mM, NaCl 350 mM, glycerol 20%, NP-40 1%, $MgCl_2$ 1 mM, EDTA 0.5 mM, EGTA 0.1 mM), to which an EDTA-free Halt protease and phosphatase inhibitor cocktail was added (Thermo Scientific). Protein content was determined via the detergent compatible (DC) protein assay (similar principle as the Lowry assay, Bio-Rad), for which absorbance was measured using the VERSAmax microplate reader (SoftMax Pro 7 software). After denaturation of 25 µg (or less) of total protein with Laemmli buffer, dithiothreitol (DTT) and heating for 5 minutes at 95°C, SDS-PAGE was performed. Afterwards, proteins were blotted on a nitrocellulose membrane (Cytiva, Amersham Protran) and the membrane was blocked with Startingblock™ blocking buffer (Thermo Scientific) mixed with tris-buffered saline (TBS) containing 0.1% Tween (1:1). The membranes were incubated with primary antibodies overnight (anti-HDAC6 (7612S, Cell Signaling Technology), anti-α-tubulin (T6793, Sigma), anti-acetylated α-tubulin (T7451, Sigma), anti-histone H3 (4499S, Cell Signaling Technology), anti-acetylated histone H3 (9649S, Cell Signaling Technology), anti-GAPDH (G8795, Sigma)). As secondary antibodies, we used species-specific HRP-conjugated antibodies (NA931, NA934, GE-Healthcare). To visualize results, chemiluminescent substrate (ECL

prime Western blotting, Amersham, Cytiva) was added and signals were imaged on a GE Amersham Imager 680 RGB. Experiments were performed in duplicate.

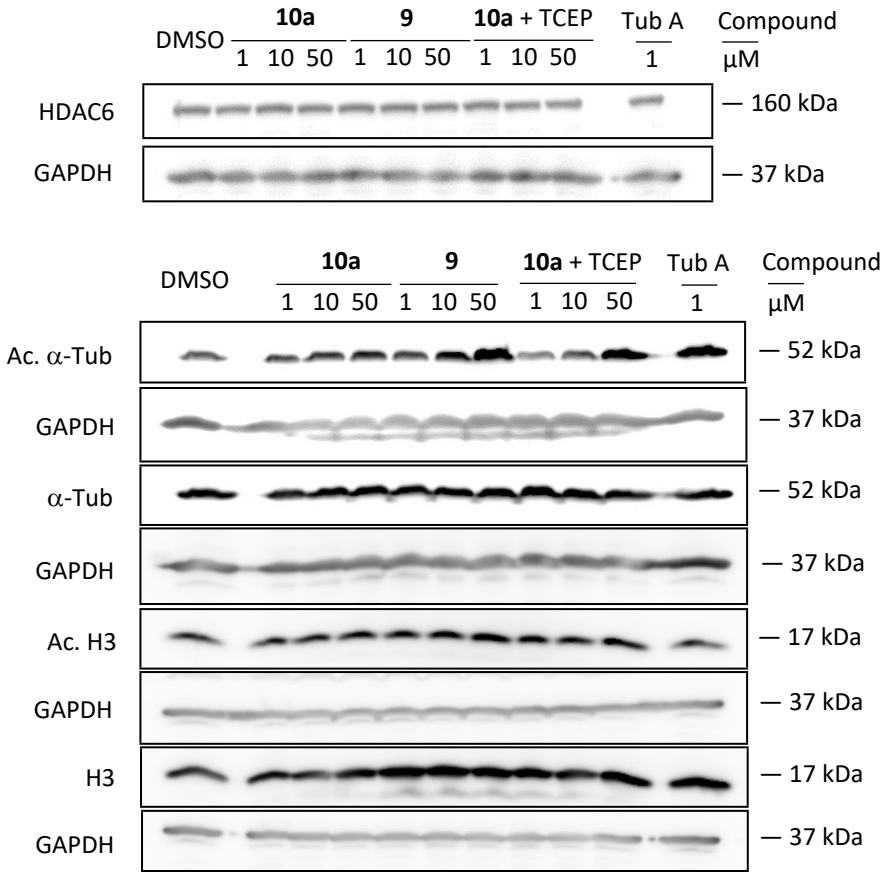


*Figure 3 Compounds **9**, **10a**, the combination of compound **10a** with TCEP and Tubastatin A differentially impact the levels of the HDAC6 substrates acetylated α-tubulin and acetylated histone H3. MM1.S cells were treated for 1h with DMSO, **10a** (1, 10, 50 µM), **9** (1, 10, 50 µM), **10a** (1, 10, 50 µM) + TCEP (1.5 equivalents) or Tubastatin A (Tub A, 1 µM, positive control). Protein lysates were subjected to Western Blot analyses, hereby assaying the protein levels of acetylated α-tubulin (Ac. Tub), α-tubulin, acetylated histone H3 (Ac.H3) and histone H3 (H3). GAPDH was used as a loading control. A representative Western blot is shown of three biological replicates. The results show that the mercaptoacetamide **10a** only slightly increased acetylated a-tubulin levels, while prodrug **9** and combination of compound **10a** and the reducing agent TCEP significantly enhanced acetylated α-tubulin levels in MM1.S cells. To confirm HDAC6 expression in MM1.S cells, HDAC6 levels for all treatment conditions were assessed as well via Western blot.*

