Development of novel β-carboline-based hydroxamate derivatives as HDAC inhibitors with DNA damage and apoptosis inducing ability

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1. Experimental procedures for chemical synthesis

$^1$H NMR spectra were recorded with a Bruker Avance 300 MHz spectrometer at 300 K, using TMS as an internal standard. MS spectra were recorded on a Mariner Mass Spectrum (ESI). High resolution mass spectra were recorded using an Agilent Technologies LC/MSD TOF. All compounds were routinely checked by TLC and $^1$H NMR. TLCs and preparative thin-layer chromatography were performed on silica gel GF/UV 254, and the chromatograms were conducted on silica gel (200–300 mesh, Merck) and visualized under UV light at 254 and 365 nm. All solvents were reagent grade and, when necessary, were purified and dried by standard methods. 4-Formylbenzoate 1, L-tryptophan 4, differently substituted primary amines 2a-d and aldehydes were commercially available. Compounds 5a-d and 4a-d were synthesized according to literature procedures.\textsuperscript{1,2} Solutions after reactions and extractions were concentrated using a rotary evaporator operating at a reduced pressure of ca. 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. All compounds were of >95% purity determined by HPLC.

Methyl 4-((methylamino)methyl)benzoate (3a)

To a solution of methyl 4-formylbenzoate 1 (6.8 mmol, 1.12 g) in 15ml anhydrous tetrahydrofuran (THF) was added ethanol amine 2a (8.2 mmol, 0.50 g) and catalytic amount of HOAc. The reaction was stirred at room temperature for 5h. To the mixture was added sodium boron hydride (17.1 mmol, 0.65 g). After the reaction was completed, the solvent was removed under reduced pressure. The crude residue was dissolved in water and was adjusted by 2M HCl to pH = 3. The solution was washed with ethyl acetate (3 × 20 mL). The water layer was adjusted with 2M NaOH to pH = 11. The solution was extracted with ethyl acetate (3 × 20 mL). The organic phase was washed with brine and concentrated to give a yellow oil product 3a, yield 76%. MS (ESI) m/z = 180 [M+H].

Methyl 4-((ethylamino)methyl)benzoate (3b)

Compound 3b was synthesized from 4-formylbenzoate 1, ethanamine 2b, and sodium boron hydride, according to the synthetic procedure of 3a in a yield of 62%, yellow oil. MS (ESI) m/z = 194 [M+H].

Methyl 4-((propylamino)methyl)benzoate (3c)

Compound 3c was synthesized from 4-formylbenzoate 1, propan-1-amine 2c, and sodium boron hydride, according to the synthetic procedure of 3a in a yield of 62%, yellow oil. MS (ESI) m/z = 208 [M+H].

Methyl 4-((butylamino)methyl)benzoate (3d)

Compound 3f was synthesized from 4-formylbenzoate 1, butan-1-amine 2d, and sodium boron hydride, according to the synthetic procedure of 3a in a yield of 62%, yellow oil. MS (ESI) m/z = 222 [M+H].

N-(4-(Hydroxycarbamoyl)benzyl)-N-methyl-9H-pyrido[3,4-b]indole-3-carboxamide (8a)

