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Supplementary Information for

Structure-Based Optimisation of Non-Steroidal Cytochrome P450 17A1 Inhibitors

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1 Computational Details

1.1 Protein preparation

The X-ray structure of CYP17A1 in complex with the inhibitor abiraterone (PDB entry 3ruk, resolution of 2.6 Å) was used.[12] Only the A monomer was used and water molecules more than 5 Å from the ligand were removed. The protein was prepared for docking using the Protein Preparation Wizard suite implemented in Maestro.[3] Hydrogen atoms and missing residues were added to the initial coordinates. The structure was protonated according to pH = 7.0 and the Fe atom assigned a charge on +3. The positions of the hydrogen atoms were initially optimized keeping the heavy atoms fixed and finally the structure was optimized allowing the hydrogen atoms to move freely and heavy atoms restrained to move max. 0.3 Å. All energy minimizations were done using the OPLS-2005 force field.[4]

1.2 Preparation of ligands

The ligands, **1 - 6**, were all generated as 2D structures and subsequently converted to 3D structures in Maestro. The LigPrep program[5] was used to obtain low energy conformations. The 3D structures were energy minimized using the MacroModel suite[6] of Maestro (OPLS-2005 force field, default settings). Proper protonation states and possible tautomers to reflect pH = 7.0 ± 2.0 were determined using the Epik program.[7].

1.3 Docking

Docking was performed with GOLD (Genetic Optimization for Ligand Docking, version 5.2.2) program[8] with the haem-tailored ChemScore scoring function developed by Kirton *et al*.[9] The protein was kept rigid, while single bonds of the ligands were treated as rotatable. The docking radius was set to 15 Å around the center of mass of the co-crystallized ligand, abiraterone, and 50 independent docking runs were performed.

1.4 SiteMap

Identification of preferred sites for hydrogen bond donors and acceptors and for hydrophobic groups is based on the original idea by Goodford[10] and, subsequently, refined and implemented in the SiteMap program by Halgren.[11] Standard settings were used and the area of interest was defined as a region on 6 Å around the docked ligands.[12]

1.5 Determination of ligand strain

The ligand strain (conformational penalty, ΔE_{conf}) was determined as defined as the energy difference between the docked (binding, $E_{binding}$) conformation and the global energy conformation (E_{global}):

$$\Delta E_{conf} = E_{binding} - E_{global}$$

A conformational penalty (ΔE_{conf}) on less than 3 kcal/mol (13 kJ/mol) is normally assumed to be a requirement for bioactive conformations.[13]

The energy of the binding conformation ($E_{binding}$) was determined by transferring the docked pose from GOLD to Maestro and then performing an energy minimization in MacroModel

using the OPLS-2005 force field with the heavy atoms constrained in a flat-bottom potential to move max. 0.3 Å and by applying a force constant on 100 kJ/mol·Å^2.

The energy of the global energy conformation (E_{global}) was determined by performing a Monte Carlo Multiple Minimum (MCMM) search, a standard operation in the MacroModel suite in Maestro.

1.6 Molecular dynamics simulations

The molecular dynamics simulations were performed with the Desmond program as implemented in the Schrodinger software system.[14] A standard setup was applied consisting of the following steps: 1) Using the system builder the protein was placed in a minimized orthorhombic SPC water box. 2) Ions were added to obtain a neutral system. 3) A minimization using the OPLS-2005 force field comprising a maximum of 2000 iterations and a convergence criterion on 1.0 kcal/mol/Å was performed. 4) The haem iron and the coordinating nitrogen atoms were constrained with a force constant and a distance calculated by density functional theory (see below). The restraints were applied using a desmond_restraint.py script. 5) After equilibration the system was simulated for 20-40 ns.

1.7 Density functional theory calculations

Force fields are generally not properly parametrized to handle coordination to metal atoms.[9] Thus, in order to maintain the correct coordination around the iron atom in the haem group in CYP17A1 it was necessary to constrain the Fe-N distance. The distance and force constant were determined by density functional theory using the Turbomole program.[15] Calculations were carried out at the B3LYP level of theory.[16-18] The Fe-N distance between an iron atom and the nitrogen atom in a pyridine ring was scanned in steps of 0.1 Å in the interval 2.0±0.2 Å and, subsequently, a parabolic equation was fitted to the distance-energy curve and the restraints derived. It was found that an optimal Fe-N distance on 2.2 Å and a force constant on 70.5 kcal/mol·Å^2 yielded heme geometries close to experimentally determined heme geometries.