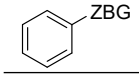
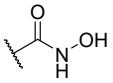
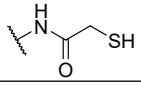
HDAC enzyme inhibition (performed by Cerep)

The enzyme inhibition assays were performed by Eurofins Cerep Panlabs. HDAC inhibition percentages were determined by using recombinant HDAC1-9 (*h*) and a fluorogenic HDAC substrate. Compound enzyme inhibition effect was calculated as a percentage inhibition of control enzyme activity. Results showing an inhibition higher than 50% are considered to represent significant effects of the test compounds. Results showing an inhibition lower than 25% are not considered significant and mostly attributable to variability of the signal around the control level. Low to moderate negative values have no real meaning and are attributable to variability of the signal around the control level.

HDAC6 inhibition by lean inhibitors

Incubation was performed for 30 minutes at room temperature and fluoro-lysine was measured via fluorimetry. Trichostatin A was used as a reference compound, lean inhibitors were tested at 10^{-5} M.

Table 1 Percentage of HDAC6 inhibition by the lean inhibitors composed of a phenyl linker and varying zinc-binding groups (ZBG) at 10 µM.

Lean inhibitor: 		**% inhibition of control values**		
Entry	**ZBG**	**1st**	**2nd**	**Mean**
1		86.8	89.2	88.0
2		-4.7	-0.5	-2.6
3		-6.5	-4.2	-5.4
4		-6.0	-4.4	-5.2
5		-8.7	-4.7	-6.7
6		-9.2	-4.5	-6.9
7		69.6	72.8	71.2
8	Trichostatin A	IC_{50}= 8.5 nM		

