Oxa Analogues of Nexturastat A Demonstrate Improved HDAC6 Selectivity and Superior Antileukaemia Activity

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1 General Information
Reaction, monitoring and purification

Chemicals and solvents were purchased from commercial suppliers (Sigma-Aldrich, Acros Organics, TCI, Fluorochem ABCR, Alfa Aesar, J&K, Carbolution) and used without further purification. Dry solvents were obtained from Acros Organics. Ambient or room temperature correspond to 22°C. The reaction progression was monitored using Thin-Layer-Chromatography plates by Macherey Nagel (ALUGRAM Xtra SIL G/UV254). Visualisation was achieved with ultraviolet irradiation (254 nm) or by staining with a KMnO₄-solution (9 g KMnO₄, 60 g K₂CO₃, 15 mL of a 5% aqueous NaOH-solution, ad 900 mL demineralised water). Purification was either performed with prepacked Silica cartridges (RediSep® Rf Normal Phase Silica, RediSep® Rf RP C18) for flash column chromatography (CombiFlashRf200, TeleDynelsoo) or by recrystallisation. Different eluent mixtures of solvents (n-hexane and ethyl acetate or dichloromethane and methanol) served as the mobile phase for flash column chromatography and are stated in the experimental procedure.

Analytics

An NMR-Spectrometer by Bruker (Bruker Avance III – 300, Bruker Avance DRX – 500 or Bruker Avance III - 600) were used to perform ¹H- and ¹³C-NMR experiments. Chemical shifts are given in parts per million (ppm), relative to residual non-deuterated solvent peak (¹H-NMR: DMSO-d₆ (2.50), ¹³C-NMR: DMSO-d₆ (39.52)). Signal patterns are indicated as: singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m). Coupling constants, J, are quoted to the nearest 0.1 Hz and are presented as observed. ESI-MS was carried out using Bruker Daltonics UHR-QTOF maXis 4G (Bruker Daltonics) under electrospray ionization (ESI). The above-mentioned characterisations were carried out by the HHU Center of Molecular and Structural Analytics at Heinrich-Heine University Düsseldorf (http://www.chemie.hhu.de/en-analytics-center-hhucemsia.html). APCI-MS was carried out with an Advion expression¹-CMS. Melting points were determined using a Büchi M-565 melting point apparatus (uncorrected). Analytical HPLC was carried out on a Knauer HPLC system comprising of an Azura P6.1L pump, an Optimas 800 autosampler, a Fast Scanning Spectro-Photometer K-2600 and a Knauer Reversed Phase column (SN: FK36). Evaluated compounds were detected at 254 nm. The solvent gradient table is shown in Table 1. The purity of all final compounds was 95% or higher.
Table 1: The solvent gradient table for analytic HPLC analysis.

| Time / min | Water + 0.1% TFA | ACN + 0.1% TFA |
|------------|------------------|-----------------|
| Initial    | 90               | 10              |
| 0.50       | 90               | 10              |
| 20.0       | 0                | 100             |
| 30.0       | 0                | 100             |
| 31.0       | 90               | 10              |
| 40.0       | 90               | 10              |
2 Synthetic procedures

2.1 Synthesis of tert-butyl propoxycarbamate (1a)

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{H} & \quad \text{H} \\
\text{EtOH} & \quad \text{EtOH} \\
1.00 \text{ eq NaOH} & \quad 1.00 \text{ eq 1-bromopropane} \\
1.00 \text{ eq } & \quad 1.00 \text{ eq } \\
\end{align*}
\]

1.00 eq (75.1 mmol, 10 g) of tert-butyl N-hydroxycarbamate was combined with 1.00 eq (75.1 mmol, 3 g) of NaOH and 1.00 eq (75.1 eq, 9.24 g, 6.82 mL) of 1-bromopropane in 250 mL of ethanol. After 6 h at 70 °C whilst stirring, the solvent was removed under reduced pressure and the product purified by flash column chromatography (n-hexane/EtOAc). 7.42 g (42.3 mmol, 56 %) of 1a was obtained as colourless oil.

