3,5-Diamino-1,2,4-triazoles as a novel scaffold for potent competitive LSD1 (KDM1A) inhibitors

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Electronic Supplemental Information (ESI): complete experimental section including synthetic details and a description of all biological procedures, and an electrostatic contact map for LSD1 bound to **6**.

EXPERIMENTAL

CHEMISTRY

All reagents and dry solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI), Sigma Chemical Co. (St. Louis, MO), VWR (Radnor, PA) or Fisher Scientific (Chicago, IL) and were used without further purification except as noted below. Pyridine was dried by passing it through an aluminum oxide column and then stored over KOH. Triethylamine was distilled from potassium hydroxide and stored in a nitrogen atmosphere. Methanol was distilled from magnesium and iodine under a nitrogen atmosphere and stored over molecular sieves. Methylene chloride was distilled from phosphorus pentoxide and chloroform was distilled from calcium sulfate. Tetrahydrofuran was purified by distillation from sodium and benzophenone. Dimethyl formamide was dried by distillation from anhydrous calcium sulfate and was stored under nitrogen. Microwave procedures were carried out on a Biotage Initiator 8 microwave synthesizer. Preparative scale chromatographic procedures were carried out on a Teledyne Isco Combi-Flash Rf200 using silica gel 60 cartridges, 230-440 mesh. Thin layer chromatography was conducted on Merck precoated silica gel 60 F-254. Compound **6** was purchased from Ryan Scientific (Mt. Pleasant, SC) and compounds **7**-**20** were purchased from Chembridge (San Diego, CA). TCP was purchased from Acros Organics (Pittsburgh, PA).

All ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Mercury 400 mHz spectrometer, and all chemical shifts are reported as d values referenced to TMS or DSS. Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; p, pentuplet; m, multiplet; br, broad peak. In all cases, ^{1}H-NMR, ^{13}C-NMR and MS spectra were consistent with assigned structures. Mass spectra were recorded by LC/MS on a Waters autopurification liquid chromatograph with a model 3100 mass spectrometer detector. All target molecules **27**-**45** were determined to be >95% pure by UPLC chromatography (95% H_2O/5% acetonitrile to 20% H_2O/80% acetonitrile over 10 minutes) using a Waters Acquity H-series ultrahigh-performance liquid chromatograph fitted with a C18 reversed-phase column (Acquity UPLC BEH C18 1.7 mM, 2.1 X 50 mm).

General procedure for the preparation of 2-chloro-6-phenoxy benzonitriles: Synthesis of 2-chloro-6-phenoxybenzonitrile **23**. To a 20 mL microwave vial containing a magnetic stir bar was added 0.78 g (5.0 mmol) of 2-fluoro-6-chlorobenzonitrile, 1.04 g (7.5 mmol) of K_2CO_3, 0.52 g (5.5mmol) of phenol and 12 mL of anhydrous DMSO. The vial was then sealed and stirred to distribute the contents evenly. The mixture was then microwaved at 190°C for 6 min at high absorption to insure even heating. The reaction mixture was then poured into a beaker containing 100 mL of crushed ice to precipitate the product. The aqueous layer was extracted with three 50 mL portions of diethyl ether, and the ether layer was washed with 25 mL of saturated NaCl, dried over anhydrous Na_2SO_4, filtered, and the ether was removed in vacuo to yield 1.11 g of the desired diaryl ether **23** (97% yield). The crude product **23** was pure enough (95%) to be used in the next reaction without further purification.

General procedure for the preparation of 2-chloro-6-phenoxy benzylamines: Synthesis of 2-chloro-6-phenoxybenzylamine **24**. A 1.11 g portion of **23** (4.8 mmol) was dissolved in 50 mL of anhydrous diethyl ether, cooled to 0°C in an ice bath and stirred while bubbling dry argon into the reaction mixture for 10 min. A 14.49 mL portion of 1.0 M $LiAlH_4$ in THF (14.49 mmol) was then added dropwise with stirring over 20 min. The resulting reaction mixture was allowed to stir for 2hrs at 0°C, and then warmed to room temperature and allowed to stir overnight. The mixture was cooled to 0°C, and the reaction was quenched by the slow addition of Na_2SO_4•10 H_2O. When the evolution of gas subsided, the reaction was stirred for 30 min at room temperature, and the mixture was filtered through a Celite pad. The filtrate was concentrated to dryness to yield crude 2-chloro-6-benzoxybenzylamine **24**.