HDAC6 inhibition by mercaptoacetamides **10a-e**

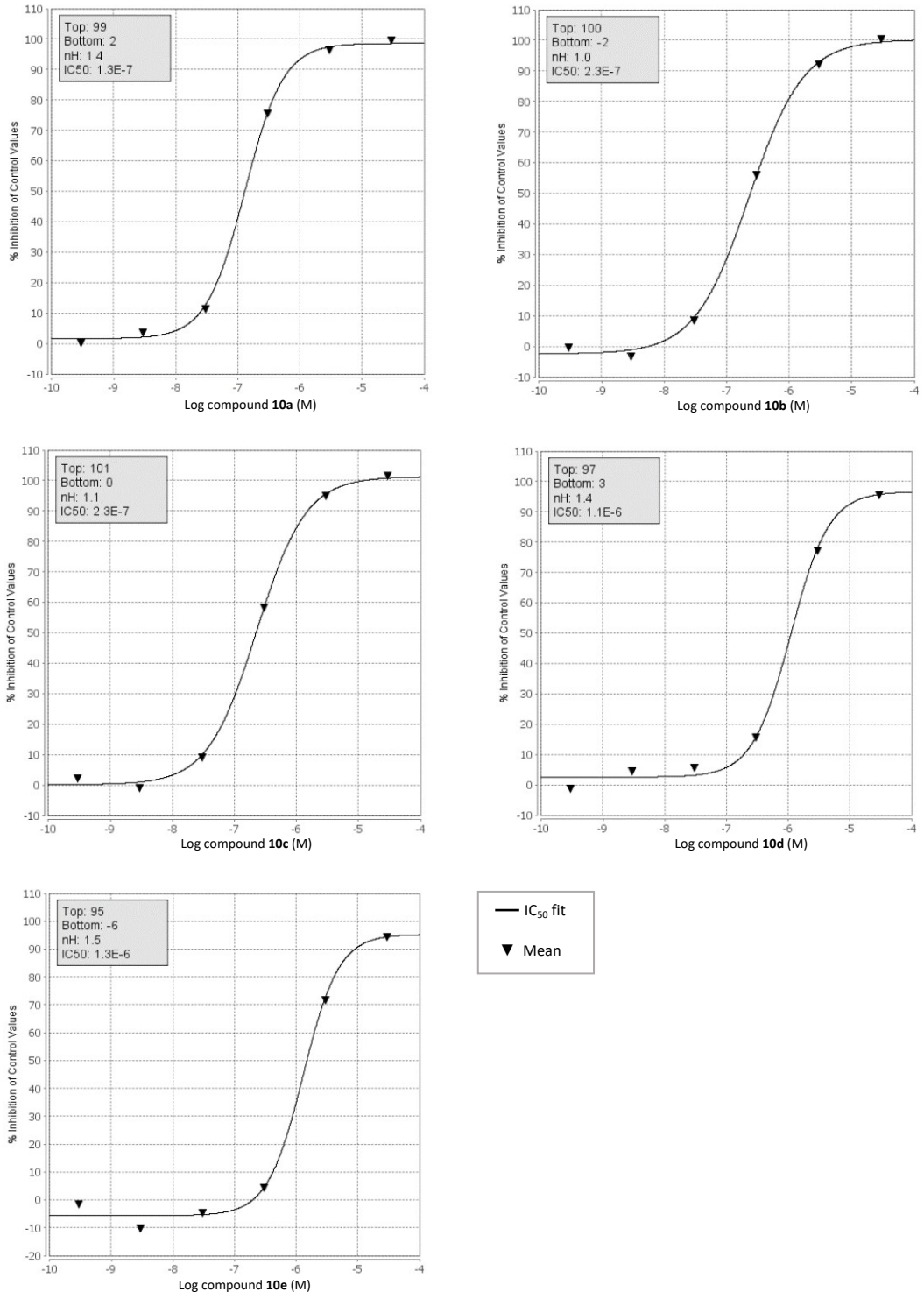
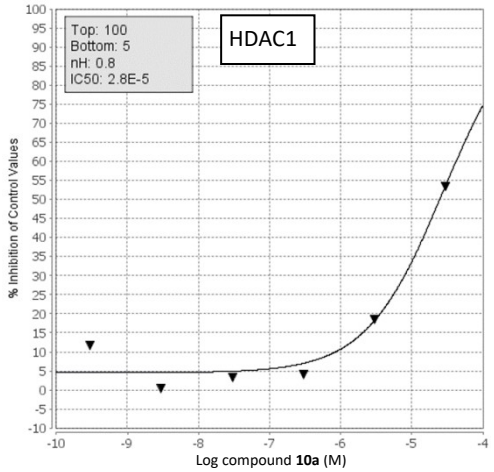
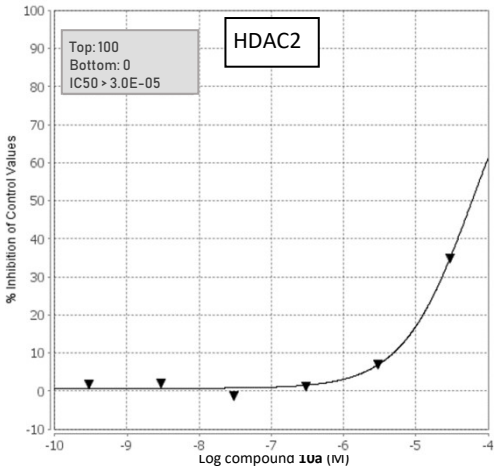


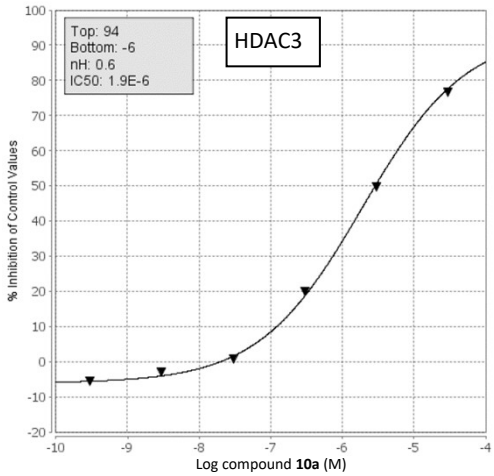
*Figure 5 HDAC6 inhibition effects of compounds **10a-e**. Result reference compound Trichostatin A: IC_{50}= 2.2 x 10^{-8} M.*

