Detailed methods for "Dihydrotanshinone as a natural product-based CYP17A1 lyase inhibitor for hyperandrogenic disorders".

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Materials and methods

2.1. Reagents and materials

Dried plant material of Salvia miltiorrhiza (SM) was purchased from Inner Mongolia Leihetang Company. The material was naturally dried at room temperature, ground into a powder, and extracted with analytical-grade acetone. The powder was soaked in acetone in an amber glass container and stored in the dark at room temperature for 24 hours with intermittent shaking. The mixture was then filtered through filter paper. The residue was re-extracted once under the same conditions, and the filtrates were combined. The solvent was removed by passive evaporation (5–7 days) in a fume hood until the crude extract was dry. The extract was weighed to determine the extraction yield and stored in sealed amber vials at 4°C (short-term) or −20°C (long-term).

Abiraterone acetate was acquired from MedChemExpress through Lucerna Chem AG, (Lucerne, Switzerland). Radiolabeled substrates Progesterone [4-^{14}C] (specific activity: 55 mCi/mmol; concentration: 0.1 mCi/mL), and 17α-Hydroxypregnenolone [21-^{3}H] (specific activity: 15 Ci/mmol; concentration: 1 mCi/mL), were purchased from American Radiolabeled Chemicals Inc., (St. Louis, MO, USA). Non-radioactive compounds such as pregnenolone, progesterone, 17α-hydroxy pregnenolone, resazurin sodium salt, and dimethyl sulfoxide (DMSO) were procured from Sigma-Aldrich, St. Louis, MO, USA. Organic solvents were obtained from Carl Roth® GmbH+ Co. KG, Karlsruhe, Germany, and activated charcoal from Merck AG, Darmstadt, Germany. Silica gel-coated aluminum-backed TLC plates were purchased from Macherey-Nagel, Oensingen, Switzerland. Tritium screens for autoradiography were provided by Fujifilm, Dielsdorf, Switzerland.

2.2. Cell cultures

Human adrenocortical NCI-H295R cells (ATCC: CRL-2128) were purchased from ATCC and cultured in DMEM/Ham's F-12 medium with L-glutamine and 15 mM HEPES (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 5% NU-I serum (Becton Dickinson, Franklin Lakes, NJ, USA), 0.1% insulin, transferrin, selenium (100 U/mL; Thermo Fisher Scientific, Waltham, MA, USA), and 1% penicillin (100 U/mL; Thermo Fisher Scientific, Waltham, MA, USA), and streptomycin (100 µg/mL; GIBCO). RWPE-1 cells (ATCC: CRL-36O7) were cultured in keratinocyte serum-free media (K-SFM) supplemented with 0.05 mg/mL bovine pituitary extract and 5 ng/mL epidermal growth factor, and 1% penicillin (100 U/mL; Thermo Fisher Scientific, Waltham, MA, USA), and streptomycin (100 µg/mL; GIBCO). HEK-293T (ATCC: CRL-3216) cells cultured in DMEM medium with L-glutamine and 15 mM HEPES (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), and 1% penicillin (100 U/mL; Thermo Fisher Scientific, Waltham, MA, USA), and streptomycin (100 µg/mL; GIBCO). Passage numbers remained below 30 as per standard procedures. All cell lines were validated and characterized before use to ensure their authenticity and reliability. Routine mycoplasma testing was conducted to confirm that the cells were free from contamination. Additionally, key functional and morphological characteristics were assessed to verify their alignment with previously reported profiles.

2.3. Alamar blue assay

RWPE-1 cells, HEK-293 cells, and NCI-H295R cells were plated in 96-well plates at 10,000 cells per well and incubated overnight at 37°C with 5% CO2. After 24 h, the medium was replaced with fresh medium containing the SM at a final concentration between 10-0.01 µg/ml. And the pure components from SM (TAI, TAII, and DT) at a final concentration between 10-0.01 µM. Cells were incubated for an additional 24 h. Cell viability was measured using the Alamar Blue assay (1-5). Post-incubation, 0.05 mg/mL Alamar Blue solution in phosphate-buffered saline (PBS) was added to each well. The plates were incubated in the dark at 37 °C with 5% CO2 for 4 h, and fluorescence was measured at an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Cell viability was calculated relative to the control sample treated with DMSO. All experiments were conducted in triplicate (1, 3).