General procedure for the preparation of target molecules 27-45: Synthesis of N^3-(2-chloro-6-phenoxybenzyl)-4*H*-1,2,4-triazole-3,5-diamine **6**. A 0.935 g portion of benzylamine **24** (4.0 mmol) was dissolved in 12 mL of diethyl ether and added to a 20 mL microwave vial equipped with a magnetic stir bar. A 0.702 g portion of dimethyl cyanodithioiminocarbonate **25** (4.8 mmol) was added and the vial was sealed. The contents were microwaved at 45°C for 5 min, cooled to room temperature, and the ether was removed *in vacuo* to yield the intermediate **26** as a white to pale yellow solid. A 0.192 g portion of hydrazine hydrate (6.0 mmol) in 12 mL of dry ethanol was then injected, the vial was stirred to break up the solid intermediate, and the resulting mixture was microwaved at 90°C for 10 min at high absorption. The ethanol was removed *in vacuo* to yield crude **6**, which was purified on silica (9% MeOH in CH_2Cl_2) to afford 1.07 g of pure **6** (85%) as an off-white, amorphous solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 4.21 (s, 2H), 6.77-6.80 (dd, 1H), 6.99-7.01 (d, 2H), 7.11-7.15 (t, 1H), 7.20-7.27 (m, 2H), 7.33-7.38 (t, 2H). UPLC retention time: 12.1 min. MS calculated 315.09, found 316.33 ($[M+1]^+$)

N^3-(2-chloro-6-(4-(trifluoromethoxy)phenoxy)benzyl)-4*H*-1,2,4-triazole-3,5-diamine **27**. Compound **27** was synthesized exactly as described above in 86% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 4.31 (s, 2H), 6.72 (s, 1H), 6.85-6.87 (dd, 2H), 7.04-

7.08 (d, 1H), 7.26-7.33 (m, 2H), 7.49 (s, 1H). UPLC retention time: 15.5 min. MS calculated 399.07, found 400.27 ([M+1]$^+$)

N^3-(2-chloro-6-(*p*-tolyloxy)benzyl)-4*H*-1,2,4-triazole-3,5-diamine **28**. Compound **28** was synthesized exactly as described above in 82% yield as a white solid.^{1}H-NMR (400MHz, CD_3OD/TMS) δ 2.17 (s, 3H), 4.39 (s, 2H), 6.71-6.78 (m, 3H), 7.19 (d, 1H), 7.37-7.45 (m, 2H). UPLC retention time 14.4 min. MS calculated 329.79, found 330.36 ([M+1]$^+$)

N^3-(2-chloro-6-(2-isopropyl-5-methylphenoxy)benzyl)-4*H*-1,2,4-triazole-3,5-diamine **29**. Compound **29** was synthesized exactly as described above in 78% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 1.15-1.21 (d, 6H), 2.26 (s, 3H), 3.09-3.15 (m, 1H), 4.07 (s, 2H), 6.56-6.58 (dd, 1H), 6.97-7.00 (1H), 7.13-7.25 (m, 3H), 7.51 (s, 1H). UPLC retention time 16.0 min. MS calculated 371.15, found 372.37 ([M+1]$^+$)

N^3-(2-chloro-6-(3-methoxyphenoxy)benzyl)-4*H*-1,2,4-triazole-3,5-diamine **30**. Compound **30** was synthesized exactly as described above in 81% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 3.80 (s, 3H), 4.29 (s, 2H), 6.56-6.59 (dd, 1H), 6.60-6.61 (t, 1H), 6.72-6.75 (dd, 1H), 6.85-6.88 (dd, 1H), 7.25-7.32 (m, 3H). UPLC retention time 15.5 min. MS calculated 345.10, found 346.30 ([M+1]$^+$)

N^3-(2-(4-(*tert*-butyl)phenoxy)-6-chlorobenzyl)-4*H*-1,2,4-triazole-3,5-diamine **31**. Compound **31** was synthesized exactly as described above in 81% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 1.35 (s, 9H), 4.02 (s, 2H), 6.78-6.81 (dd, 1H), 6.95-6.98 (dt, 2H), 7.21-7.28 (m, 2H), 7.42-7.45 (dt, 2H). UPLC retention time 16.1 min. MS calculated 371.15, found 372.37 ([M+1]$^+$)