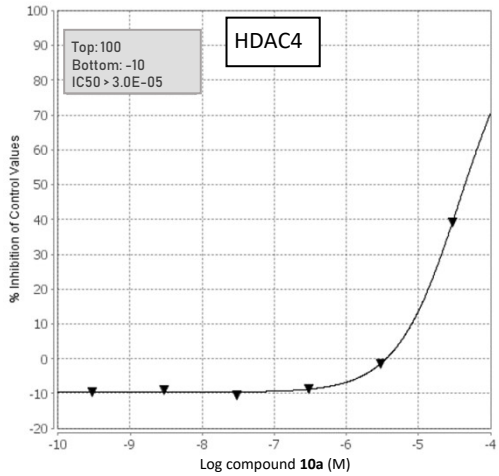
HDAC1-9 inhibition by compound **10a**

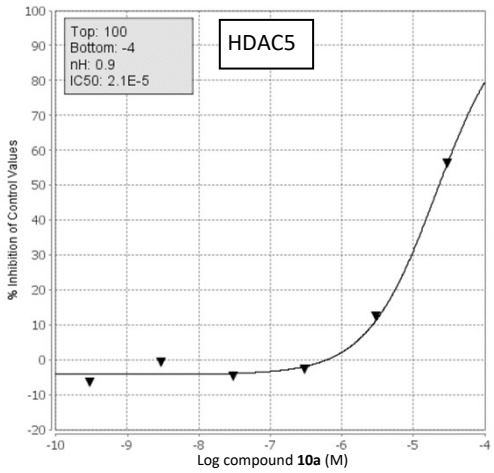
Effect of compound **10a** on HDAC1-9 was determined and IC_{50} values calculated when possibble. Incubation was performed for 15-60 minutes at room temperature and fluoro-lysine was measured via fluorimetry. Trichostatin A was used as a reference compound.

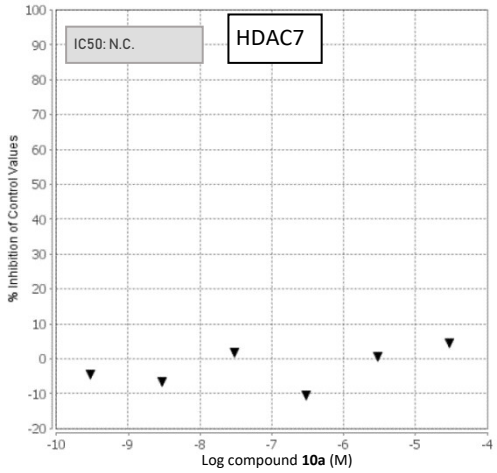












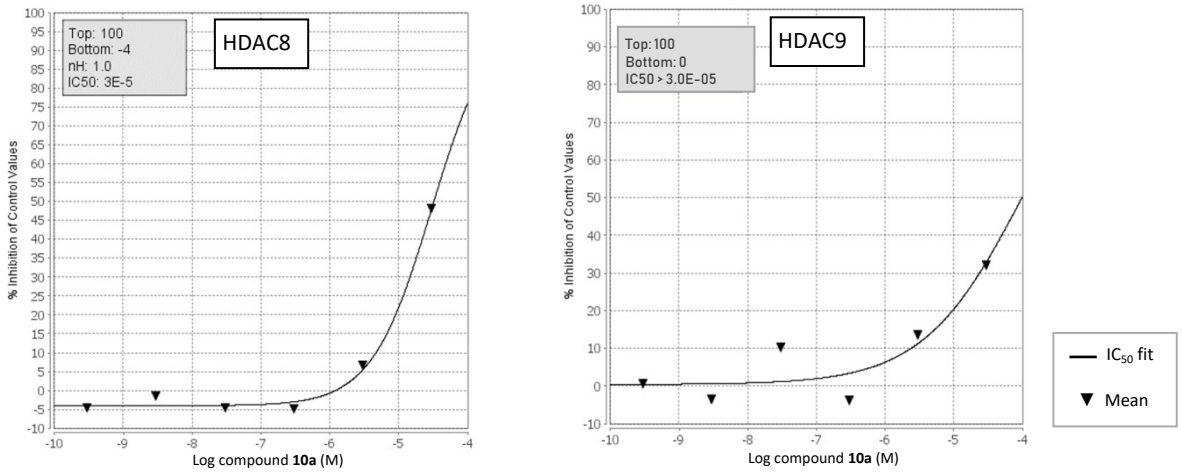


*Figure 6 Determination of IC_{50} of compound **10a** on HDAC1-5 and HDAC7-9.*

Table 2 Results of IC_{50} and Hill coefficient (n_H) determination of reference compound Trichostatin A on HDAC1-5 and HDAC7-9.

Enzyme	IC_{50} (nM)	n_H
HDAC1	3.5	1.5
HDAC2	22	1.2
HDAC3	10	1.5
HDAC4	3200	1.0
HDAC5	1800	1.0
HDAC7	2400	1.3
HDAC8	930	2.0
HDAC9	3200	1.3

Evaluation of compound 10a in a genetic toxicity assay (performed by Eurofins)

Compound SG008c was tested in the Ames fluctuation test (detection method: photometry) against four strains of Salmonella typhimurium (TA98, TA100, TA1535, and TA1537), with and without metabolic activation by rat liver S9 (denoted as '+ S9' and '- S9', respectively). Incubation took place for 96 hours at 37°C. Wells that displayed bacteria growth due to the reversion of the histidine mutation (as judged by the ratio of OD_{430}/OD_{570} being greater than 1.0) are counted and recorded as positive counts. The significance of the positive counts between the treatment (in the presence of test compound) and the control (in the absence of test compound) are calculated using the one-tailed Fisher's exact test. Three significance levels are reported as follows:

- Weak positive, if $0.01 \leq p < 0.05$, denoted as "+"
- Strong positive, if $0.001 \leq p < 0.01$, denoted as "++"
- Very strong positive, if $p < 0.001$, denoted as "+++"

No positive effects could be detected at the concentrations tested (5, 10, 50 and 100 µM), indicating that compound SG008c is not mutagenic toward the strains tested.