\(^1\text{H} \text{ NMR} \) (300 MHz, DMSO-\(d_6\)) \(\delta\) 9.89 (s, 1H), 3.63 (t, \(J = 6.5\) Hz, 2H), 1.64 – 1.43 (m, 2H), 1.40 (s, 8H), 0.87 (t, \(J = 7.4\) Hz, 3H).

\(^{13}\text{C} \text{ NMR} \) (75 MHz, DMSO-\(d_6\)) \(\delta\) 156.1, 79.4, 76.8, 28.1, 20.9, 10.4.

\(\text{MS (+APCI): 176 [M+H]}^+\)

2.2 Synthesis of tert-butyl (benzyloxy)carbamate (1b)[1]

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{H} & \quad \text{H} \\
\text{DCM/H}_2\text{O} & \quad \text{DCM/H}_2\text{O} \\
2.00 \text{ eq NaHCO}_3 & \quad 1.20 \text{ eq Boc}_2\text{O} \\
1.20 \text{ eq } & \quad 1.20 \text{ eq } \\
\end{align*}
\]

1.00 eq (70.0 mmol, 11.2 g) O-benzylhydroxylamine was dissolved in 80 mL of a mixture of DCM and dH\(_2\)O (1:1). After the reaction was cooled on ice, 2.00 eq (140 mmol, 11.8 g) of NaHCO\(_3\) and 1.20 eq (84.0 mmol, 18.3 g) of Boc\(_2\)O was added and stirred for 3 h at ambient temperature. The reaction mixture was diluted with 40 mL of sat. aq. NaHCO\(_3\) solution and the product extracted with DCM. The combined org. layers were dried over Na\(_2\)SO\(_4\) and the solvent removed under reduced pressure. After crystallisation from \(n\)-hexane, 5.83 g (26.1 mmol, 37 %) of 1b was obtained as colourless crystals.

All spectroscopic data agreed with literature.[1]
2.3 General procedure for the synthesis of methyl 4-((alkoxyamino)methyl)benzoate hydrochloride (2)

1.00 eq of tert-butyl alkoxy carbamate was dissolved in 4 mL/mmol of THF and cooled to 0°C. After 30°C whilst stirring, 1.10 eq of methyl 4-(bromomethyl)benzoate was added and stirred for 16 h to RT. The solvent was removed under reduced pressure and the precipitate resuspended in 50 mL of EtOAc. Subsequently, the organic layer was washed with dH$_2$O (3x), brine (1x) and dried over Na$_2$SO$_4$. After the solvent was removed under reduced pressure, the product was dissolved in 10 mL/mmol DCM and 5.00 eq of 5 M HCl in dioxane added. The product was collected by filtration, washed with DCM (3x).

2.3.1 Synthesis of methyl 4-((propoxyamino)methyl)benzoate hydrochloride (2a)

2a was synthesised from 7.00 g (39.9 mmol) of 1a, 10.1 g (43.9 mmol) of methyl 4-(bromomethyl)benzoate and 1.92 g (47.9 mmol) of NaH (60% mineral oil) in 160 mL of THF according to general procedure 2.9. 9.49 g (36.6 mmol, 92%) of 2a was obtained as a colourless solid over two steps.

$^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ 8.03 – 7.92 (m, 2H), 7.78 – 7.66 (m, 2H), 4.46 (s, 2H), 4.05 (t, $J$ = 6.5 Hz, 2H), 3.86 (s, 3H), 1.55 (dt, $J$ = 13.8, 7.3, 6.4 Hz, 2H), 0.82 (t, $J$ = 7.4 Hz, 3H).

$^{13}$C NMR (75 MHz, DMSO-d$_6$) $\delta$ 165.8, 135.9, 130.8, 129.9, 129.0, 74.3, 52.2, 51.2, 20.7, 10.0.

