

Supporting Information 1

Design, synthesis and docking study of acyl thiourea derivatives as possible histone deacetylase inhibitors with novel zinc binding group

Duraïd H. Al-Amily* and Mohammed H. Mohammed

Address: Pharmaceutical Chemistry Department, College of Pharmacy-University of
Baghdad, Baghdad, Iraq.

Email: Duraïd H. Al-Amily – colrelated@copharm.uobaghdad.edu.iq

* Corresponding author

Contents

Materials and Methods	S2
Chemical synthesis	S3 – S6
<i>In vitro</i> cytotoxicity study	S7 – S8
Molecular docking	S9
References	S10

Materials and Methods

Reagents and solvents were used for chemical synthesis as obtained from the supplier (Sigma-Aldrich, Fluka, Romil, GCC Diagnostics, ReagentWorld, and Thomas-Baker). Melting points were measured using Stuart SMP3 melting point apparatus (UK) and are uncorrected. Thin-layer chromatography was achieved using 0.2 mm pre-coated TLC-sheets Alugram® Xtra SIL G/UV254 (Macherey-Nagel, Germany) and the visualization was under a 254 nm UV lamp. FT-IR spectroscopy was done using Shimadzu IRAffinity-1 Spectrometer (Shimadzu, Japan) and Specac® Quest ATR (diamond-UK) at the University of Baghdad-College of Pharmacy. ¹H-NMR and ¹³C-NMR analysis was performed at 400 MHz and 100 MHz respectively (*d*₆-DMSO as the solvent) using The Bruker Avance III, 400 MHz spectrometer (USA) at Sophisticated Test & Instrumentation Centre (Cochin University of Science and Technology-India), with the chemical shifts (δ) expressed in parts per million.

Molecular docking was performed using AutoDock Vina software [1] that is integrated into USCF Chimera software [2]. Enzymes crystallized structures were retrieved from the protein data bank (PDB, www.rcsb.org) [3].

Cytotoxicity assay was performed at the iRAQ Biotech laboratories using trypsin/EDTA, RPMI 1640, fetal bovine serum (Capricorn, Germany); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT stain- Bio-World, USA); dimethyl sulfoxide (DMSO- Santacruz Biotechnology, USA); CO₂ incubator and laminar flow hood (Cypress Diagnostics, Belgium); microtiter reader (Gennex lab., USA); cell culture plates (Santa Cruz Biotechnology, USA).

Chemical synthesis

General synthesis of adipic and pimelic anhydrides (4b and 5b)

Adipic acid (**4a**) or pimelic acid (**5a**) (27.4 mmol of each) was suspended in acetic anhydride (3 mL/g) and refluxed for 1 hour. The solvent was then evaporated under reduced pressure to obtain a semisolid mixture of **4b**, **4c**, **5b**, and **5c**, which was used as such in the next step. FT-IR (ATR; ν , cm^{-1}): 1801, 1739 for **4b** and 1813, 1743 for **5b** (sym. and asym. C=O respectively).

General synthesis of monosodium adipic monoanilide and monosodium pimelic monoanilide (4d and 5d)

The obtained mixture of compounds **4b**, **4c**, **5b**, and **5c** was dissolved in 10 mL of dry DMF in a round bottom flask that was equipped with calcium chloride tube and cooled to 0 °C. Then 3 mL of aniline (32.88 mmol) were added gradually with continuous stirring and cooling. The mixture was then kept stirred at room temperature for 24 hours. The mixture was then acidified with 5 N hydrochloric acid, diluted to about 300 mL with cold water, and filtered. The precipitate was washed with water (3*50 mL). Then it was suspended in no more than 50 mL of ice-cooled water and the pH of this mixture was raised to 7 by a drop wise addition of 0.5 N sodium hydroxide solution at 0 °C. The mixture was stirred for 30 minutes at 0 °C while maintaining this pH by addition of 0.5 N sodium hydroxide solution as needed. Then the mixture was filtered and the filtrate was evaporated to dryness.