A bacterial cytotoxicity assay was conducted in parallel to rule out false negative Ames results. Reverted Salmonella typhimurium strains TA98, TA100, TA1535 and TA1537 were incubated for 96 hour at 37°C and optical density was measured via photometry. The results for cytotoxicity are expressed as percent of control growth (OD_{650}). A cytotoxicity value of less than 60 % of control growth is flagged, and the compound is considered as toxic at the respective concentration. Compound SG008c showed no cytotoxicity at the concentrations tested (0.6, 1.2, 2.5, 10, 25, 50 and 100 µM).

Table 3 Results of the Ames fluctuation test against four strains of Salmonella typhimurium (TA98, TA100, TA1535, and TA1537), with and without metabolic activation by rat liver S9. Wells that displayed bacteria growth due to the reversion of the histidine mutation are counted and recorded as positive counts.

ASSAY NAME	COMPOUND CONCENTRATION	MEASUREMENT	VALUE	Flag
Ames fluctuation test (TA100 - S9)	5.0E-06	Count (# of wells)	1	
Ames fluctuation test (TA100 - S9)	1.0E-05	Count (# of wells)	3	
Ames fluctuation test (TA100 - S9)	5.0E-05	Count (# of wells)	0	
Ames fluctuation test (TA100 - S9)	1.0E-04	Count (# of wells)	1	
Ames fluctuation test (TA100 - S9)	5.0E-06	Count flag		-
Ames fluctuation test (TA100 - S9)	5.0E-05	Count flag		-
Ames fluctuation test (TA100 - S9)	1.0E-04	Count flag		-
Ames fluctuation test (TA100 - S9)	5.0E-06	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA100 - S9)	1.0E-05	Fisher Exact Test (p-value)	0.30849	
Ames fluctuation test (TA100 - S9)	5.0E-05	Fisher Exact Test (p-value)	0.5	
Ames fluctuation test (TA100 - S9)	1.0E-04	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA100 - S9)	5.0E-06	Positive Significance (- to +++)		-
Ames fluctuation test (TA100 - S9)	1.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA100 - S9)	5.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA100 - S9)	1.0E-04	Positive Significance (- to +++)		-
Ames fluctuation test (TA100 + S9)	5.0E-06	Count (# of wells)	1	
Ames fluctuation test (TA100 + S9)	1.0E-05	Count (# of wells)	0	
Ames fluctuation test (TA100 + S9)	5.0E-05	Count (# of wells)	4	
Ames fluctuation test (TA100 + S9)	1.0E-04	Count (# of wells)	0	
Ames fluctuation test (TA100 + S9)	5.0E-06	Count flag		<
Ames fluctuation test (TA100 + S9)	1.0E-05	Count flag		<<
Ames fluctuation test (TA100 + S9)	5.0E-05	Count flag		-
Ames fluctuation test (TA100 + S9)	1.0E-04	Count flag		<<
Ames fluctuation test (TA100 + S9)	5.0E-06	Fisher Exact Test (p-value)	0.01526	
Ames fluctuation test (TA100 + S9)	1.0E-05	Fisher Exact Test (p-value)	0.00285	
Ames fluctuation test (TA100 + S9)	5.0E-05	Fisher Exact Test (p-value)	0.17764	
Ames fluctuation test (TA100 + S9)	1.0E-04	Fisher Exact Test (p-	0.00285	

		value)		
Ames fluctuation test (TA100 + S9)	5.0E-06	Positive Significance (- to +++)		-
Ames fluctuation test (TA100 + S9)	1.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA100 + S9)	5.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA100 + S9)	1.0E-04	Positive Significance (- to +++)		-
Ames fluctuation test (TA1535 - S9)	5.0E-06	Count (# of wells)	0	
Ames fluctuation test (TA1535 - S9)	1.0E-05	Count (# of wells)	0	
Ames fluctuation test (TA1535 - S9)	5.0E-05	Count (# of wells)	0	
Ames fluctuation test (TA1535 - S9)	1.0E-04	Count (# of wells)	0	
Ames fluctuation test (TA1535 - S9)	5.0E-06	Count flag		-
Ames fluctuation test (TA1535 - S9)	1.0E-05	Count flag		-
Ames fluctuation test (TA1535 - S9)	5.0E-05	Count flag		-
Ames fluctuation test (TA1535 - S9)	1.0E-04	Count flag		-
Ames fluctuation test (TA1535 - S9)	5.0E-06	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA1535 - S9)	1.0E-05	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA1535 - S9)	5.0E-05	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA1535 - S9)	1.0E-04	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA1535 - S9)	5.0E-06	Positive Significance (- to +++)		-
Ames fluctuation test (TA1535 - S9)	1.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA1535 - S9)	5.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA1535 - S9)	1.0E-04	Positive Significance (- to +++)		-
Ames fluctuation test (TA1535 + S9)	5.0E-06	Count (# of wells)	0	
Ames fluctuation test (TA1535 + S9)	1.0E-05	Count (# of wells)	0	
Ames fluctuation test (TA1535 + S9)	5.0E-05	Count (# of wells)	0	
Ames fluctuation test (TA1535 + S9)	1.0E-04	Count (# of wells)	0	
Ames fluctuation test (TA1535 + S9)	5.0E-06	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA1535 + S9)	1.0E-05	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA1535 + S9)	5.0E-05	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA1535 + S9)	1.0E-04	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA1535 + S9)	5.0E-06	Positive Significance (- to +++)		-
Ames fluctuation test (TA1535 + S9)	1.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA1535 + S9)	5.0E-05	Positive Significance		-