To a solution of 6a (0.25 g, 1.2 mmol), EDCI (0.27 g, 1.44 mmol) and catalytic amount of DMAP in 10 mL anhydrous CH$_2$Cl$_2$, was added methyl 4-((methylamino)methyl)benzoate 3a (0.21 g, 1.2 mmol). The mixture was stirred at room temperature overnight. Then 20 mL of CH$_2$Cl$_2$ was added and the mixture was washed with water (30 mL × 3) and brine. The organic phase was dried over
anhydrous sodium sulfate, filtered and evaporated in vacuo, and the crude product was purified by column chromatography to give 7a, which was then dissolved in 3 mL anhydrous methanol, and poured into a solution of NH₂OK (0.09 g, 4 mmol) in 3 mL of anhydrous methanol. The mixture was stirred for 10-15 h and the solvent was evaporated in vacuo. The residue was diluted with saturated NH₄Cl aqueous solution, and then extracted with ethyl acetate (6 mL × 5). The organic layers were combined, dried over anhydrous Na₂SO₄ and evaporated. The resulting residue was purified by column chromatography (eluting with EA followed by 20:1 CHCl₃/MeOH followed by 10:1 CHCl₃/MeOH) on silica gel to afford 8a as a pale yellow solid in a yield of 65%. Analytical data for 8a: ¹H NMR (DMSO-d₆, 300 MHz): δ10.62 (s, 1H, NH), 9.05 (s, 1H, NH), 8.87 (s, 1H, Ar-H), 8.72 (s, 1H, Ar-H), 8.21 (m, 1H, Ar-H), 8.07 (d, 2H, J = 7.5 Hz, Ar-H), 7.72 (m, 1H, Ar-H), 7.53-7.58 (m, 3H, Ar-H), 7.31 (m, 1H, Ar-H), 5.03 (s, 1H, NCH₂), 4.90 (s, 1H, NCH₂), 3.73 (s, 3H, NCH₃); MS (ESI) m/z = 375 [M+H]+; HRMS (ESI): m/z calcd for C₂₁H₁₉N₄O₃: 375.1379; found: 375.1393 [M+H]+.

N-Ethyl-N-(4-(hydroxycarbamoyl)benzyl)-9H-pyrido[3,4-b]indole-3-carboxamide (8b)

Compound 8b was synthesized from 6a, methyl 4-((ethylamino)methyl)benzoate 3b, EDCI, and NH₂OK, according to the synthetic procedure of 8a in a yield of 63%, a pale yellow solid. Analytical data for 8b: ¹H NMR (DMSO-d₆, 300 MHz): δ11.02 (s, 1H, NH), 9.11 (s, 1H, NH), 8.87 (s, 1H, Ar-H), 8.75 (s, 1H, Ar-H), 8.19 (m, 1H, Ar-H), 8.05 (d, 2H, J = 7.5 Hz, Ar-H), 7.72 (m, 1H, Ar-H), 7.51-7.57 (m, 3H, Ar-H), 7.26 (m, 1H, Ar-H), 5.03 (s, 1H, NCH₂), 4.87 (s, 1H, NCH₂), 3.57-3.66 (m, 2H, NCH₂CH₃), 1.25-1.30 (m, 2H, NCH₂CH₂CH₃); MS (ESI) m/z = 389 [M+H]+; HRMS (ESI): m/z calcd for C₂₂H₂₁N₄O₄: 389.1535; found: 389.1551 [M+H]+.

N-(4-(Hydroxycarbamoyl)benzyl)-N-propyl-9H-pyrido[3,4-b]indole-3-carboxamide (8c)

Compound 8c was synthesized from 6a, methyl 4-((propylamino)methyl)benzoate 3c, EDCI, and NH₂OK, according to the synthetic procedure of 8a in a yield of 62%, a pale yellow solid. Analytical data for 8c: ¹H NMR (DMSO-d₆, 300 MHz): δ11.21 (s, 1H, NH), 8.89 (s, 1H, Ar-H), 8.71 (s, 1H, Ar-H), 8.27 (m, 1H, Ar-H), 8.07 (d, 2H, J = 7.5 Hz, Ar-H), 7.53-7.61 (m, 3H, Ar-H), 7.32-7.39 (m, 2H, Ar-H), 5.06 (s, 1H, NCH₂), 4.91 (s, 1H, NCH₂), 3.43-3.55 (m, 2H, NCH₂CH₂CH₃), 1.67-1.71 (m, 2H, NCH₂CH₃), 0.87-0.95 (m, 2H, NCH₂CH₂CH₃); MS (ESI) m/z = 403 [M+H]+; HRMS (ESI): m/z calcd for C₂₃H₂₃N₄O₄: 403.1692; found: 403.1705 [M+H]+.