2 Experimental Details

2.1 Compounds

Compound **2** and **4** were obtained from Otava Chemicals.[19] Compound **3**, **5** and **6** were obtained from Vitas-M Laboratory.[20] The purity for all five compounds was 90-95%.

2.2 Recombinant CYP17A1 assay

The CYP17A1 assay was developed using a similar protocol as for the CYP19 assay as described by Jacobsen *et al.*[21] and according to the protocol supplied for the CYP17A1 assay described by Hutschenreuter *et al.*[22] Surplus amounts of NADPH were added instead of the NADPH generating system used by Hutschenreuter and colleagues. Two separate experiments were conducted, one experiment for both the CYP17A1 hydroxylase reaction and the lyase reaction and one experiment for the lyase reaction. For the hydroxylase experiments, progesterone (PRO) was used as substrate, and the production of 17-hydroxyprogesterone (OHPRO) and androstenedione (AN) were determined. For the lyase reaction, OHPRO was

used as substrate and the formation of AN was determined. In order to ensure maximum velocity of the enzyme (V_{max}), an excess of substrate was used in the assay. The amount of PRO was 150 µg/mL, whereas the concentration of OHPRO was 100 µg/mL. Abiraterone (ABI) was used as positive control for the inhibition of the recombinant CYP17A1 enzyme along with zero inhibition control and absolute inhibition control samples, to ensure that the enzyme was active.

Abiraterone and the six investigated compounds **1** - **6** were first diluted in DMSO. The maximum DMSO concentration in the experiments was 0.16% (v/v). Test compounds were then diluted in phosphate buffer (50 mM potassium phosphate, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, pH 7.4). 50 µL of each test solution in phosphate buffer was transferred to 600 µL tubes. For each test compound and substrate, control samples were prepared with 50 µL phosphate buffer. All analyses were carried out in triplicates.

CYP17A1 enzyme was kept in -80°C freezer and thawed on ice. An enzyme-substrate (E/S) solution was prepared in phosphate buffer by the addition of the CYP17A1 substrates, PRO and OHPRO, and the CYP17A1 enzyme and mixed gently. Final concentrations in the E/S solution were 1.2 µg/mL PRO, 0.8 µg/mL OHPRO and 40 µg protein/mL enzyme.[22] 50 µL of E/S solution was transferred to all test tubes and pre-incubated for 10 minutes at 37°C. The CYP17A1 enzyme was activated by the addition of 50 µL 750 µM NADPH in phosphate buffer to each test tube and gently mixed. In control tubes, absolute inhibition of the enzyme was obtained by adding 75 µL of 2 M NaOH immediately after the addition of NADPH. The reaction mixture was incubated for 30 minutes at 37°C. The final concentration in a 150 µL reaction mixture was 0.4 µg/mL PRO, 0.27 µg/mL OHPRO, 10 µg protein/mL and 250 µM NADPH.

Reactions were stopped by adding 75 µL of 2 M NaOH to each test tube. The samples were then neutralized by adding 75 µL of 2 M HCl to each tube. 200 µL internal standard mixture in heptane containing d_9-PRO and d_7-AN was transferred to each test tube with a final concentration of 0.025 µg/mL of each. Liquid-liquid extraction was performed by shaking test tubes for ½-1 min on a whirl mixer and centrifuging for 5 minutes at 9500 G. 100 µL of the heptane phase was transferred to a LC vial with insert. Samples were evaporated to dryness under a stream of nitrogen at 60°C and re-dissolved in 200 µL 1:9 (v/v) methanol in water. Calibrations curves were made PRO, OHPRO, and AN in a concentration range of 0.1-100 ng/mL with internal standards of d_9-PRO and d_7-AN added in a concentration corresponding to the concentrations used in the assay procedure. Samples were then analysed on LCMS using the method described below (**2.4**).