		(- to +++)		
Ames fluctuation test (TA1535 + S9)	1.0E-04	Positive Significance (- to +++)		-
Ames fluctuation test (TA1537 + S9)	5.0E-06	Count (# of wells)	0	
Ames fluctuation test (TA1537 + S9)	1.0E-05	Count (# of wells)	0	
Ames fluctuation test (TA1537 + S9)	5.0E-05	Count (# of wells)	0	
Ames fluctuation test (TA1537 + S9)	1.0E-04	Count (# of wells)	2	
Ames fluctuation test (TA1537 + S9)	5.0E-06	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA1537 + S9)	1.0E-05	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA1537 + S9)	5.0E-05	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA1537 + S9)	1.0E-04	Fisher Exact Test (p-value)	0.24737	
Ames fluctuation test (TA1537 + S9)	5.0E-06	Positive Significance (- to +++)		-
Ames fluctuation test (TA1537 + S9)	1.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA1537 + S9)	5.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA1537 + S9)	1.0E-04	Positive Significance (- to +++)		-
Ames fluctuation test (TA1537-S9)	5.0E-06	Count (# of wells)	1	
Ames fluctuation test (TA1537-S9)	1.0E-05	Count (# of wells)	0	
Ames fluctuation test (TA1537-S9)	5.0E-05	Count (# of wells)	0	
Ames fluctuation test (TA1537-S9)	1.0E-04	Count (# of wells)	1	
Ames fluctuation test (TA1537-S9)	5.0E-06	Fisher Exact Test (p-value)	0.5	
Ames fluctuation test (TA1537-S9)	1.0E-05	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA1537-S9)	5.0E-05	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA1537-S9)	1.0E-04	Fisher Exact Test (p-value)	0.5	
Ames fluctuation test (TA1537-S9)	5.0E-06	Positive Significance (- to +++)		-
Ames fluctuation test (TA1537-S9)	1.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA1537-S9)	5.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA1537-S9)	1.0E-04	Positive Significance (- to +++)		-
Ames fluctuation test (TA98 - S9)	5.0E-06	Count (# of wells)	0	
Ames fluctuation test (TA98 - S9)	1.0E-05	Count (# of wells)	0	
Ames fluctuation test (TA98 - S9)	5.0E-05	Count (# of wells)	0	
Ames fluctuation test (TA98 - S9)	1.0E-04	Count (# of wells)	0	
Ames fluctuation test (TA98 - S9)	5.0E-06	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA98 - S9)	1.0E-05	Fisher Exact Test (p-	1	

		value)		
Ames fluctuation test (TA98 - S9)	5.0E-05	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA98 - S9)	1.0E-04	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA98 - S9)	5.0E-06	Positive Significance (- to +++)		-
Ames fluctuation test (TA98 - S9)	1.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA98 - S9)	5.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA98 - S9)	1.0E-04	Positive Significance (- to +++)		-
Ames fluctuation test (TA98 + S9)	5.0E-06	Count (# of wells)	1	
Ames fluctuation test (TA98 + S9)	1.0E-05	Count (# of wells)	1	
Ames fluctuation test (TA98 + S9)	5.0E-05	Count (# of wells)	0	
Ames fluctuation test (TA98 + S9)	1.0E-04	Count (# of wells)	0	
Ames fluctuation test (TA98 + S9)	5.0E-06	Fisher Exact Test (p-value)	0.5	
Ames fluctuation test (TA98 + S9)	1.0E-05	Fisher Exact Test (p-value)	0.5	
Ames fluctuation test (TA98 + S9)	5.0E-05	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA98 + S9)	1.0E-04	Fisher Exact Test (p-value)	1	
Ames fluctuation test (TA98 + S9)	5.0E-06	Positive Significance (- to +++)		-
Ames fluctuation test (TA98 + S9)	1.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA98 + S9)	5.0E-05	Positive Significance (- to +++)		-
Ames fluctuation test (TA98 + S9)	1.0E-04	Positive Significance (- to +++)		-

Notes:
1. Weak positive, if $p < 0.05$, denoted as "+"
Strong positive, if $p < 0.01$, denoted as "++"
Very strong positive, if $p < 0.001$, denoted as "+++"
2. When possible, compounds which score significantly below background are flagged.
This may indicate low level cytotoxicity undetectable by the growth assay.
The compounds are flagged as described below.
if $p < 0.05$, flagged as "<"
if $p < 0.01$, flagged as "<<"
if $p < 0.001$, flagged as "<<<"
3. Hyphens (-) indicate negative results.