N-Butyl-N-(4-(hydroxycarbamoyl)benzyl)-9H-pyrido[3,4-b]indole-3-carboxamide (8d)

Compound 8d was synthesized from 6a, methyl 4-((butylamino)methyl)benzoate 3d, EDCI, and NH₂OK, according to the synthetic procedure of 8a in a yield of 66%, a pale yellow solid. Analytical data for 8d: ¹H NMR (DMSO-d₆, 300 MHz): δ11.08 (s, 1H, NH), 8.89 (s, 1H, Ar-H), 8.71 (s, 1H, Ar-H), 8.28 (m, 1H, Ar-H), 8.07 (d, 2H, J = 7.5 Hz, Ar-H), 7.55-7.63 (m, 3H, Ar-H), 7.30-7.37 (m, 3H, Ar-H), 5.06 (s, 1H, NCH₂), 4.92 (s, 1H, NCH₂), 3.48-3.56 (m, 2H, NCH₂CH₂CH₃), 1.67-1.70 (m, 2H, NCH₂CH₂CH₃), 1.38-1.43 (m, 2H, NCH₂CH₂CH₂CH₃), 0.69-0.75 (m, 2H, NCH₂CH₂CH₂CH₃); MS (ESI) m/z = 417 [M+H]+; HRMS (ESI): m/z calcd for C₂₄H₂₅N₄O₄: 417.1862; found: 417.1862 [M+H]+.

N-(4-(Hydroxycarbamoyl)benzyl)-N,9-dimethyl-9H-pyrido[3,4-b]indole-3-carboxamide (8e)

Compound 8e was synthesized from 6b, methyl 4-((methylamino)methyl)benzoate 3a, EDCI, and NH₂OK, according to the synthetic procedure of 8a in a yield of 61%, a pale yellow solid. Analytical data for 8e: ¹H NMR (DMSO-d₆, 300 MHz): δ9.13 (s, 1H, NH), 8.72 (s, 1H, Ar-H), 8.29 (m, 1H, Ar-H), 8.03-8.07 (m, 3H, Ar-H), 7.52-7.59 (m, 2H, Ar-H), 7.28 (m, 1H, Ar-H), 5.08 (s, 1H, NCH₂), 4.92 (s, 1H, NCH₂), 3.76 (s, 1H, NCH₃), 2.89 (s, 3H, CH₃); MS (ESI) m/z = 389 [M+H]+; HRMS
N-Ethyl-N-(4-(hydroxycarbamoyl)benzyl)-9-methyl-9H-pyrido[3,4-b]indole-3-carboxamide (8f)

Compound 8f was synthesized from 6b, methyl 4-((ethylamino)methyl)benzoate 3b, EDCI, and NH₄OK, according to the synthetic procedure of 8a in a yield of 65%, a pale yellow solid. Analytical data for 8f: ¹H NMR (DMSO-d₆, 300 MHz): δ 9.16 (s, 1H, NH), 8.76 (m, 1H, Ar-H), 8.28 (m, 1H, Ar-H), 8.04-8.10 (m, 3H, Ar-H), 7.52-7.56 (m, 1H, Ar-H), 7.30 (m, 1H, Ar-H), 5.05 (s, 1H, NCH₂), 4.91 (s, 1H, NCH₂), 3.56-3.67 (m, 2H, NCH₂CH₂), 2.91 (s, 3H, CH₃), 1.25-1.33 (m, 3H, NCH₂CH₂); MS (ESI) m/z = 403 [M+H]⁺; HRMS (ESI): m/z calcd for C₂₉H₂₃N₄O₃: 403.1692; found: 403.1677 [M+H]⁺.

N-(4-(Hydroxycarbamoyl)benzyl)-9-methyl-N-propyl-9H-pyrido[3,4-b]indole-3-carboxamide (8g)

Compound 8g was synthesized from 6b, methyl 4-((propylamino)methyl)benzoate 3c, EDCI, and NH₄OK, according to the synthetic procedure of 8a in a yield of 70%, a pale yellow solid. Analytical data for 8g: ¹H NMR (DMSO-d₆, 300 MHz): δ 9.26 (s, 1H, NH), 8.70 (m, 1H, Ar-H), 8.30 (m, 1H, Ar-H), 8.03-8.09 (m, 3H, Ar-H), 7.63 (m, 1H, Ar-H), 7.51 (d, 2H, J = 7.5 Hz, Ar-H), 7.28 (m, 1H, Ar-H), 5.03 (s, 1H, NCH₂), 4.87 (s, 1H, NCH₂), 2.89 (s, 3H, CH₃), 3.38-3.53 (m, 2H, NCH₂CH₂CH₂), 1.63-1.70 (m, 2H, NCH₂CH₂CH₂), 0.85-0.91 (m, 3H, NCH₂CH₂CH₂); MS (ESI) m/z = 417[M+H]⁺; HRMS (ESI): m/z calcd for C₂₉H₂₅N₄O₃: 417.1848; found: 417.1861 [M+H]⁺.