N^3-(2-chloro-6-(3,5-dimethylphenoxy)benzyl)-4*H*-1,2,4-triazole-3,5-diamine **32**. Compound **32** was synthesized exactly as described above in 74% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 2.30 (s, 6H), 4.41 (s, 2H), 6.64 (s, 2H), 6.81 (s, 2H), 7.21-7.29 (m, 2H). UPLC retention time 14.5 min. MS calculated 343.82, found 344.33 ([M+1]$^+$)

N^3-(2-(3,5-bis(trifluoromethyl)phenoxy)-6-chlorobenzyl)-4*H*-1,2,4-triazole-3,5-diamine **33**. Compound **33** was synthesized exactly as described above in 77% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 4.01 (S, 2H), 7.14 (S, 1H), 7.25 (S, 2H), 7.33 (S, 1H), 7.49 (S, 1H), 7.57 (S, 1H). UPLC retention time 15.4 min. MS calculated 451.76, found 452.63 ([M+1]$^+$)

N^3-(2-chloro-6-(4-((trifluoromethyl)thio)phenoxy)benzyl)-4*H*-1,2,4-triazole-3,5-diamine **34**. Compound **34** was synthesized exactly as described above in 79% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 4.22 (s, 2H), 7.14 (d, 1H), 7.28-7.39 (m, 5H), 7.40 (d, 1H). UPLC retention time 14.0 min. MS calculated 415.05, found 416.24 ([M+1]$^+$)

N^3-(2-(4-bromo-2-(trifluoromethyl)phenoxy)-6-chlorobenzyl)-4*H*-1,2,4-triazole-3,5-diamine **35**. Compound **35** was synthesized exactly as described above in 61% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 4.28 (s, 2H), 7.04 (d, 1H), 7.22 (d, 1H), 7.27 (dd, 1H), 7.44 (d, 1H), 7.55 (d, 1H), 7.71 (s, 1H). UPLC retention time 15.4 min. MS calculated 460.99, found 462.18 ([M+1]$^+$)

N^3-(2-chloro-6-(4-((methyl)thio)phenoxy)benzyl)-4*H*-1,2,4-triazole-3,5-diamine **36**. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 2.32 (s, 3H), 4.07 (s, 2H), 7.14 (d, 1H), 7.31 (m, 2H), 7.39-7.47 (m, 2H), 7.55 (d, 1H). UPLC retention time 14.6 min. MS calculated 361.08, found 362.23 ([M+1]$^+$)

N^3-(2-chloro-6-(2-methoxy-4-methylphenoxy)benzyl)-4*H*-1,2,4-triazole-3,5-diamine **37**. Compound **37** was synthesized exactly as described above in 40% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 2.40 (s, 3H), 3.75 (s, 3H), 6.54-6.57 (m, 3H), 6.83-6.88 (d, 1H), 7.06-7.18 (m, 2H). UPLC retention time 14.7 min. MS calculated 359.11, found 360.29 ([M+1]$^+$)

N^3-(2-chloro-6-(3,5-dimethoxyphenoxy)benzyl)-4*H*-1,2,4-triazole-3,5-diamine **38**. Compound **38** was synthesized exactly as described above in 76% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 3.75 (s, 6H), 6.14 (s, 1H), 6.22 (s, 2H), 6.90 (d, 1H), 7.26-7.33 (m, 2H). UPLC retention time 14.1 min. MS calculated 375.11, found 376.29 ([M+1]$^+$)

N^3-(2-chloro-6-(2,3-dimethylphenoxy)benzyl)-4*H*-1,2,4-triazole-3,5-diamine **39**. Compound **39** was synthesized exactly as described above in 43% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 2.14-2.21 (m, 3H), 2.35-2.37 (m, 3H), 6.52 (d, 1H), 6.73-6.79 (m, 3H), 7.01-7.20 (m, 2H). UPLC retention time 15.0 min. MS calculated 343.12, found 344.33 ([M+1]$^+$)

N^3-(2-(benzo[*d*][1,3]dioxol-5-yloxy)-6-chlorobenzyl)-4*H*-1,2,4-triazole-3,5-diamine **40**. Compound **40** was synthesized exactly as described above in 80% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 4.41 (s, 2H), 5.51 (s, 2H), 6.00 (s, 1H), 6.99-7.11 (m, 3H), 7.33-7.39 (m, 2H). UPLC retention time 13.7 min. MS calculated 359.08, found 360.25 ([M+1]$^+$)