*Table 4 Bacterial cytotoxicity results of compound **10a** and reference compound Mitomycin C for four Salmonella typhimurium strains.*

Compound	Concentration	Effect			Mean Effect
		1	2	3	
TA98					
Mitomycin C (p1) T4	0.009 µM	83.9	82.8	81.6	82.8
Mitomycin C (p1) T4	0.0188 µM	62.3	66.9	68.0	65.7
Mitomycin C (p1) T4	0.0375 µM	41.9	38.4	41.9	40.7
Mitomycin C (p1) T4	0.075 µM	48.7	46.4	40.7	45.3
Mitomycin C (p1) T4	0.15 µM	4.4	25.9	(75.9)	15.2
Mitomycin C (p1) T4	0.3 µM	3.2	4.4	3.2	3.6
Mitomycin C (p1) T4	0.6 µM	7.8	4.4	4.4	5.5
Mitomycin C (p1) T4	1.2 µM	5.5	6.6	6.6	6.3
10a	0.63 µM	100.9	96.4	97.5	98.3
10a	1.25 µM	94.1	94.1	94.1	94.1
10a	2.5 µM	89.6	93.0	88.4	90.3
10a	5.0 µM	90.7	93.0	90.7	91.5
10a	10.0 µM	86.2	89.6	88.4	88.1
10a	25.0 µM	103.2	96.4	95.3	98.3
10a	50.0 µM	95.3	75.9	65.7	79.0
10a	100.0 µM	111.2	83.9	108.9	101.3
TA100					
Mitomycin C (p2) T4	0.009 µM	80.1	90.6	68.1	79.6
Mitomycin C (p2) T4	0.0188 µM	54.5	44.0	38.0	45.5
Mitomycin C (p2) T4	0.0375 µM	51.5	50.0	50.0	50.5
Mitomycin C (p2) T4	0.075 µM	33.5	9.5	15.5	19.5
Mitomycin C (p2) T4	0.15 µM	5.0	5.0	3.5	4.5
Mitomycin C (p2) T4	0.3 µM	5.0	3.5	3.5	4.0
Mitomycin C (p2) T4	0.6 µM	8.0	6.5	5.0	6.5
Mitomycin C (p2) T4	1.2 µM	6.5	5.0	6.5	6.0
10a	0.63 µM	90.6	113.1	93.6	99.1
10a	1.25 µM	101.1	104.1	96.6	100.6
10a	2.5 µM	102.6	96.6	87.6	95.6

10a	5.0 µM	105.6	93.6	99.6	99.6
10a	10.0 µM	101.1	104.1	95.1	100.1
10a	25.0 µM	93.6	(141.6)	93.6	93.6
10a	50.0 µM	132.6	144.6	(86.1)	138.6
10a	100.0 µM	110.1	(186.7)	86.1	98.1
		TA1535			
Mitomycin C (p3) T4	0.009 µM	138.8	131.2	131.2	133.8
Mitomycin C (p3) T4	0.0188 µM	104.8	112.4	112.4	109.9
Mitomycin C (p3) T4	0.0375 µM	72.1	82.2	77.1	77.1
Mitomycin C (p3) T4	0.075 µM	47.0	48.2	49.5	48.2
Mitomycin C (p3) T4	0.15 µM	10.5	24.3	33.1	22.6
Mitomycin C (p3) T4	0.3 µM	9.2	10.5	9.2	9.6
Mitomycin C (p3) T4	0.6 µM	10.5	10.5	9.2	10.1
Mitomycin C (p3) T4	1.2 µM	9.2	10.5	10.5	10.1
10a	0.63 µM	103.6	102.3	107.3	104.4
10a	1.25 µM	107.3	108.6	104.8	106.9
10a	2.5 µM	106.1	111.1	99.8	105.7
10a	5.0 µM	98.5	(214.3)	111.1	104.8
10a	10.0 µM	91.0	92.2	103.6	95.6
10a	25.0 µM	99.8	122.4	131.2	117.8
10a	50.0 µM	(72.1)	135.0	138.8	136.9
10a	100.0 µM	89.7	84.7	(160.2)	87.2
		TA16537			
Mitomycin C (p4) T4	0.009 µM	116.2	130.5	122.7	123.1
Mitomycin C (p4) T4	0.0188 µM	107.1	103.2	95.4	101.9
Mitomycin C (p4) T4	0.0375 µM	53.8	86.3	87.6	75.9
Mitomycin C (p4) T4	0.075 µM	43.4	47.3	44.7	45.1
Mitomycin C (p4) T4	0.15 µM	9.5	42.1	43.4	31.6
Mitomycin C (p4) T4	0.3 µM	3.0	4.3	-7.4	0.0
Mitomycin C (p4) T4	0.6 µM	4.3	5.6	3.0	4.3
Mitomycin C (p4) T4	1.2 µM	5.6	4.3	6.9	5.6
10a	0.63 µM	101.9	101.9	96.7	100.1

