

Supporting Information

Discovery of a VHL and HIF1 α interaction inhibitor with in vivo angiogenic activity by structure-based virtual screening

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Materials and reagents

Compounds **1-16** (Commercial available, purity > 90%) were purchased from J&K Scientific Ltd., Hong Kong. Compound **51** was a gift from Prof. C. M. Crews (Departments of Chemistry, Molecular, Cellular & Developmental Biology and Pharmacology and Center for Molecular Discovery, Yale University, New Haven, USA). Dual-Luciferase® Reporter Assay System was purchased from Promega (Madison, US). HRE-Luciferase plasmid was purchased from Addgene (Addgene plasmid 26731). pRL-TK plasmid was purchased from Promega Corporation (Madison, WI, USA). Transfection Reagent obtained from Thermo Scientific (Thermo Scientific, St Leon-Rot, Germany). VEGF antibody, HIF1 α antibody, VHL antibody and β -actin antibody were purchased from Abcam (Abcam Inc., Cambridge, MA, USA). All the complexes were immersed in dimethyl sulfoxide (DMSO). XTT kit from Sigma-Aldrich (Santa Clara, CA).

Molecular docking and virtual screening.

Model construction. The initial model of VHL/HIF1 α was derived from the X-ray crystal structure of the VHL-HIF1 α complex co-crystallized with the a tri-acylated lipopeptide (PDB: 3ZRC),¹ using the molecular conversion procedure implemented in the ICM-pro 3.6-1d program (Molsoft).² The molecular conversion procedure implemented in ICM-pro 3.6-1d program can read, build, convert, refine, analyze and superimpose molecules, plus provide target evaluation to generate three dimensional models. Hydrogen and missing heavy atoms were added to the receptor structure, also atom types and partial charges were assigned. The model was then subjected to local energy minimization to identify the optimal position by using the ICM biased probability Monte Carlo algorithm³ and analytical derivatives in the internal coordinates. The optimization gradient was 45 kcal/mol/Å³.

High throughput molecular docking. A chemical library containing over 90,000 natural product or natural product-like compounds (ZINC natural product database) was docked to

the molecular model of VHL-HIF1 α *in silico*. Molecular docking was performed using the virtual library screening (VLS) module in the ICM-Pro 3.6-1d program (Molsoft). In the ICM fast docking and VLS procedure, the receptor all-atom model was converted into energy potential maps calculated on a fine 3D grid (0.5 Å cell). The grid potential maps account for van der Waals, hydrogen-bonding, hydrophobic, and electrostatic interactions between ligand and receptor. The search area for molecular docking was restricted to the interaction domain of VHL-HIF1 α . Each compound in the library was assigned the MMFF⁴ force field atom types and charges then subjected to Cartesian minimization. During the docking analysis, the ligand was represented by an all-atom model and considered fully flexible in the potential field of the receptor, the binding pose and internal torsions were sampled by the BPMC minimization procedure, which involved local energy minimization after each random move. Each compound was docked to the protein complex binding pocket and a score from the docking was assigned to each compound according to the weighed component of the ICM scoring function (see below). Each compound was docked three times to ensure the convergence of the Monte Carlo optimization, and the minimum score of each ligand from the three independent docking experiments was retained and used for ranking. The docking procedure takes about 30 s of time per compound on an Intel Xeon 2.8 GHz CPU using a 100 processor Linux cluster. A permissive cut-off score of -30.0 was chosen in order to weed out low-affinity ligands and to reduce the number of compounds tested *in vitro*. 16 compounds were purchased for in vitro biological testing.

ICM full-atom ligand-receptor complex refinement and scoring. Once the ligand-receptor complexes are generated by molecular docking, they have to be subjected to complex refinement and scoring. According to the ICM method,⁵ the molecular system was described using internal coordinates as variables. Energy calculations were based on the ECEPP/3 force field with a distance-dependent dielectric constant. The biased probability Monte Carlo