N^3-(2-chloro-6-(phenylthio)benzyl)-4*H*-1,2,4-triazole-3,5-diamine **41**. Compound **41** was synthesized exactly as described above in 91% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 4.31 (s, 2H), 7.19 (m, 1H), 7.36-7.44 (m, 6H), 7.61 (d, 1H). UPLC retention time 14.2 min. MS calculated 331.07, found 332.28 ([M+1]$^+$)

N^3-(2-chloro-6-((3,4-dimethoxyphenyl)thio)benzyl)-4*H*-1,2,4-triazole-3,5-diamine **42**. Compound **42** was synthesized exactly as described above in 82% yield as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 3.67 (s, 6H), 4.40 (s, 2H), 6.56 (m, 2H), 7.04 (m, 2H), 7.39 (d, 1H), 7.49 (d, 1H). UPLC retention time 13.7 min. MS calculated 391.09, found 392.29 ([M+1]$^+$)

N^3,N^5-bis(2-methoxybenzyl)-1*H*-1,2,4-triazole-3,5-diamine (commercial compound **7**). Compound **7** was purchased from Chembridge (San Diego, CA) as a white solid. ^{1}H-NMR (400MHz, CD_3OD/TMS) δ 3.64 (s, 6H), 4.17 (s, 2H), 6.86-6.90 (t, 2H), 6.94-6.96 (m, 4H), 7.21-7.27 (m, 2H). UPLC retention time 13.7 min. MS calculated 339.17, found 340.28 ([M+1]$^+$)

Cell Culture and Reagents. Calu-6 cells (human lung adenocarcinoma ATCC-HTB-56) were purchased from ATCC, and cultured in EMEM growth medium containing 10% (v/v) fetal bovine serum and 5% penicillin and streptomycin. Cultures were grown at 37°C in a humidified environment containing 5% CO_2. For each experiment, cells were seeded at a starting density of 400,000 cells per T25 flask.

Determination of cell viability. For the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) reduction assay, 4000 cells/well were seeded in 100 µl medium in a 96¯well plate and the cells were allowed to attach at 37oC in 5% CO2 for one day. The medium was aspirated and cells were treated with 100 µl of fresh medium containing appropriate concentrations of each test compound. The cells were incubated for 4 days at 37°C in 5% CO2. After 4 days 20 µL of the MTS reagent solution (Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay) was added to the medium. The cells were incubated for another 2 hours at 37°C under 5% CO2 environment. Absorbance was measured at 490 nm on a microplate reader equipped with SOFTmax PRO 4.0 software to determine the cell viability. Cell counts were confirmed by counting DAPI stained nuclei as previously mentioned.Absorbance was measured at 490 nm on a microplate reader equipped with SOFTmax PRO 4.0 software to determine the cell viability.

In vitro LSD1 demethylation assay and kinetics. Inhibition assays and kinetics were performed using LSD1 Inhibitor Screening Assay Kit (Cayman Chemical, #700120). The substrate and all compounds were incubated in assay buffer from 30 min up to 4 hr at 37°C as described in the commercial protocol. The volume of each reaction well was 50 µl, containing 5 ml of a 200 mM solution of substrate peptide and 20 ml of a 15ng/ml enzyme solution. All compounds were diluted in 1% DMSO with assay buffer to a final volume of 50 mM. Fluorescence was measured at the recommended wavelengths of λ_{ex}=530 nm, λ_{em}-590 nm. IC_{50} determinations were performed using serial dilutions at 10, 5, 2.5, 1.25. 0.625, 0.3125 and 0.156 mM)..

In silico molecular modeling. LSD1 active site (PDB #3ZMT, LSD1-CoREST in complex with PRSFLV peptide) was defined as a sphere enclosing residues within 10Å around the crystallographic peptide ligand. Prior to energy minimization, LSD1 was protonated and the PH was set to 7.4. The 3D structure of inhibitors was energy minimized using MM94x force field for 1000 iterations and a convergence value of 0.001 kcal/mol/Å as the termination criterion. Initial docking results yielded 60 poses of each structure bound the active site of LSD1. The top 5 poses that yielded the lowest E-score were chosen for further analysis. The best fit for binding was analyzed for interacting residues. Key interactions with compound **6** include two hydrogen bonds with aspartate 555 and another hydrogen bond with the carbonyl of alanine 539. In addition, the compound participates in pi-stacking with the flavin ring of the FAD cofactor within 2.98Å. Thus, compound **6** shows close association with the active site and effectively prohibits substrate binding.