M.p.: 227.8 °C, HPLC: $R_t$=7.95, purity ≥ 99 %, MS (+APCI): 224 [M+H]$^+$
2.3.2 Synthesis of methyl 4-(((benzylxoy)amino)methyl)benzoate hydrochloride (2b)

\[ \text{2b was synthesised from 7.50 g (51.0 mmol) of 1b, 8.46 g (37.0 mmol) of methyl 4-(bromomethyl)benzoate and 1.61 g (40.3 mmol) of NaH (60\%, mineral oil) in 134 mL of THF according to general procedure 2.3. 9.46 g (30.7 mmol, 91\%) of 2b was obtained as a colourless solid over two steps.} \]

\[ \text{^1H NMR (300 MHz, DMSO-d}_6\text{)}\ \delta 8.05 - 7.89 (m, 2H), 7.79 - 7.60 (m, 2H), 7.37 (h, J = 1.2 Hz, 5H), 5.13 (s, 2H), 4.51 (s, 2H), 3.86 (s, 3H).} \]

\[ \text{^13C NMR (75 MHz, DMSO-d}_6\text{)}\ \delta 166.2, 144.7, 138.2, 128.9, 128.8, 128.1, 128.1, 128.1, 127.4, 75.0, 54.9, 52.0.}\]

\[ \text{M.p.: 194.2 °C, HPLC: R}_t=11.3, \text{ purity } = 95.0 \%, \text{ MS (+APCI): 272 [M+H]^+}} \]

2.4 General procedure for the synthesis of alkoxyurea derivatives (3)

1.00 eq of methyl 4-((alkoxyamino)methyl)benzoate hydrochloride was suspended in 25 mL/mmol of DCM. Subsequently, 1.00 eq of DIPEA and 1.00 eq of the respective isocyanate were added and stirred for 16 h at ambient temperature. The solvent was removed under reduced pressure, the residue resuspended in EtOAc and washed with dH2O (3x) and brine (1x). After drying over Na2SO4, the crude product was purified by flash column chromatography (n-hexane/EtOAc).
2.4.1 Synthesis of methyl 4-((3-phenyl-1-propoxyureido)methyl)benzoate (3a)

3a was synthesised from 200 mg (0.779 mmol) of 2a, 92.8 mg (0.779 mmol) of phenyl isocyanate in 19.4 mL of DCM. The product was obtained as a colourless solid with a yield of 73 % (0.570 mmol, 195 mg).

$^1$H NMR (600 MHz, DMSO- $d_6$) δ 8.88 (s, 1H), 8.00 – 7.87 (m, 2H), 7.63 – 7.53 (m, 2H), 7.52 – 7.42 (m, 2H), 7.33 – 7.20 (m, 2H), 7.02 (tt, $J$ = 7.4, 1.2 Hz, 1H), 4.77 (s, 2H), 3.84 (s, 3H), 3.82 (t, $J$ = 6.8 Hz, 2H), 1.63 (h, $J$ = 7.3 Hz, 2H), 0.86 (t, $J$ = 7.4 Hz, 3H).

$^{13}$C NMR (151 MHz, DMSO- $d_6$) δ 166.5, 157.0, 143.4, 139.3, 129.7, 129.1, 128.9, 123.4, 120.6, 76.3, 52.6, 51.2, 21.2, 10.7.m.p.: °C; HPLC: $R_t$=17.37 min, purity ≥ 99 %, ESI-MS: .

M.p.: 55.8°C; HPLC: $R_t$=15.53 min, purity = 95.2 %, MS (+APCI): 343 [M+H]$^+$

2.4.2 Synthesis of methyl 4-((3-(3,5-dimethylphenyl)-1-propoxyureido)methyl)benzoate (3b)

3b was synthesised from 200 mg (0.776 mmol) of 2a, 114 mg (0.776 mmol) of 3,5-dimethylphenyl isocyanate in 19.0 mL of DCM. The product was obtained as a colourless solid with a yield of 73 % (0.565 mmol, 209 mg).