(BPMC) minimization procedure was used for global energy optimization. This procedure consisted of four iterative steps. The BPMC global-energy-optimization method consists of 1) a random conformation change of the free variables according to a predefined continuous probability distribution; 2) local-energy minimization of analytical differentiable terms; 3) calculation of the complete energy including non-differentiable terms such as entropy and solvation energy; 4) acceptance or rejection of the total energy based on the Metropolis criterion and return to step (1). The binding between the small molecules and VHL-HIF1 α were evaluated with a full-atom ICM ligand binding score⁶ from a multi-receptor screening benchmark as a compromise between approximated Gibbs free energy of binding and numerical errors. The scoring function should give a good approximation of the binding free energy between a ligand and a receptor, as well as a function of different energy terms based on a force-field. The ICM scoring function is weighed according to the following parameters (i) internal force-field energy of the ligand, (ii) entropy loss of the ligand between bound and unbound states, (iii) ligand-receptor hydrogen bond interactions, (iv) polar and non-polar solvation energy differences between bound and unbound states, (v) electrostatic energy, (vi) hydrophobic energy and (vii) hydrogen bond donor or acceptor desolvation. The lower the ICM score, the higher the chance the ligand is a binder. The score was calculated by:

$$S_{\text{bind}} = E_{\text{int}} + T\Delta S_{\text{Tor}} + E_{\text{vw}} + \alpha_1 E_{\text{el}} + \alpha_2 E_{\text{hb}} + \alpha_3 E_{\text{hp}} + \alpha_4 E_{\text{sf}}$$

whereas E_{vw} , E_{el} , E_{hb} , E_{hp} , and E_{sf} are van der Waals, electrostatic, hydrogen bonding, and nonpolar and polar atom solvation energy differences between bound and unbound states, respectively. E_{int} is the ligand internal strain, ΔS_{Tor} is its conformational entropy loss upon binding, and $T = 300$ K, and α_i are ligand- and receptor independent constants.⁷

Cell culture

Human embryonic kidney (HEK) 293T cells were cultivated in DMEM medium with 1% penicillin (100 units/ml)/streptomycin (100 $\mu\text{g/ml}$) and 10% fetal bovine serum (FBS). Cells

were maintained at a cell density of $1-2 \times 10^6$ cells/mL. Cells were cultured in an atmosphere of 5% CO₂ at 37 °C.

Fluorescence polarization assay

VHL complex, fluorescent ligand FAM-DEALA-Hyp-YIPD and compounds were diluted with VHL buffer (50 mM Tris, 200 mM NaCl, 2 mM DTT, pH 7.5). For fluorescence polarization assay, per well of a 384 well plate containing 9 µL of 1µM VHL complex (450 nM final), 9 µL of 278 nM FAM-DEALAHyp-YIPD. Before read fluorescence polarization on a SpectraMax M5 microplate reader (Molecular Devices, excitation 485 nM, emission 520 nM), the plate was shaken 1 minute, then centrifuged 1 minute.

Transient transfection

HEK293T cells were seeded in six well plates 24 h before transfection. HRE-luciferase plasmid, pRL-TK plasmid and TurboFect reagent were mixed together in DMEM medium and the resulting solution was incubated for 20 min at room temperature. The mixture was added dropwise to the HEK293T cells in the wells. The cells were incubated for 32 h at 37 °C in a CO₂ incubator before use.

Dual luciferase reporter assay

The inhibition of HIF1 α activity was assayed by a reporter assay using a dual luciferase reporter assay system (Promega, Madison, WI, USA), as previously described.⁸ Transiently transfected cells were treated with compounds or **51** in normoxic conditions for 8 h before measurement. Luciferase activity was integrated over a 10 second period and measured using a spectrophotometer (Spectra-max M5, Molecular Devices, USA). The results were standardized with the activity of Renilla luciferase. All data are expressed as mean \pm SD.

Western blotting

Cells harvested from six-well plates, washed with ice-cold PBS, and lysed with RIPA buffer (Millipore). Cell extracts were prepared and protein samples were collected. Western blotting analysis was performed as described⁹.

Co-IP assay

The co-IP assay was performed as previous described¹⁰. Briefly, HEK293T cells were seeded at the density of 1×10^6 cells in a six-well palte. Cells were treated with the indicated concentrations of compound **1** for 8 h under normoxia conditions (5% CO₂). Cells were lysed and collected the portein samples. The concentration of protein samples was calculated using the Pierce BCA protein assay kit. 30 µg of each protein sample were incubated 12 h with 10 µL pre-incubated anti-HIF1α magnetic beads according to the manufacturer's protocol. The complex was washed 5 times to elute non-specific and non-cross-linked antibodies. Then, the precipitated proteins were subjected to SDS-PAGE and analysed by Western blotting with anti-VHL (1:1,000, Abcam, Cambridge, MA) or anti-HIF1α (1:1,000, Abcam, Cambridge, MA).