Nanoisothermal calorimetry (ITC). Experiment was performed on Nano-ITC Low Volume (TA Instruments). Initial setup protocols were run with 55.6M H_20/H_20 titrations and run to remove any background heat release upon injection. Using 50mM PBS, buffer was added to the system with 20 injections to remove any buffer background. Compound **6** was then diluted to 1µM with <1% DMSO, and injected into the system to eliminate ligand background peaks. After equilibrating the system, LSD1 was diluted to 500 nM, added to the cell, and titrated in with 10 injections of compound **6**.

Monoamine oxidase A and B activity assay. MAO/A/B activity was measured with the luminescent MAO-Glo assay kit (Promega, #V1452) according to the manufacturer's instructions. In brief, total MAO activity was assayed by incubation of compounds with MAO/A or MAO/B enzyme solution containing MAO substrate according to the suppliers directions. Reconstituted luciferin detection reagent was added, and the resulting luminescent signal was detected with 0.5 integration time after 20 mins.

Immunofluorescence staining of global methylation. Cells were seeded at $1x10^3$ cells/well and were then stained for Immunofluorescence (IF) imaging using fluorescently labeled secondary antibodies. Cells were fixed and permeabalized as mentioned above, and then blocked with 10% Normal Donkey Serum (NDS) for 1hr, washed with PBS, and incubated overnight at 4°C with the H3K4me2 antibody (Cell Signaling, #2139S). Fluorescent secondary antibodies were added to corresponding wells at 1:500 dilutions in 1% NDS for 2 hours. Cells were washed, left in 1X PBS and imaged using Hermes WiScan (IDEA Biomedical). The imaging system is able to view 10-40x pictures as well as quantify average intensity on a per-cell basis, eliminating any bias towards IF staining. Quantification of H3K4me2 marks was presented as a frequency distribution of the cell count population.

Cell Viability/Cytotoxicity Assay. For the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) reduction assay, $3x10^3$ cells/well were seeded in 100µl medium in a 96-well plate and allowed to attach at 37°C in 5% CO2 for one day. The medium was aspirated and cells were treated with 100µl of fresh medium containing appropriate dose-response concentrations of each test compound. The cells were incubated from 48-72hrs at 37°C in 5% CO2. After, 20µL of the MTS reagent solution (Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay) was added to the medium. The cells were incubated for another 2 hours at 37°C. Absorbance was measured at 490 nm on a microplate reader equipped with SOFTmax PRO 4.0 software to determine the cell viability. Cell counts were confirmed by counting DAPI stained nuclei as previously mentioned.