2.3 H295R cell assay

The H295R steroid hormone synthesis assay was performed according to the OECD validation guideline with minor modifications.[23] In brief, cells were cultured in DMEM/F12 media supplemented with 1% ITS-premix and 2.5% Nu-serum at 37 °C with a 5% CO_2 atmosphere. The cells were only used for experiments between passage 4-12°. During exposure experiments, cells were grown in 24 well plates with a density of 3×10^5 cells/mL. Cells were allowed to settle for 24 hours after which the medium was changed and compound **2**, **3**, **4**, **5** and **6**, respectively, added in the following concentrations: 0.001, 0.01, 0.1, 0.314, 1, 3.14, 10 µM. To avoid interference from low levels of steroid hormones present in the Nu-serum, the exposure experiment was conducted with serum-free media. Each compound was tested at a minimum of seven concentration levels using three or more replicates and the experiment was repeated on two different days (n = 6-12). On each test plate a solvent control

(SC) (medium with 0.1% DMSO) was included in triplicate and the maximal concentration of DMSO in the cell medium was 0.1%.[23] After 48 hours of incubation in presence of the test compounds, 950 µL of the medium was carefully removed and internal standards (50 µl of 0.1 µg/µl solution containing deuterated steroid analogues) were added. Samples were stored at -20 ˚C for later analysis.

Cell viability was confirmed with the resazurin assay, as described by Nielsen *et al.*[24] All tested concentrations confirmed viable cells with the exception of cells exposed to 10 µM of compound **4**.

Steroid extraction and clean-up were performed by double protein precipitation. First, 900 µL of cold acetonitrile were added to the samples, which were then vortexed and centrifuged at ~ 9500 x g for 10 minutes. Second, 900 µL cold methanol was added to the supernatant and the mixture was vortexed and centrifuged at 1500 x g for 10 minutes. Finally, the supernatant was collected and concentrated to ~1 mL under a gentle stream of nitrogen at 60 °C.[25]

2.4 Analysis of H295R samples using LC and MS

The analysis of the samples was performed using LCMS as described by Weisser *et al.*[26] For on-line solid phase extraction (SPE) of steroids, a total of 100 µL was injected on a binary 1290 Agilent Infinity Series system and a binary 1100 Agilent HPLC pump were used in combination.[27] For online clean-up, a C_{18} enrichment column (µbondapak® C_{18}, 3.9 × 20 mm, 10 µm)[28] was used. The enrichment column was connected to the autosampler through the TTC switching valve (two positions, 6 ports). Between the autosampler and the TTC switching valve a 0.3 µm in-line filter (Agilent 1290 infinity in-line filter) was installed. Separation of steroid hormones was performed using a C_{18} analytical column (Kinetex, 2.6 µm C_{18} 100 Å, 75 x 2.1 mm).[29] An isocratic flow of 1 mL/min H_2O:methanol:formic acid at 90:10:0.1 (v/v/v) was generated by the 1100 pump which was connected to the autosampler. The 1290 pump performed a gradient elution with a flow rate of 0.3 mL, which was connected to the TTC switching valve. Mobile phase A and B were composed of H_2O with 0.1% formic acid (v/v) and pure methanol, respectively.

An AB SCIEX 4500 QTRAP mass spectrometer[30] equipped with an atmospheric pressure chemical ionization (APCI) Turbo V source was used for detection.

LC and MS optimizations were conducted using Analyst software package[31] and obtained chromatographic peaks and quantification were processed in the MultiQuant program.[32] Calculations and graphics were performed using Microsoft Office Excel 2010 and GraphPad Prism.[33]

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4 Table S1

IC_{50} values (nM) and standard deviations for inhibition of CYP17A1 products for control compound abiraterone (ABI) using the recombinant CYP17A1 assay using progesterone as substrate.

CYP17A1 assay Control compound	Hydroxylase IC_{50} (nM)	Lyase IC_{50} (nM)
ABI1[a]	1.56±0.50	2.16±0.73
ABI2[b]	2.05±0.58	4.23±2.73
ABI3[c]	2.80±0.49	4.72±1.62

[a] **ABI1** was performed while testing compound **2** and **3** in CYP17A1 assay.
[b] **ABI2** was performed while testing compound **4** and **5** in CYP17A1 assay.
[c] **ABI3** was performed while testing compound **1** and **6** in CYP17A1 assay.
IC_{50} values were the same for all data sets (p=0.2503, F-test).

5 Figure S1

Dose-response curves from 3 experiments for the reference compounds, compound **1** and abiraterone, in the CYP17A1 assay. x-axis: Concentration of compound in µM for compound **1** and in nM for abiraterone; y-axis: Relative concentration of the compound in percent of the solvent control.