$^1$H NMR (300 MHz, DMSO- $d_6$) δ 8.70 (s, 1H), 8.02 – 7.84 (m, 2H), 7.55 – 7.40 (m, 2H), 7.29 – 7.14 (m, 2H), 6.66 (tt, $J$ = 1.6, 0.8 Hz, 1H), 4.75 (s, 2H), 3.84 (s, 3H), 3.80 (t, $J$ = 6.8 Hz, 2H), 2.29 – 2.17 (m, 6H), 1.63 (h, $J$ = 7.2 Hz, 2H), 0.85 (t, $J$ = 7.4 Hz, 3H).

$^{13}$C NMR (75 MHz, DMSO- $d_6$) δ 166.0, 156.5, 143.0, 138.6, 137.3, 129.2, 128.7, 128.6, 124.4, 117.8, 75.8, 52.1, 50.8, 21.0, 20.7, 10.2.

M.p.: 99.3 °C; HPLC: $R_t$=17.37 min, purity ≥ 99 %, MS (+APCI): 371 [M+H]$^+$
2.4.3 Synthesis of methyl 4-((3-(4-(dimethylamino)phenyl)-1-propoxyureido)methyl)benzoate (3c)

1.00 eq (0.963 mmol, 250 mg) of 2a was combined with 1.00 eq of (0.963 mmol, 131 mg) N,N-dimethyl-p-phenylenediamine, 1.00 eq (0.963, 200 mg) of 4-nitrophenyl chloroformate and 2.00 eq (1.93 mmol, 250 mg, 0.337 mL) of DIPEA in 20 mL of ACN. After the reaction was refluxed for 16 h, the solvent was removed, and the precipitate resuspended in EtOAc. The resulting solution was washed with dH2O (3x) and brine (1x) and dried over Na2SO4. 3c was obtained as yellow solid after recrystallisation from n-hexane/EtOAc with a yield of 75 % (0.718 mmol, 277 mg).

1H NMR (600 MHz, DMSO-d6) δ 8.63 (s, 1H), 8.07 – 7.89 (m, 2H), 7.58 – 7.42 (m, 2H), 7.42 – 7.30 (m, 2H), 6.82 – 6.47 (m, 2H), 4.74 (s, 2H), 3.85 (s, 3H), 3.79 (t, J = 6.8 Hz, 2H), 2.84 (s, 6H), 1.62 (p, J = 7.2 Hz, 2H), 0.85 (t, J = 7.4 Hz, 3H).

13C NMR (151 MHz, DMSO-d6) δ 166.5, 157.5, 147.6, 143.6, 129.6, 129.2, 129.0, 128.8, 122.6, 113.0, 76.3, 52.5, 51.6, 41.0, 21.2, 10.7.

M.p.: 86.7 °C; HPLC: R_t=10.08 min, purity ≥ 99 %, MS (+APCI): 386 [M+H]^+

2.4.4 Synthesis of methyl 4-((1-(benzyloxy)-3-(3,5-dimethylphenyl)ureido)methyl)benzoate (3d)

3d was synthesised from 158 mg (0.583 mmol) of 2b, 85.5 mg (0.583 mmol) of 3,5-dimethylphenyl Isocyanate in 15 mL of DCM. The product was obtained as a colourless solid with a yield of 85 % (0.495 mmol, 207 mg).

1H NMR (300 MHz, DMSO-d6) δ 8.69 (s, 1H), 7.98 – 7.86 (m, 2H), 7.52 – 7.27 (m, 7H), 7.13 (dd, J = 1.6, 0.9 Hz, 2H), 6.65 (tt, J = 1.6, 0.8 Hz, 1H), 4.90 (s, 2H), 4.72 (s, 2H), 3.83 (s, 3H), 2.25 – 2.17 (m, 6H).

13C NMR (75 MHz, DMSO-d6) δ 166.0, 156.4, 142.8, 138.5, 137.3, 135.5, 129.6, 129.2, 128.7, 128.6, 128.5, 128.3, 124.4, 117.7, 76.0, 52.1, 50.9, 21.0.