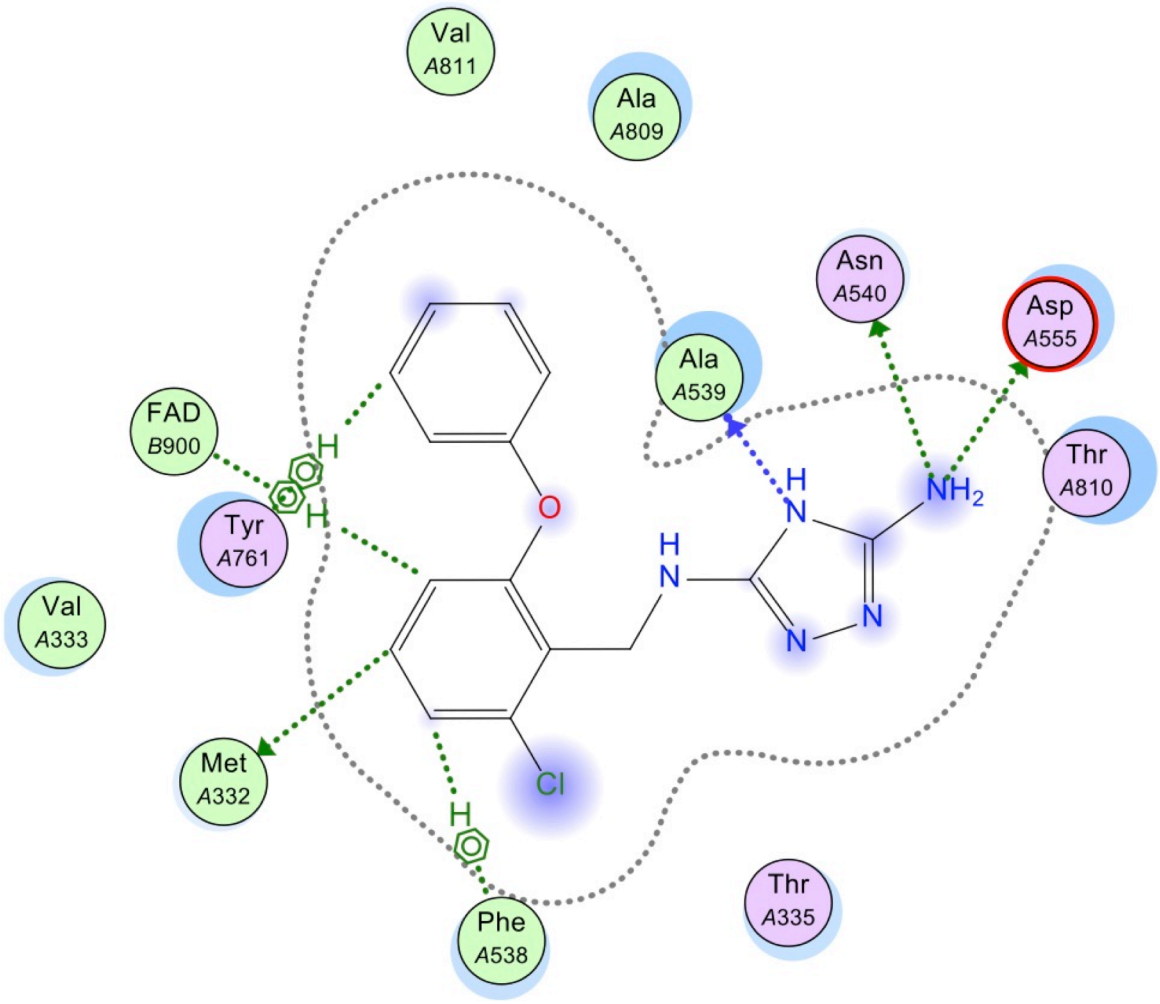

Figure S1. Electrostatic contacts for the least energy pose of compound **6** in the active site of LSD1/CoREST. Key interactions with compound **6** include two hydrogen bonds with aspartate 555 and another hydrogen bond with the carbonyl of alanine 539. In addition, the compound participates in pi-stacking between the phenoxy ring of compound **6** and the flavin ring of the FAD cofactor within 2.98Å.

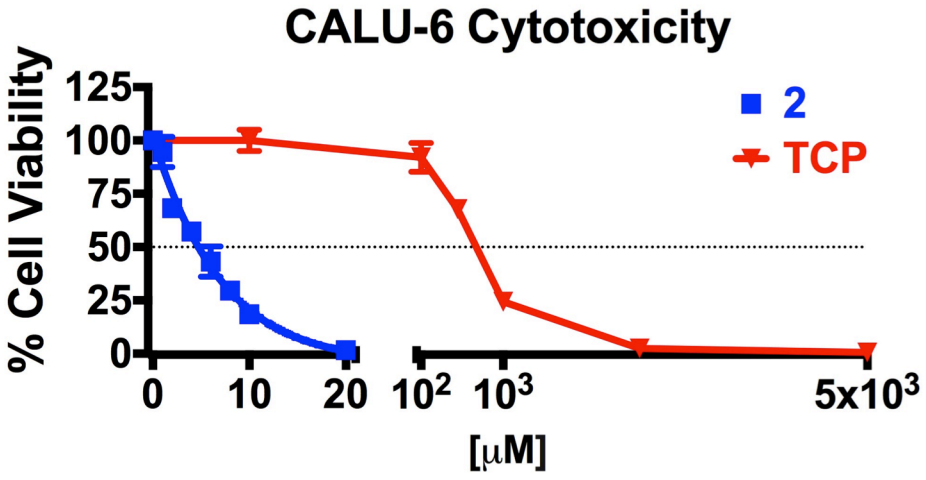

Figure S2. Comparison of the cytotoxic effects of verlindamycin (**2**) and TCP in Calu-6 human lung adenocarcinoma cells in vitro.