Cytotoxicity experiment

HEK293T cells were seeded at the density of 5,000 cells per well in 96-well plates and incubated for 48 h. Compound **1** dissolved in DMSO was added to cells at final concentrations ranging from 10 nM to 3000 µM for 48 h. Added 10 µL of the prepared XTT mixture in per well with mix gently for a further 2 h. Before starting test, shake the plate one mins at room temperature in the dark. The cytotoxicity of compound **1** was exhibited as the percentage of absorbance in SpectraMax M5 microplate reader at a wavelength of 450 nm.

VEGF detected by ELISA

The experiment was performed as previously described.¹¹ The concentration of VEGF in the given condition from hepatoma cell line HEK293T was measured using a ELISA kit (sigma).

The HEK293T cells (5×10^5 /well) were incubated 16 h in six-well culture dishes in DMEM medium with 1% penicillin (100 units/ml)/streptomycin (100 μ g/ml) and 10% fetal bovine serum (FBS). Compound **1** dissolved in DMSO was added to cells with various concentrations for 8 h.

Zebrafish experiments

Zebrafish embryo preparation was performed as previously described.¹² Transgenic Tg(fli-1:EGFP) zebrafish were kept separately with a 14 h light/10 h dark cycle under standard conditions. Zebrafish embryos were generated by natural pair-wise mating (3–12 months old) and were raised at 28.5 °C in embryo water. 24 hpf zebrafish embryos were collected, distributed into a 12-well microplate with 6 fish in each well and co-treated with 300 nM VRI (VEGFR tyrosine kinase inhibitor II) and indicated concentration compound for 24 h. Embryos receiving embryo water with 0.1% DMSO served as a vehicle control and were equivalent to no treatment. All of these experiments were repeated three times, with 8 embryos per group.

Statistical analysis

For statistical analysis, all data were analyzed with one-way analysis of variance (ANOVA) followed by the Dunnett's method for multiple comparisons by using GraphPad Prism 6.0. A significant difference was defined as $P < 0.05$.

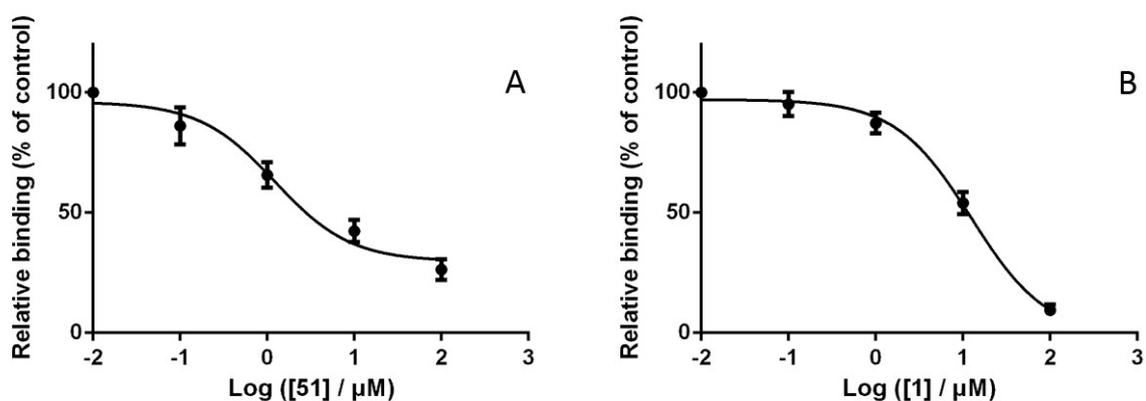


Fig. S2 Dose-dependent effect of compound **1** or **51** on inhibition of VHL-HIF1 α as determined by a fluorescence polarization assay. IC₅₀ values of **1** and **51** are *ca.* 2.29 μ M and 10.08 μ M, respectively. Error bars represent the standard deviations of results obtained from three independent experiments.