M.p.: 116.9 °C; HPLC: R_t=17.68 , purity = 95.8 %, MS (+APCI): 419 [M+H]^+
2.5 General procedure for the synthesis of alkoxyurea based hydroxamic acids (4)

\[
\text{R}^1\text{N}-\text{O}-\text{N}^{+}\text{H} - \text{O}-\text{Ar} \\
\text{DCM/MeOH (2:1)} \\
\text{10.0 eq NaOH} \\
\text{30.0 eq H}_2\text{NOH}_{(aq)}
\]

1.00 eq of 3 was dissolved in 30 mL of DCM/MeOH (2:1) and cooled on ice. Afterwards, 30.0 eq of H\textsubscript{2}NOH\textsubscript{(aq)} and 10.0 eq of freshly grinded NaOH was added to the reaction solution and stirred for 16 h to ambient temperature. The reaction was neutralised with a 1 M HCl solution and the product extracted with EtOAc. After the combined organic layers were dried over Na\textsubscript{2}SO\textsubscript{4}, the product was purified by flash column chromatography (DCM/30 % MeOH in DCM).

2.5.1 Synthesis of \(N\)-hydroxy-4-((3-phenyl-1-propoxyureido)methyl)benzamide (4a)

\[
\text{N} - \text{O} - \text{N}^{+}\text{H} - \text{O}-\text{Ar} \\
\text{DCM/MeOH (2:1)} \\
\text{30.0 eq H}_2\text{NOH}_{(aq)} \\
\text{10.0 eq NaOH}
\]

4a was synthesised according to general procedure 2.5 on a 0.538 mmol scale and obtained as a white solid with a yield of 67 % (0.390 mmol, 145 mg).

\(^1\text{H NMR} (300 MHz, DMSO-d_6) \delta 11.16 (s, 1H), 9.00 (s, 1H), 8.86 (s, 1H), 7.77 – 7.66 (m, 2H), 7.61 – 7.52 (m, 2H), 7.42 – 7.34 (m, 2H), 7.32 – 7.23 (m, 2H), 7.02 (ddt, \(J = 7.7, 6.9, 1.2 \text{ Hz}, 1H\)), 4.73 (s, 2H), 3.82 (t, \(J = 6.8 \text{ Hz}, 2H\)), 1.64 (h, \(J = 7.2 \text{ Hz}, 2H\)), 0.87 (t, \(J = 7.4 \text{ Hz}, 3H\)).

\(^{13}\text{C NMR} (151 MHz, DMSO-d_6) \delta 164.5, 157.0, 140.9, 139.3, 132.3, 128.9, 128.8, 127.3, 123.3, 120.5, 76.2, 51.1, 21.2, 10.7.

\text{M.p.:} 151.4 °C; \text{HPLC:} \(R_t=14.48 \text{ min, purity} \geq 99 \%. \text{HRMS (+ESI):} \text{calc. for } C_{18}H_{22}N_3O_4 344.1605 [M+H]^+, \text{found 344.1605. EA calc. for } C_{18}H_{22}N_3O_4 \text{ C 62.96, H 6.16, N 12.24, found C 63.18, H 6.13, N 12.19.}
2.5.2 Synthesis of N-hydroxy-4-((3-(3,5-dimethylphenyl)-1-propoxyureido)methyl)benzamide (4b)

4b was synthesised according to general procedure 2.5 on a 0.776 mmol scale and obtained as a white solid with a yield of 76 % (0.587 mmol, 218 mg).

$^1$H NMR (300 MHz, DMSO-$d_6$) δ 11.16 (s, 1H), 9.01 (s, 1H), 8.67 (s, 1H), 7.77 – 7.62 (m, 2H), 7.44 – 7.29 (m, 2H), 7.20 (dt, $J$ = 1.5, 0.7 Hz, 2H), 6.67 (td, $J$ = 1.6, 0.9 Hz, 1H), 4.71 (s, 2H), 3.80 (t, $J$ = 6.8 Hz, 2H), 2.29 – 2.15 (m, 6H), 1.63 (h, $J$ = 7.2 Hz, 2H), 0.86 (t, $J$ = 7.4 Hz, 3H).