Compound **4d**: off white powder, yield 35% relative to **4a**, m. p. 259.5-263.8 °C. FT-IR (ATR; ν , cm^{-1}): 3332 (aromatic NH), 3062 (aromatic C-H), 2939 (asym. CH₂), 2866 (sym. CH₂), 1666 (amide C=O), 1600 (aromatic C-C), 1562 (asym. carboxylate C=O), 1539 and 1500 (aromatic C-C), 1431 (sym. carboxylate C=O).

Compound **5d**: off white powder, yield 25.5% relative to **5a**, m. p. 214-217 °C. FT-IR (ATR; ν , cm⁻¹): 3290 (aromatic NH), 3078 (aromatic C–H), 2920 (asym. CH₂), 2858 (sym. CH₂), 1654 (amide C=O), 1597 (aromatic C–C), 1558 (asym. carboxylate C=O), 1543 and 1496 (aromatic C–C), 1435 (sym. carboxylate C=O).

General synthesis of adipic monoanilide acid chloride and pimelic monoanilide acid chloride (4e and 5e)

Compound **4d** or **5d** (4 mmol) was suspended in 15 mL dry dichloromethane in a round bottom flask that was equipped with calcium chloride tube and cooled in an ice bath. 0.32 mL of pyridine (4 mmol) was added followed by a drop wise addition of 0.34 mL of thionyl chloride (4.8 mmol) with continuous stirring and cooling for 10 minutes. The mixture was then allowed to warm to room temperature with continuous stirring for 30 minutes. Then the precipitate formed was collected using Büchner funnel. The precipitate was washed with dry dichloromethane (3*10 mL) during filtration. The white solid mass obtained (in both cases of **4e** and **5e**) was used as such in the next step.

Compound **4e** FT-IR (ATR; ν , cm⁻¹): 3313 (aromatic NH), 3066 (aromatic C–H), 2935 (asym. CH₂), 2870 (sym. CH₂), 1797 (acid chloride C=O), 1662 (amide C=O), 1600, 1535 and 1500 (aromatic C–C).

Compound **5e** FT-IR (ATR; ν , cm⁻¹): 3313 (aromatic NH), 3043 (aromatic C–H), 2939 (asym. CH₂), 2870 (sym. CH₂), 1797 (acid chloride C=O), 1662 (amide C=O), 1597, 1527 and 1500 (aromatic C–C).

General synthesis of adipic monoanilide isothiocyanate and pimelic monoanilide isothiocyanate (4f and 5f)

A solution of ammonium thiocyanate (4 mmol, 0.3 g), dissolved in 5 mL dry acetone, was added gradually with stirring to 10 mL of a suspension of the precipitate

obtained from the previous step (**4e** or **5e**) in dry acetone. The reaction flask, equipped with a calcium chloride tube, was refluxed for two hours. Then the solvent was evaporated under reduced pressure and the residue was suspended in 10 mL dry dichloromethane and filtered. After evaporation of the filtrate under reduced pressure, a yellow oil was obtained for both **4f** and **5f** and was used directly in the next step.

General synthesis of N-adipoyl monoanilide thiourea and N-pimeloyl monoanilide thiourea (4 and 5)

The oily residue from the previous step (**4f** or **5f**) was dissolved in 150 mL dry chloroform and transferred into a Drechsel gas bottle containing a stirring bar and connected to a gas delivery tube the other end of which was connected to a drying column containing calcium oxide. This column was fit through its ground glass ending onto a flask containing 100 mL of strong ammonia solution. On gently warming the flask (35-40 °C), a continuous stream of dry ammonia gas was bubbled in to the chloroform solution of the isothiocyanate (**4f** or **5f**) for 1 hour with continuous stirring. Then the solvent was removed under reduced pressure and the residue was washed with 10 mL ice-cooled water, filtered, and the precipitate was washed again with 10 mL of 5% sodium bicarbonate solution and filtered (twice) yielding a yellow precipitate for both **4** and **5**.