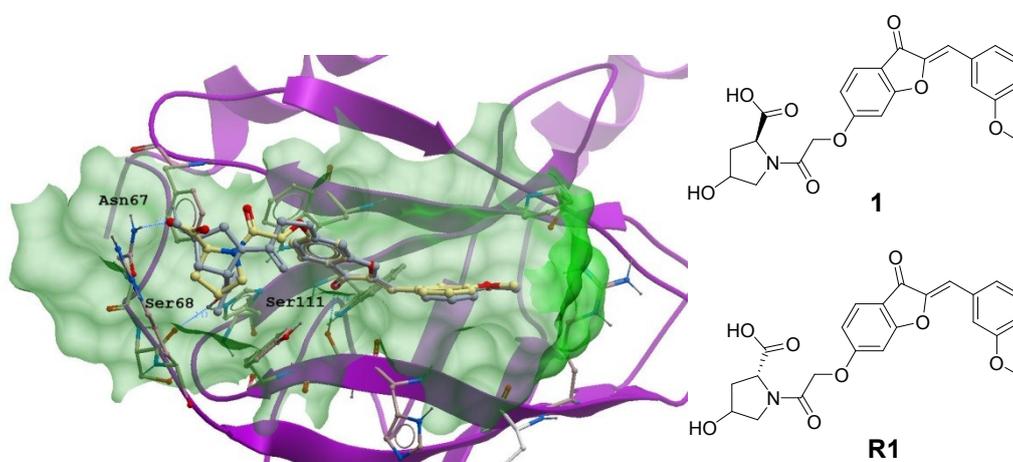


Fig. S3 Overlay of the docking poses of **1** and its enantiomer **R1** to the VHL-HIF1 α heterodimer. Compound **1** is shown in gold color and compound **R1** is shown in gray color.

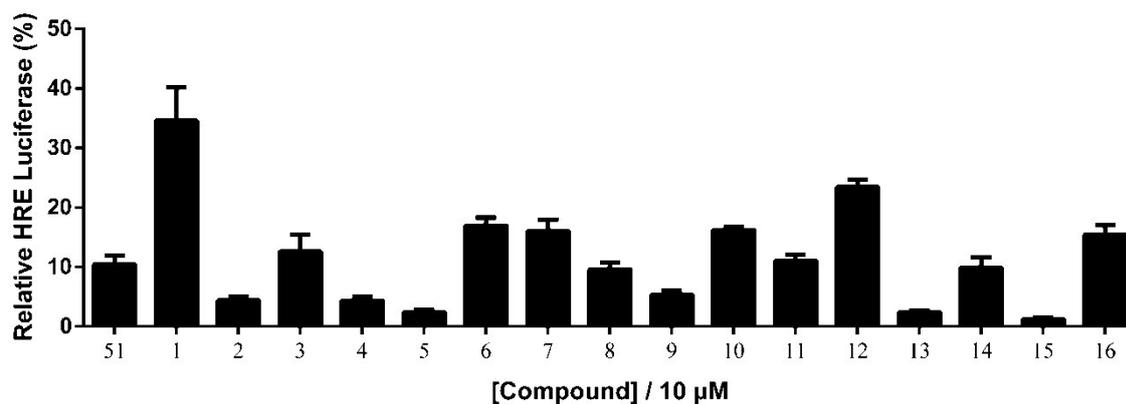


Fig. S4 Effect of compounds **1–16** on HRE activity as determined by a dual luciferase reporter assay. HEK293T cells were treated with 10 μM of compounds or **51** for 8 h under normoxic conditions (5% CO_2). Error bars represent the standard deviations of results obtained from three independent experiments.

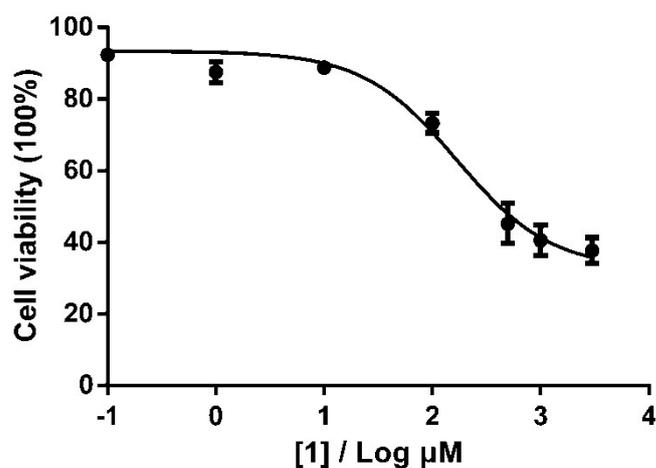


Fig. S5 The cytotoxicity effect of compound **1** on HEK293T cells as determined by an XTT assay. HEK293T cells were exposed to the indicated concentrations of **1** for 48 h. The IC_{50} value of **1** is *ca.* 724.3 μM . Error bars represent the standard deviations of the results from three independent experiments.

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