$^{13}$C NMR (151 MHz, DMSO-$d_6$) δ 164.1, 156.5, 140.5, 138.6, 137.4, 131.8, 128.4, 126.9, 124.4, 117.8, 75.8, 50.7, 21.1, 20.7, 10.2.

M.p.: 164.5°C; HPLC: R$_t$=12.37 min, purity ≥ 99 %, MS (+APCI): 372 [M+H]$^+$.  

2.5.3 Synthesis of N-hydroxy-4-((3-(4-(dimethylamino)phenyl)-1-propoxyureido)methyl)benzamide (4c)

4c was synthesised according to general procedure 2.5 on a 0.623 mmol scale and obtained as a white solid with a yield of 34 % (0.215 mmol, 83 mg).

$^1$H NMR (300 MHz, DMSO-$d_6$) δ 11.17 (s, 1H), 9.00 (s, 1H), 8.59 (s, 1H), 7.70 (d, $J$ = 7.9 Hz, 2H), 7.38 (d, $J$ = 8.0 Hz, 2H), 7.32 (d, $J$ = 8.7 Hz, 2H), 6.67 (d, $J$ = 8.7 Hz, 2H), 4.69 (s, 2H), 3.78 (t, $J$ = 6.7 Hz, 2H), 2.83 (s, 6H), 1.62 (q, $J$ = 7.1 Hz, 2H), 0.86 (t, $J$ = 7.4 Hz, 3H).

$^{13}$C NMR (151 MHz, DMSO-$d_6$) δ 164.1, 157.1, 147.1, 140.7, 131.8, 128.4 (d, $J$ = 8.7 Hz), 126.8, 122.0, 112.6, 75.7, 51.1, 40.6, 20.8, 10.3.

M.p.: 71.4°C; HPLC: R$_t$=12.68 min, purity ≥ 99 %, MS (+APCI): 387 [M+H]$^+$.  

2.5.4 Synthesis of N-hydroxy-4-((3-(3,5-dimethylphenyl)-1-benzoylureido)methyl)benzamide (4d)

4d was synthesised according to general procedure 2.5 on a 0.776 mmol scale and obtained as a white solid with a yield of 76% (0.587 mmol, 218 mg).

$^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 11.18 (s, 1H), 9.03 (s, 1H), 8.67 (s, 1H), 7.81 – 7.65 (m, 2H), 7.47 (dq, $J$ = 4.4, 2.4 Hz, 2H), 7.38 (td, $J$ = 8.4, 4.0 Hz, 5H), 7.14 (d, $J$ = 1.5 Hz, 2H), 6.65 (s, 1H), 4.91 (s, 2H), 4.68 (s, 2H), 2.21 (s, 6H).

$^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 164.1, 156.5, 140.4, 138.5, 137.4, 135.6, 131.9, 129.7, 128.5, 128.4, 128.3, 126.9, 124.5, 117.7, 76.0, 50.8, 21.1.

M.p.: 254.1°C; HPLC: $R_t$=12.68 min, purity = 95.5 %, MS (+APCI): 420 [M+H]$^+$.
3 Nuclear magnetic resonance (NMR)

3.1 NMR of 1a
3.2 NMR of 1b
3.3 NMR of 1c
3.4 NMR of 2a
3.5 NMR of 2b
3.6 NMR of 2c
3.7 NMR of 3a
3.8 NMR of 3b
3.9 NMR of 3c
3.10 NMR of 3d
3.11 NMR of 4a
3.12 NMR of 4b
3.13 NMR of 4c
3.14 NMR of 4d
4 HDAC isozyme profiling
4.1 HDAC1-3 and HDAC6 inhibition assays