2.4. CYP17A1 assays

Assay of CYP17A1 17α-hydroxylase and 17,20 lyase activities were performed as described previously (1, 3, 6-14). The 17α-hydroxylase activity was measured by conversion of Prog to 17OH-Prog, while production of DHEA from 17OH-Preg was used for monitoring the 17,20 lyase activity of CYP17A1. In vitro CYP17A1 assays were performed using a liposome system consisting of POR and CYP17A1 at a ratio of 3:4 (POR: CYP17A1). The final assay mixture consisted of proteins (37.5 µmol POR: 50umol CYP17A1), 10 mM $MgCl_2$, 6mM KOAC 0.05 mg/ml DLPC,1mM reduced Glutathione in 50 mM HEPES buffer, and the reaction volume was 200 µL. The drugs were added to the final mixture and incubated for 1h before the catalytic reaction was initiated by the addition of NADPH to 1 mM final concentration. Radiolabeled [^{14}C]-PROG (10,000 cpm/µM) was used as a tracer. Steroids were extracted and separated by thin-layer chromatography (TLC) on silicagel (SIL G/UV254) TLC plates (Macherey-Nagel, Oensingen, Switzerland) as previously described (1, 2).

2.4.2 CYP17A1 lyase activity

Assay of CYP17A1 Lyase activity:. In vitro CYP17A1 assays were performed using POR, CYP17A1 and cytochrome b_5 in the ratio 4:3:3. The final assay mixture consisted proteins , 10 mM $MgCl_2$, 6mM KOAC 0.05 mg/ml DLPC,1mM reduced Glutathione in 50 mM HEPES buffer, and the reaction volume was 200 µL. The drugs were added to the final mixture and incubated for 1hr before the catalytic reaction was initiated by the addition of NADPH to 1 mM final concentration. Radiolabeled [^{3}H]-17OH-pregnenolone (50,000 cpm/µM) and the enzyme activity measured by using the Scintillating counter in the water release assay (1, 3, 10, 15-19).

2.5. CYP21A2 assays

Transient transfections were performed using the CYP21A2 WT vector (20). HEK293 cells were seeded in six-well plates (3×10^5 cells/well). After 24 hours, the growth medium was replaced, and cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Bedford, MA, USA) with 12.5 µg of plasmid (plasmid concentration: 1068 µg/ml) . Twenty-four hours after transfection, transfected cells were replated in 24-well plates (1.5×10^5 cells/well) to ensure homogeneity of the cell population across the wells. Six hours after transfection, 2 mL of fresh medium was replaced, and the cells were cultured for 48 hours. Cell growth was examined. If cells were growing well, SM, TAI, TAII, and DT were added at a concentration of 10 µg/mL for SM and 10 µM for TAI, TAII, and DT. DMSO was added to the negative control, and 10 µM abiraterone was added to the positive control, and the cells were incubated for 4 hours. Functional assays were initiated by adding 1 µM unlabeled progesterone (using 10,000 cpm

of [^{14}C]-progesterone as a tracer). After incubation at 37°C for 1 hour, the culture medium and cells were harvested. Steroids were extracted from the culture medium with ethyl acetate and isooctane (1:1 volume ratio), dried, and dissolved in dichloromethane. Steroids were separated by thin-layer chromatography (TLC), placed on a phosphor screen, and visualized using a Typhoon PhosphorImager FLA-7000 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Image intensity was measured and quantified using ImageQuant TL v8 (Cytiva, MA, USA). The CYP21A2 enzyme activity was expressed as relative steroid conversion. Steroids were quantified as a percentage of radioactivity incorporated into 11-deoxyprogesterone to the total radioactivity measured in the whole sample and compared between the WT and variants. Cells were collected with trypsin and washed with 1× PBS to quantify the amount of protein. Results were analyzed from three technical replicates. To ensure a similar amount of CYP21A2 in each reaction, a western blot analysis was performed to normalize the enzyme activity with the relative CYP21A2 expression.