Compound **4**: yellow powder, yield 15% relative to **4d**, m. p. 159.6-166.6 °C. R_f 0.63 (dichloromethane: ethyl acetate; 6:4). FT-IR (ATR; ν , cm⁻¹): 3387 (asymmetric NH₂) 3329 (*sec*-amide NH), 3197 (symmetric NH₂), 3043 (aromatic C-H), 2920 (asymmetric CH₂), 2850 (symmetric CH₂), 1705 (acyl thiourea C=O), 1662 (aromatic amide C=O), 1589, 1523 and 1500 (aromatic C-C), 1149 (C=S). ¹H-NMR (δ , ppm): 1.56 (m, -CH₂-CH₂-, 4H), 2.07 (t, aliphatic -CH₂-C(O)-, 2H), 2.3 (t,

aromatic -CH₂-C(O)-, 2H), 7.02 (t, *p*-CH, 1H), 7.28 (t, *m*-CH, 2H), 7.58 (d, *o*-CH, 2H), 9.34 (br, NH₂, 1H *d*₆-DMSO exchangeable), 9.64 (br, NH₂, 1H *d*₆-DMSO exchangeable), 9.86 (s, -NH-, 1H), 11.06 (-CONHCS-, 1H *d*₆-DMSO exchangeable). ¹³C-NMR (δ): 24.56, 24.83, 35.53, 36.07, 119.03, 122.95, 128.60, 139.22, 170.96, 174.43, 174.49.

Compound **5**: yellow powder, yield 18% relative to **5d**, m. p. 144.9-152.4 °C. R_f 0.69 (dichloromethane: ethyl acetate; 6:4) FT-IR (ATR; ν, cm⁻¹): 3352 (asymmetric NH₂) 3313 (*sec*-amide NH), 3190 (symmetric NH₂), 3043 (aromatic C-H), 2939 (asymmetric CH₂), 2850 (symmetric CH₂), 1705 (acyl thiourea C=O), 1662 (aromatic amide C=O), 1589, 1523 and 1500 (aromatic C-C), 1149 (C=S). ¹H-NMR (δ, ppm): 1.28 (m, -CH₂-, 2H), 1.54 (m, -CH₂-, 2H), 2.04 (m, -CH₂-, 2H), 2.29 (t, aliphatic -CH₂-C(O)-, 2H), 2.37 (t, aromatic -CH₂-C(O)-, 2H), 7.02 (t, *p*-CH, 1H), 7.28 (t, *m*-CH, 2H), 7.58 (d, *o*-CH, 2H), 9.32 (br, NH₂, 1H *d*₆-DMSO exchangeable), 9.65 (br, NH₂, 1H *d*₆-DMSO exchangeable), 9.85 (s, -NH-, 1H), 11.04 (-CONHCS-, 1H *d*₆-DMSO exchangeable). ¹³C-NMR (δ): 24.77, 24.9, 28.32, 34.94, 36.27, 119.02, 122.92, 128.60, 139.26, 171.23, 174.37, 174.69.

***In vitro* cytotoxicity study**

Maintenance of cell cultures

HRT-18 (human colon adenocarcinoma), HC-04 (mouse hepatic carcinoma), and HBL-100 (epithelial cells obtained from healthy human breast milk) cell lines were maintained in RPMI-1640 medium that was fortified with solutions of 10% fetal bovine, 100 IU/mL penicillin, and 100 µg/mL streptomycin. The cells were subcultured with Trypsin-EDTA, re-seeded at 80% confluence two times a week, and incubated at 37 °C [4].

Cytotoxicity assay

MTT cell viability assay was done using 96-well plates. Cell lines were seeded at 1×10^4 cells/well. After either 24 hours or a confluent monolayer was achieved, cells were treated with compounds **4** and **5** separately at different concentration (6.25, 12.5, 25, 50, 100 µM). Cell viability was measured after 72 hours of treatment by removing the medium, adding 28 µL of 2 mg/mL solution of MTT stain and incubating the cells for 2.5 hours at 37 °C. After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 130 µL of dimethyl sulfoxide followed by 37 °C incubation for 15 minutes with shaking [5]. The absorbency was determined on a microplate reader at 492 nm; the assay was performed in triplicate.