The in vitro inhibitory activity of compounds 4a-d and Nexturastat A against human HDAC1, HDAC2, HDAC3/NcoR2 and HDAC6 were measured using a previously published protocol.[2] OptiPlate-96 black microplates (Perkin Elmer) were used with an assay volume of 50 µL. 5.0 µL test compound or control, diluted in assay buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂, 0.1 mg/mL BSA), were incubated with 35 µL of the fluorogenic substrate ZMAL (Z-Lys(Ac)-AMC)[3] (21.43 µM in assay buffer) and 10 µL of human recombinant HDAC1 (BPS Bioscience, Catalog# 50051), HDAC2 (BPS Bioscience, Catalog# 50052), HDAC3/NcoR2 (BPS Bioscience, Catalog# 50003) or HDAC6 (BPS Bioscience, Catalog# 50006) at 37 °C. After an incubation time of 90 min, 50 µL of 0.4 mg/mL trypsin in trypsin buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl) were added, followed by further incubation at 37 °C for 30 min. Fluorescence was measured with an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Fluoroskan Ascent microplate reader (Thermo Scientific). All compounds were evaluated in duplicates in at least two independent experiments.

4.2 HDAC8 inhibition assay

HDAC8 recombinant enzyme (cat nr. KDA-21-481) was purchased from Reaction Biology Corp. (Malvern, PA, USA). The HDAC activity assay was performed in 96-well plates (Corning, Germany). Briefly, 20 ng of HDAC8 was used and diluted in assay buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, and 1 mg/mL BSA). After a 5 min incubation step the reaction was started with 10 µL of 60 µM Boc-Lys-(TFα)-AMC (Bachem, Germany). The reaction was stopped after 90 min by adding 100 µL stop solution (16 mg/mL trypsin, 2 µM panobinostat in 50 mM Tris-HCl, pH 8.0, and 100 mM NaCl). 15 min after the addition of the stop solution, fluorescence intensity was measured at excitation wavelength of 355 nm and emission wavelength of 460 nm in a NOVOstar microplate reader (BMG LabTech, Offenburg, Germany).

5 Cellular HDAC evaluation

5.1 Cell culture

The leukemic cell lines, HAL-01 (B-cell acute lymphoblastic leukemia or B-ALL), HL60 (acute myeloid leukemia or AML), SUPB15 (B-ALL), K562 (chronic myeloid leukemia or CML) and MOLM13 (AML) were cultured in RPMI 1640 GlutaMax (Life Technology, California, USA, Catalog#61870036) supplemented with 10% FCS. Jurkat (T-cell acute lymphoblastic
leukemia or T-ALL) was cultured in RPMI 1640 GlutaMax supplemented with 20% FCS. All cells were cultured in a 37°C humidified incubator with 5% CO₂ according to the suggested culture conditions from DSMZ (https://www.dsmz.de), with the addition of 1% penicillin-streptomycin (Life Technologies, California, USA, Catalog#15070063). All cell lines were bought from the DSMZ, Braunschweig, Germany.

5.2 Cell viability assay
The assay was performed to determine the IC₅₀ values for the leukemic cell lines. The experimental compounds and nexturastat A (Selleckchem, Houston, USA, Catalog#S7473) were first dissolved in DMSO with an initial stock concentration of 10mM and printed on white 384-well plates (Thermo Fisher Scientific, Waltham, USA, Catalog#3570) with increasing concentrations (5nM - 25μM) by using a digital dispenser (D300e, Tecan, Männedorf, Switzerland). Afterwards, 30 µl of cell suspension/well were seeded with a concentration of 0.04 x 10⁶ cells /ml and incubated under standard culture conditions. After 72 h the cell viability was measured utilizing the ATP based CellTiter-Glo luminescent assay (Promega, Madison, USA, Catalog#G7573) with a microplate reader (Spark, Tecan). The obtained raw data were normalised to DMSO treated controls (DMSO < 0.5%) and the IC₅₀ values calculated using the sigmoid dose curve (Hill slope) and nonlinear regression (GraphPad Prism Inc., San Diego, CA)(n=3). The IC₅₀ data were plotted as a clustered heat map, followed by unsupervised hierarchical clustering. Each box of the heatmap represents the mean of three independent experiments (n=3). The average IC₅₀ values of each compound across all tested cell lines were used for statistical analysis, *, ** and n.s. indicate significant one-way ANOVA P values of < 0.05, < 0.01 and > 0.05 respectively. The IC₅₀ values ± SD that were used for statistical analysis and the heatmap are shown in table 2.