2.5.1 Western blot

CYP21A2 expression was determined from total protein extracts. Cells were incubated with the previously described lysis buffer for 1 h and centrifuged at 10,000 × g for 10 min at 4°C (20). The supernatant was collected, and total protein was determined using the Pierce Coomassie Plus (Bradford) assay kit (Thermo Fisher Scientific, Hanover Park, IL, USA). 50 μg of total protein was loaded onto an SDS-PAGE gel (GeneScript, Piscataway, NJ, USA) and then transferred to a PVDF membrane as previously described . Two primary antibodies were used: a mouse DKY-Tag monoclonal antibody (GenScript, Catalog No. A00187) at a dilution of 1:1000 and a mouse anti-β-actin monoclonal antibody (Sigma Aldrich, St. Louis, MO, USA, Catalog No. SAB3500350) at a dilution of 1:1500. The secondary antibody, IRDye 800CW-conjugated donkey anti-mouse antibody (LI-COR, NE, USA, Catalog No. 926-32212), was used at a dilution of 1:15,000. Fluorescence signals were detected using an Odyssey SA infrared imaging system (LI-COR Bioscience Inc., Lincoln, NE, USA).

2.7 Computational details

All calculations were done with the Schodinger software system (Schrödinger Release 2025-2, Schrödinger, LLC, New York, NY, USA, 2025). Structures of the tanshinones were extracted from the PubChem database and subjected to the Ligand Preparation procedure in Maestro (v. 14.4.133) prior to the docking studies (21, 22). Protein structures were extracted from the Protein Data Bank and subjected to the Protein Preparation Procedure in Maestro (21, 23). Only the A-chain was used except for the 5IRQ structure, where both the A- and C-chains were used, since they contained different enantiomers of the ligand, *(R)*- and *(S)*-orteronel, respectively (24). The GLIDE docking program (v2025-2 build 133) was used for the docking studies using the default setup. Receptor Grid Generation was defined by the ligand present in the CYP17A1 active site. The standard precision (SP) mode for scoring the poses was applied. For each compound up to 100 poses were collected and further refined by the default post-docking minimization available in GLIDE. The ten best scoring poses were visually inspected and subjected to MM-GBSA (Molecular Mechanics/Generalized Born Surface Area) energy calculation. The binding modes were described by a combination of the subjective terms “down/up” and “left/right” with reference to the orientation of the furane oxygen atom relative to the heme group (see Figure 4). The MM-GBSA energy calculations were done with the Prime module (v2025-2 build 133) in Maestro. This method calculates both the free energy of binding and the individual energy contributions to the free energy. The numbers in Table 1 refer to the free energy of binding. One representative of each binding mode of each compound was selected for MD simulation. The MD simulations were performed with the Desmond program (v8.2.133) and

comprised a default seven step equilibration procedure followed by a 50 ns production run at 300 K using the OPLS4 force field. For each simulation 50 frames were collected and analysed. Structures were displayed with the Pymol program (v.3.1.5.1).

2.8. Data analysis

Data analysis was conducted using RStudio (version 3.6.0+) and GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) to ensure a thorough evaluation. Results are expressed as the mean ± standard deviation (SD) from three independent experiments to account for variability and reproducibility. One-way analysis of variance (ANOVA) was used to evaluate differences between treatment groups and controls, with post-hoc analyses performed using Tukey's Honest Significant Difference (HSD) test to identify specific differences. Significance was determined with thresholds of $*p < 0.05$ and $**p < 0.001$. All tests were two-tailed, and assumptions of normality and variance homogeneity were checked prior to analysis to validate the ANOVA results.

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