N-Butyl-N-(4-(hydroxycarbamoyl)benzyl)-9-methyl-9H-pyrido[3,4-b]indole-3-carboxamide (8h)

Compound 8h was synthesized from 6b, methyl 4-((butylamino)methyl)benzoate 3f, EDCI, and NH₄OK, according to the synthetic procedure of 8a in a yield of 68%, a pale yellow solid. Analytical data for 8h: ¹H NMR (DMSO-d₆, 300 MHz): δ 9.12 (s, 1H, NH), 8.75 (s, 1H, Ar-H), 8.28 (m, 1H, Ar-H), 8.03-8.10 (m, 3H, Ar-H), 5.51-5.76 (m, 3H, Ar-H), 7.30 (m, 1H, Ar-H), 5.09 (s, 1H, NCH₂), 4.95 (s, 1H, NCH₂), 3.52-3.61 (m, 2H, NCH₂CH₂), 2.91 (s, 3H, CH₃), 1.65-1.71 (m, 2H, NCH₂CH₂CH₂), 1.40-1.44 (m, 2H, NCH₂CH₂CH₂), 0.67-0.72 (m, 3H, NCH₂CH₂CH₂); MS (ESI) m/z =431 [M+H]⁺; HRMS (ESI): m/z calcd for C₃₅H₃₇N₄O₃: 431.2050; found: 431.1989 [M+H]⁺.

N-(4-(Hydroxycarbamoyl)benzyl)-9-(4-methoxyphenyl)-N-methyl-9H-pyrido[3,4-b]indole-3-carboxamide (8i)

Compound 8i was synthesized from 6c, methyl 4-((methylamino)methyl)benzoate 3a, EDCI, and NH₂OK, according to the synthetic procedure of 8a in a yield of 62%, a pale yellow solid. Analytical data for 8i: ¹H NMR (DMSO-d₆, 300 MHz): δ 11.06 (s, 1H, NH), 8.79 (s, 1H, Ar-H), 8.44 (m, 1H, Ar-H), 8.16 (m, 1H, Ar-H), 7.96-8.05 (m, 3H, Ar-H), 7.53-7.59 (m, 3H, Ar-H), 7.46 (d, 2H, J = 7.5 Hz, Ar-H), 7.35 (m, 1H, Ar-H), 7.12 (d, 2H, J = 7.5 Hz, Ar-H), 5.07 (s, 1H, NCH₂), 4.91 (s, 1H, NCH₂), 3.82 (s, 1H, NCH₂); MS (ESI) m/z = 481 [M+H]⁺; HRMS (ESI): m/z calcd for C₃₈H₃₅N₄O₄: 481.1798; found: 481.1814 [M+H]⁺.

N-Ethyl-N-(4-(hydroxycarbamoyl)benzyl)-9-(4-methoxyphenyl)-9H-pyrido[3,4-b]indole-3-carboxamide (8j)