|        | 4a       | 4b       | 4c       | 4d       | Nexturastat A |
|--------|----------|----------|----------|----------|---------------|
| SUPB15 | 6783 ± 391 | 2162 ± 124.5 | 6617 ± 180.5 | 3671 ± 62 | 8663 ± 804    |
| HL60   | 1691 ± 116 | 443 ± 23.5  | 12935 ± 757.5 | 2909 ± 124.5 | 894 ± 111    |
| K562   | 1770 ± 92.5 | 1617 ± 35   | 7684 ± 797   | 5787 ± 107.5 | 2255 ± 128   |
| MOLM13 | 239 ± 18   | 108 ± 14    | 7190 ± 310.5 | 1699 ± 69   | 220 ± 22.5   |
| Jurkat | 7087 ± 263 | 3042 ± 142  | 5216 ± 129.5 | 4679 ± 197  | 7412 ± 259   |
| HAL01  | 6653 ± 209.5 | 2515 ± 122.5 | 13778 ± 901.5 | 2821 ± 94 | 9047 ± 400.5 |

Table 2: IC₅₀ values of cell viability screen in nM
5.3 Immunoblot

Cells (0.5 x 10^6 cells/ml) were treated with the indicated concentration of the compound or vehicle (DMSO) for 24 h under standard culture conditions. Cell pellets were lysed with 300 µl RIPA buffer (50 mM Tris-HCl pH 8.0, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1% SDS, 150 mM sodium chloride, 2 mM EDTA, supplemented with protease and phosphatase inhibitors (Pierce protease (Catalog#A32959) and phosphatase inhibitor (Catalog#A32957) mini tablets, Thermo Scientific, Wesel, Germany) according to manufactures guidelines. After centrifugation, the protein concentration of the whole cell extracts was determined using the Pierce BCA protein assay kit (Thermo Scientific, Wesel, Germany, Catalog#23225), according to manufactue guidelines. 20 µg of total protein extracts were resolved by a 12% SDS-PAGE at 60 mA for 60 min and transferred at 100 V for 90 min to polyvinylidene fluoride or PVDF membranes (Merck Millipore, Darmstadt, Germany) utilizing the wet mini trans-blot electrophoretic transfer cell system (Bio-Rad, California, USA, Catalog#1703930). PageRuler Prestained Protein Ladder, 10 to 180 kDa (Thermofisher Scientific, Wesel, Germany, Catalog#26616) was used as protein molecular weight marker. First, Blots were incubated in 5% BSA/ TBS-T blocking solution for 30 min under slight agitation at room temperature and washed three times for 5 min in TBS-T. Afterwards, the blots were incubated over night at 4 C° with anti-α-tubulin (Catalog#2144), anti-acetyl-histone H3 (Catalog#9677S), anti-histone H3 (Catalog#9715) and anti-GAPDH (Catalog#97166) antibodies (Cell Signaling Technology, Danvers, MA). All primary antibodies were diluted 1:1000 in 5% BSA/ TBS-T as suggested by the manufacturer’s guidelines. Afterwards, blots were washed three times for 5 min in TBS-T. Next, blots were incubated with 1:2000 dilution of secondary horseradish peroxidase-conjugated antibodies (Cell Signalling Technology, Danvers, MA, Catalog#7074) for 2 h at room temperature. Blots were washed three times with TBS-T and developed with the ECL system (GE Healthcare, Solingen, Germany, Catalog#GERPN2109), according to the manufacturer’s guidelines. Blots were detected and analysed with the Jess western blot system (Protein simple, California, USA).

6 References

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