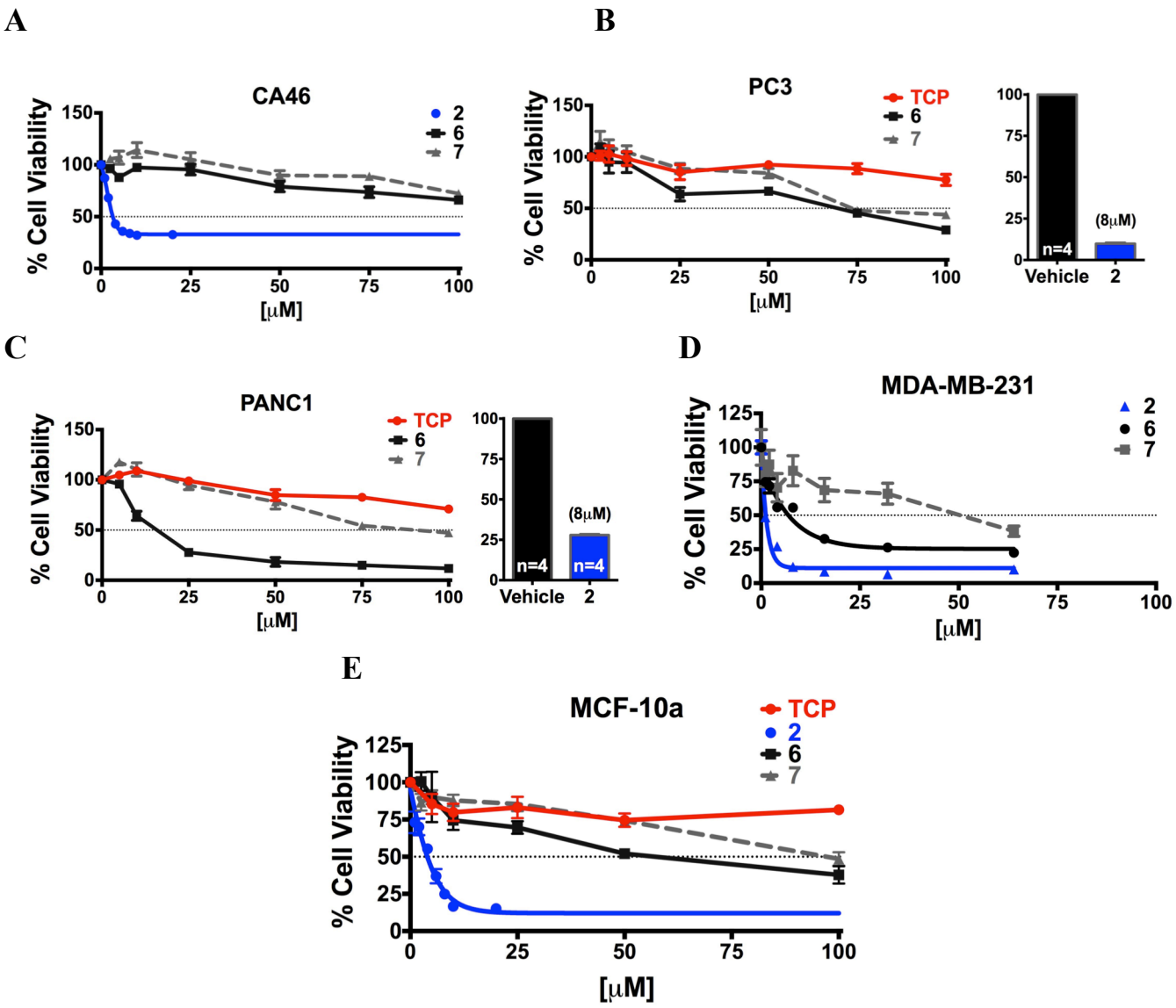

Figure S3. Comparison of the cytotoxicity of compounds **6** and **7** to known agents verlindamycin **2** and TCP in 5 cell lines in vitro using a standard MTS reduction assay. Panel A: CA46 Burlitt's Lymphoma cell line; Panel B: PC3 human prostate cancer cell line; Panel C: PANC-1 human pancreatic cancer cell line; Panel D: MDA-MB-231 estrogen receptor negative breast cancer cell line; Panel E: MCF-10A human breast epithelial cell line. In Panels B and C, verlindamycin **2** was run at 8 μM as a positive control, while in Panels A, D and E a dose-response curve was generated for **2**. Each data point is the average of 3 determinations ± standard error.