Compound 8j was synthesized from 6c, methyl 4-((ethylamino)methyl)benzoate 3b, EDCI, and NH₂OK, according to the synthetic procedure of 8a in a yield of 62%, a pale yellow solid. Analytical data for 8j: ¹H NMR (DMSO-d₆, 300 MHz): δ 11.15 (s, 1H, NH), 8.76 (s, 1H, Ar-H), 8.48 (s, 1H, Ar-H), 8.16 (m, 1H, Ar-H), 8.05 (d, 1H, J = 6.3 Hz, Ar-H), 8.01 (d, 1H, J = 6.3 Hz, Ar-H), 7.94 (m, 1H, Ar-H), 7.50-7.60 (m, 4H, Ar-H), 7.34 (m, 1H, Ar-H), 7.11 (d, 2H, J = 7.5 Hz, Ar-H), 6.91 (m, 1H, NH), 5.04 (s, 1H, NCH₂), 4.93 (s, 1H, NCH₂), 3.86 (s, 3H, OCH₃), 3.59-3.68 (m, 2H,
Compound 8k was synthesized from 6c, methyl 4-((propylamino)methyl)benzoate 3c, EDCI, and NH₂OK, according to the synthetic procedure of 8a in a yield of 62%, a pale yellow solid. Analytical data for 8k: ¹H NMR (DMSO-d₆, 300 MHz): δ8.63 (s, 1H, Ar-H), 8.40 (m, 1H, Ar-H), 7.91-8.09 (m, 3H, Ar-H), 7.84 (m, 1H, Ar-H), 7.39-7.50 (m, 3H, Ar-H), 7.32 (m, 1H, Ar-H), 7.06 (d, 2H, J = 7.5 Hz, Ar-H), 6.85 (m, 1H, NH), 5.06 (s, 1H, NCH₂), 4.94 (s, 1H, NCH₂), 3.86 (s, 3H, OCH₃), 3.40-3.54 (m, 2H, NCH₂), 1.65-1.70 (m, 2H, NCH₂), 0.88-0.93 (m, 3H, NCH₂CH₂CH₃); MS (ESI) m/z = 509 [M+H]+; HRMS (ESI): m/z calcd for C₂₀H₂₉N₄O₄: 509.2111; found: 509.2130 [M+H]+.

N-Butyl-N-(4-(hydroxycarbamoyl)benzyl)-9-(4-methoxyphenyl)-9H-pyrido[3,4-b]indole-3-carboxamide (8l)

Compound 8l was synthesized from 6c, methyl 4-((butylamino)methyl)benzoate 3d, EDCI, and NH₂OK, according to the synthetic procedure of 8a in a yield of 62%, a pale yellow solid. Analytical data for 8l: ¹H NMR (DMSO-d₆, 300 MHz): δ8.79 (s, 1H, Ar-H), 8.46 (m, 1H, Ar-H), 8.15 (m, 1H, Ar-H), 7.95-8.05 (m, 3H, Ar-H), 7.53-7.59 (m, 3H, Ar-H), 7.48 (d, 2H, J = 7.5 Hz, Ar-H), 7.35 (m, 1H, Ar-H), 7.12 (d, 2H, J = 7.5 Hz, Ar-H), 6.91 (m, 1H, NH), 5.11 (s, 1H, NCH₂), 4.97 (s, 1H, NCH₂), 3.92 (s, 3H, OCH₃), 3.51-3.58 (m, 2H, NCH₂CH₂CH₃), 1.67 (m, 2H, NCH₂CH₂CH₃), 1.41 (m, 2H, NCH₂CH₂CH₂CH₃), 0.7-0.74 (m, 3H, NCH₂CH₂CH₂CH₃); MS (ESI) m/z = 523 [M+H]+; HRMS (ESI): m/z calcd for C₃₁H₃₃N₄O₄: 523.2267; found: 523.2288 [M+H]+.

N-(4-(Hydroxycarbamoyl)benzyl)-9-(3-methoxyphenyl)-N-propyl-9H-pyrido[3,4-b]indole-3-carboxamide (8m)

Compound 8m was synthesized from 6d, methyl 4-((propylamino)methyl)benzoate 3c, EDCI, and NH₂OK, according to the synthetic procedure of 8a in a yield of 62%, a pale yellow solid. Analytical data for 8m: ¹H NMR (DMSO-d₆, 300 MHz): δ8.86 (s, 1H, Ar-H), 8.67 (m, 1H, Ar-H), 8.49 (d, 1H, J = 8.4 Hz, Ar-H), 8.16 (m, 1H, Ar-H), 8.03 (d, 1H, J = 6.0 Hz, Ar-H), 7.96 (m, 1H, Ar-H), 7.46-7.58 (m, 4H, Ar-H), 7.23-7.37 (m, 3H, Ar-H), 6.96 (m, 1H, NH), 5.06 (s, 1H, NCH₂), 4.92 (s, 1H, NCH₂), 3.91 (s, 3H, OCH₃), 3.46-3.49 (m, 2H, NCH₂CH₂CH₃), 1.73 (m, 2H, NCH₂CH₂CH₂CH₃), 0.87 (m, 3H, NCH₂CH₂CH₃); MS (ESI) m/z = 509 [M+H]+; HRMS (ESI): m/z calcd for C₂₉H₂₉N₄O₄: 509.2111; found: 509.2098 [M+H]+.