10a	1.25 µM	99.3	101.9	109.7	103.6
10a	2.5 µM	94.1	98.0	100.6	97.5
10a	5.0 µM	96.7	96.7	117.5	103.6
10a	10.0 µM	86.3	90.2	81.1	85.8
10a	25.0 µM	96.7	88.9	78.5	88.0
10a	50.0 µM	66.8	100.6	83.7	83.7
10a	100.0 µM	103.2	70.7	104.5	92.8

Notes:
1. Cytotoxicity is presented as % of control growth (OD_{650}).
2. A cytotoxicity value of less than 60 % is flagged, and the compound is considered as toxic at the respective concentration.

Table 5 Background results Ames fluctuation test.

Ames fluctuation test	Count (number of wells)
T98 - S9	0
T98 + S9	0
TA100 - S9	2
TA100 + S9	9
TA1535 - S9	1
TA1535 + S9	0
TA1537 - S9	0
TA1537 + S9	0

Table 6 Reference compound results in Ames fluctuation assay.

Compound	Test concentration (M)	Count (number of wells)	Positive significance (- to +++)	Fisher exact Test (p-value)	Count flag
		TA98 - S9			
2-Aminoanthracene	1.0E-05	0	-	1.0000	
9-Aminoacridine	1.0E-05	0	-	1.0000	
Quercetin	3.0E-05	10	+++	0.0006	
Streptozotocin	2.5E-06	0	-	1.0000	
		TA98 + S9			
2-Aminoanthracene	1.0E-05	48	+++	0.0000	
9-Aminoacridine	1.0E-05	1	-	0.5000	
Quercetin	3.0E-05	44	+++	0.0000	
Streptozotocin	2.5E-06	0	-	1.0000	
		TA100 - S9			
2-Aminoanthracene	1.0E-05	2	-	0.5000	
9-Aminoacridine	1.0E-05	0	-	0.5000	-
Quercetin	3.0E-05	0	-	0.5000	-
Streptozotocin	2.5E-06	24	+++	0.0000	
		TA100 + S9			
2-Aminoanthracene	6.0E-06	23	+++	0.0010	
9-Aminoacridine	1.0E-05	1	-	0.0153	<
Quercetin	3.0E-05	10	-	0.3972	
Streptozotocin	2.5E-06	40	+++	0.0000	

		TA1535 - S9			
2-Aminoanthracene	1.0E-05	0	-	1.0000	-
9-Aminoacridine	1.0E-05	1	-	0.5000	
Quercetin	3.0E-05	0	-	1.0000	-
Streptozotocin	2.5E-06	43	+++	0.0000	
		TA1535 + S9			
2-Aminoanthracene	2.0E-06	10	+++	0.0006	
9-Aminoacridine	1.0E-05	1	-	0.5000	
Quercetin	3.0E-05	1	-	0.5000	
Streptozotocin	2.5E-06	48	+++	0.0000	
		TA1537 - S9			
2-Aminoanthracene	1.0E-05	1	-	0.5000	
9-Aminoacridine	1.0E-05	44	+++	0.0000	
Quercetin	3.0E-05	3	-	0.1211	
Streptozotocin	2.5E-06	7	++	0.0062	
		TA1537 + S9			
2-Aminoanthracene	2.0E-06	21	+++	0.0000	
9-Aminoacridine	1.0E-05	6	+	0.0132	
Quercetin	3.0E-05	1	-	0.5000	
Streptozotocin	2.5E-06	7	++	0.0062	

Notes:
1. Weak positive, if $p < 0.05$, denoted as "+"
Strong positive, if $p < 0.01$, denoted as "++"
Very strong positive, if $p < 0.001$, denoted as "+++"
2. When possible, compounds which score significantly below background are flagged.
This may indicate low level cytotoxicity undetectable by the growth assay.
The compounds are flagged as described below.
if $p < 0.05$, flagged as "<"
if $p < 0.01$, flagged as "<<"
if $p < 0.001$, flagged as "<<<"
3. Hyphens (-) indicate negative results.

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