The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated according to the following equation:

$$\text{Cytotoxicity (\%)} = \frac{A - B}{A} \times 100$$

where *A* is the optical density of the control and *B* is the optical density of the sample.

To visualize the shape of cells under an inverted microscope, 200 µL of cell suspensions were seeded in 96-well micro-titration plates at density 1×10^4 cells/ mL

and incubated for 48 hours at 37 °C. Then the medium was removed and each of compounds **4** and **5** was added at its (IC₅₀) separately. After the exposure time, crystal violet solution (50 µL) was used to stain the plates. These plates were then incubated at 37 °C for 15 minutes followed by gentle washing with water to remove the dye. A 40x field magnification of an inverted microscope was chosen to examine the cells. Photography was carried out using a digital camera.

Statistical analysis

The obtained data were statically analyzed using an unpaired t-test with GraphPad Prism 6. The values were presented as the mean ± SEM of triplicate measurements.

Molecular docking

The chemical structures of the docked ligands were drawn with ChemBioDraw Ultra 12.0 software and converted into 3D structure with ChemBio3D Ultra 12.0 software. Then the ligands were subjected to energy minimization with the latter using its MM2 job and saved as mol2 files. This latter file was further processed by USCF Chimera software by the addition of Gasteiger charges if applicable.

Only the chain to which the co-crystallized ligand is bound was kept. All unnecessary ions, water molecules, and bound ligands were deleted too. Then hydrogen atoms were added and Gasteiger charges were assigned to simulate *in vivo* conditions. The prepared protein was then saved as a mol2 file.

Configuring AutoDock Vina software involved setting the path for the output files (pdbqt and pdb files), assigning center and size values for the search volume (25.709, -15.81, 1.122 for 4LXZ and -6.497, 2.775, -15.65 for 3ZNR), and setting options for both the enzyme and the ligand into their default values. After completion of the docking runs, the scores of enzyme-ligand free energies of binding were obtained with the best pose of each being of the lowest free energy (ΔG). The inhibition constant (K_i) for the best pose of each enzyme-ligand docking was calculated by the equation:

$$K_i = e^{\Delta G/RT}$$

where ΔG is the binding free energy (kcal. mol⁻¹), R is the gas constant (1.987 cal. mol⁻¹. K⁻¹), and T is the absolute temperature (298.15 K) [6].

References

1. TROTT, O.; OLSON, A. J. AutoDock Vina: Improving the Speed and Accuracy of Docking with a New Scoring Function, Efficient Optimization, and Multithreading. *J. Comput. Chem.* **2010**, *31*, 455–461. <https://doi.org/10.1002/jcc>.
2. Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera--a Visualization System for Exploratory Research and Analysis. *J. Comput. Chem.* **2004**, *25* (13), 1605–1612. <https://doi.org/10.1002/jcc.20084>.
3. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. The Protein Data Bank. *Nucleic Acids Res.* **2000**, *28* (1), 235–242. <https://doi.org/10.1093/nar/28.1.235>.
4. Sulaiman, G. M.; Jabir, M. S.; Hameed, A. H. Nanoscale Modification of Chrysin for Improved of Therapeutic Efficiency and Cytotoxicity. *Artif. Cells Nanomed. and Biotechnol.* **2018**, *46* (sup1), 708–720. <https://doi.org/10.1080/21691401.2018.1434661>.
5. Jabir, M. S.; Taha, A. A.; Sahib, U. I.; Taqi, Z. J.; Al-Shammari, A. M.; Salman, A. S. Novel of Nano Delivery System for Linalool Loaded on Gold Nanoparticles Conjugated with CALNN Peptide for Application in Drug Uptake and Induction of Cell Death on Breast Cancer Cell Line. *Mater. Sci. Eng. C* **2019**, *94* (March 2018), 949–964. <https://doi.org/10.1016/j.msec.2018.10.014>.
6. PATRICK J. SINKO. *Martin's Physical Pharmacy and Pharmaceutical Sciences. Physical Chemical and Biopharmaceutical Principles in the Pharmaceutical Sciences.*; Lippincott Williams & Wilkins: Baltimore, 2011.