N-Butyl-N-(4-(hydroxycarbamoyl)benzyl)-9-(3-methoxyphenyl)-9H-pyrido[3,4-b]indole-3-carboxamide (8n)

Compound 8n was synthesized from 6d, methyl 4-((butylamino)methyl)benzoate 3d, EDCI, and NH₂OK, according to the synthetic procedure of 8a in a yield of 62%, a pale yellow solid. Analytical data for 8n: ¹H NMR (DMSO-d₆, 300 MHz): δ8.75 (s, 1H, Ar-H), 8.51 (d, 1H, J = 8.4 Hz, Ar-H), 8.15 (m, 1H, Ar-H), 8.02-8.06 (m, 2H, Ar-H), 7.46-7.58 (m, 5H, Ar-H), 7.26-7.37 (m, 3H, Ar-H), 6.96 (m, 1H, NH), 5.06 (s, 1H, NCH₂), 4.91 (s, 1H, NCH₂), 3.91 (s, 3H, OCH₃), 3.48-3.56 (m, 2H, NCH₂CH₂CH₂CH₃), 1.67 (m, 2H, NCH₂CH₂CH₂CH₃), 1.08 (m, 2H, NCH₂CH₂CH₃), 0.88 (m, 3H,
NCH₂CH₂CH₃); MS (ESI) m/z =523 [M+H]+; HRMS (ESI): m/z calcd for C₃₁H₃₁N₄O₄: 523.2267; found: 523.2283 [M+H]+.  

2. UV-visible absorption spectra of SAHA and harmine in the presence of increasing amounts of CT DNA. Arrows indicate the changes in absorbance with increasing the concentration of DNA.  

![SAHA Absorption Spectrum](image1)

![Harmine Absorption Spectrum](image2)

3. Experimental procedures for biological evaluation  
**Cell Culture and Reagents.** Human hepatocellular carcinoma cells (HepG2, SMMC-7721), human colon cancer cells (HCT116, LOVO), and Hela cells were maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml of penicillin, and 0.1 µg/ml of streptomycin in a humid atmosphere incubator with 5% CO₂ at 37 °C. All cell lines were originally from the Shanghai Institute of Cell Biology (Shanghai, China). Cells were routinely subcultured twice weekly. PARP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), monoclonal anti-actin antibody, and goat peroxidase-conjugated anti-rabbit IgG antibody, and goat peroxidase-conjugated anti-mouse IgG antibody were purchased from Sigma-Aldrich (St. Louis, MO). FITC-Annexin V and PI are from (BioVision). The chemiluminescence (ECL) kit was purchased from Thermo Fisher Scientific (Rockford, IL). Antibodies such as acetyl-H3, acetyl-tubulin, Bax, Bel-2, and cleaved-caspase 3 are from Cell Signaling Technology (Danvers, MA).
Cell viability assay. Anti-proliferative activities of synthesized compounds were evaluated in vitro against four human cancer cell lines (HepG2, SMMC-7721, HCT116, and LOVO). Briefly, 100 μl of different colon cancer cells were plated in a 96-well flat bottom tissue culture plate at a density of 10^4 cells/ml, respectively, in DMEM medium and 10% fetal bovine serum and allowed to adhere overnight at 37°C in 5% CO_2. The cells were treated by adding 100 μL of different compounds at various concentrations into the respective well. The reagent DMSO (0.1%) was used as a negative control. The cell viability assay (MTT assay) was carried out at 48 hours after drug treatment. The concentration, which inhibited 50% of cellular growth (IC50 value), was calculated by the following formula: Cell inhibition rate (%) = (1 − OD of treatment group/OD of control group) × 100%. The cytotoxicity potency of tested compounds on colon cancer cells was expressed as IC50 values or by a histogram (The bars are the mean ± SD). All the data were derived from three independent measurements.

HDAC activity assay. HDAC activity assays were performed as previously reported.3 HeLa cell nuclear extract which is a rich source of HDACs was prepared using the EpiQuik nuclear extraction kit (OP-0002, Epigentek Group Inc). The HDAC activity was determined using the HDAC fluorimetric activity assay kit (Enzo Life Sciences Inc.) according to the manufacturer’s instructions. Briefly, HDAC enzyme solution (HeLa nuclear extract) was incubated with test compounds at different concentrations in the presence of HDAC substrate (Boc-Lys (Ac)-AMC) at 37 °C for 60 min. Then the lysine developer was added to stop the reaction. After 30 min, the data was recorded in a fluorescence plate reader with excitation at 355 nm and emission at 460 nm. The HDAC activity was calculated as a percentage of activity compared with the control group. The concentration required for 50% inhibition (IC50) was calculated using the software GraphPad Prism (Version 4.03).

UV–visible spectroscopy titrations. UV–visible spectroscopy titrations were performed on an ultraviolet spectrophotometer (UV 2500, Shimadzu, Tokyo, Japan) at 25 °C. Stock solutions of 20 μM of CT DNA (calf thymus DNA, which can form perfect double stranded DNA structure) were prepared in 5mM Tris buffer (60.50 mg Tris, 292.5 mg NaCl, Ph 7.0, with 100 ml Milli Q water). Stock solutions of 20 μM synthesized derivatives were prepared by dissolving them in 1:1000 DMSO/Milli Q water. UV–visible absorption titrations were done by adding 100 μl CT DNA solution each time to the quartz cuvette containing about 4 ml active compound solution. Titrations were carried out until the complex absorption band remains at a fixed wavelength upon five successive additions of CT DNA. Absorption spectra were recorded from 200 nm to 500 nm.

Flow cytometry assay of cell apoptosis. HepG2 cells cells were cultured overnight and incubated in triplicate with different concentrations of 8k (1.0, 2.0, and 4.0 μM), SAHA (5.0 μM), or vehicle for 48 h. The cells were harvested and stained with FITC-Annexin V and PI at room temperature for 15 min. The percentage of apoptotic cells was determined by flow cytometry (Epics XL-MCL, Beckman Coulter, Indianapolis, USA) analysis. The FITC signal detector (FL1) and PI staining signal detector (FL3) were used to detect the cells with the flow cytometer (Ex = 488 nm; Em = 530 nm). Ten thousand cells were counted for three independent experiments. The data were analyzed using WinList 3D (version7.1) and the histogram was plotted using Excel 2010.
**Western Blot Analysis.** HepG2 cells with or without 8k, or SAHA treatment at indicated time and doses were washed with PBS and lysed on ice for 30 minutes in PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF, and 20 mM leupeptin. The protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Up to 50 µg of total protein were separated onto an SDS-PAGE and transferred to polyvinylidenedifluoride membranes. After blocking with 5% fat free milk for 2 h, membranes were incubated overnight at 4 °C with a primary antibody in TBS-T and then reacted with a peroxidase-conjugated secondary antibody for 1 h. Immuno reactive proteins were detected with the ECL Western blotting Detection System.

**Metabolic stability**

Stock solutions of studied compounds were prepared at concentration of 10 mM in DMSO. Working solutions were prepared by dilution of stock with reaction buffer or acetonitrile, final concentration of organic solvent did not exceed 1%. Incubation mixture contained 1.0 µM of a studied derivative, 1 mM of NADPH (Sigma-Aldrich) and 0.5 mg/mL of rat liver microsomes (BD Gentest) in potassium phosphate buffer (0.1 M, pH 7.4). Incubation was carried out in thermostat at 37 °C and started by addition of studied compound. 50 µL samples were taken after 5, 15, 30, and 45 min. Enzymatic reaction was terminated by the addition of the equal volume of ice-cold acetonitrile containing 1.0 µM of ketanserin (Sigma-Aldrich) serving as internal standard (IS). Control incubations were performed without NADPH to assess chemical instability. After collection, samples were immediately centrifuged (10 min, 10,000 rpm) and resulted supernatant was directly analyzed or kept in −80 °C until LC-MS analysis. Natural logarithm of a compound over IS peak area ratio was plotted versus incubation time. Metabolic half-life (t1/2) was calculated from the slope of the linear regression.

**Reference**