SUPPLEMENTAL DATA

A NEW MODE OF MINERALOCORTICOID RECEPTOR ANTAGONISM BY A POTENT AND SELECTIVE NON-STEROIDAL MOLECULE*  
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SUPPLEMENTAL FIGURES

**Supplemental Figure 1.** Superimposition of MR LBD/BR-4628 complex with the crystal structure of the MR LBD complexed with deoxycorticosterone. Picture showing the minimized complex between BR-4628 (gold) and the MR LBD deleted of the H12 helix (grey) superimposed to the structure of the full length MR LBD (blue) associated with deoxycorticosterone (pink). Only residues that form critical contacts with the ligands are shown. Hydrogen bonds are depicted as dashed red lines. This figure was produced using DINO (http://www.dino3d.org).

**Supplemental Figure 2.** Still image from Supplemental Video 1. Picture showing the complex between BR-4628 (green) and the MR LBD at the beginning of the 4 ns molecular dynamic simulation.
Supplemental Figure 3. Transactivation properties of the wild type and mutant MRs in response to BR-4628 and spironolactone. HEK-293T cells transiently expressing the MR, MR_{N770A}, MR_{A773G}, MR_{Q776A}, MR_{S810A}, MR_{S810M}, MR_{R817A}, MR_{M852A}, MR_{C942A}, MR_{T945A} or MR_{A773G-S810M} were incubated for 16h with increasing concentrations of BR-4628 or spironolactone (Spiro) in the presence of $10^{-9}$ M aldosterone (MR, MR_{A773G}, MR_{S810A}, MR_{S810M} and MR_{A773G-S810M}), $10^{-7}$ M aldosterone (MR_{Q776A}, MR_{R817A}, MR_{C942A} and MR_{T945A}), $10^{-7}$ M 18OVP (MR_{N770A}) or $10^{-8}$ M spironolactone (MR_{M852A}). In the cases of MR_{S810M}, MR_{M852A} and MR_{A773G-S810M}, spironolactone acts as an agonist and therefore its curves are not shown in these diagrams showing inhibition of transactivation. The cell extracts were assayed for luciferase and β-galactosidase activities as previously reported (1). IC$_{50}$ values were calculated using the GraphPad Prism Software and are the mean ± S.E.M. of three independent experiments performed in triplicate.
SUPPLEMENTAL METHODS

Synthesis of ethyl (4R)-5-acetyl-2,6-dimethyl-4-(2-methyl-4-oxo-4H-chromen-8-yl)-1,4-dihydropyridine-3-carboxylate] (BR-4628, 5)

General methods and materials: $^1$H NMR and $^{13}$C NMR spectra were recorded in [D$_6$]DMSO at room temperature on Bruker Avance spectrometers operating at 400 MHz and 500 MHz for $^1$H NMR, and at 125 MHz for $^{13}$C NMR. Column chromatography was performed on silica gel 60 (0.063–0.200 mm) purchased from Merck KGaA, Darmstadt, Germany. Solvents for extraction and chromatography were reagent grade and used as received. Commercial reagents were used without purification. The starting material 1-\{2-hydroxy-3-[prop-1-en-1-yl]phenyl\}ethanone (compound 1) is accessible from 1-(3-allyl-2-hydroxyphenyl)ethanone that was prepared from commercially available 1-(2-hydroxyphenyl)ethanone in two steps via O-allylation and Claisen rearrangement as published lately (2). The synthesis of 1 from 1-(3-allyl-2-hydroxyphenyl)ethanone has furthermore been described by Ford and coworkers (3).

Analytical HPLC/MS methods are as follows:

Method 1: Instrument: Micromass Quattro LCZ with HPLC Agilent Serie 1100; column: Phenomenex Synergi 2µ Hydro-RP Mercury 20 mm x 4 mm; eluent A: 1 l water + 0.5 ml formic acid (50% in water), eluent B: 1 l acetonitrile + 0.5 ml formic acid (50% in water); gradient: 0.0 min 90% A $\rightarrow$ 2.5 min 30% A $\rightarrow$ 3.0 min 5% A $\rightarrow$ 4.5 min 5% A; flow: 0.0 min 1 ml/min, 2.5 min/3.0 min/4.5 min 2 ml/min; oven: 50°C; UV detection: 208–400 nm.

Method 2: Instrument MS: Micromass ZQ; instrument HPLC: Waters Alliance 2795; column: Phenomenex Synergi 2µ Hydro-RP Mercury 20 mm x 4 mm; eluent A: 1 l water + 0.5 ml formic acid (50% in water), eluent B: 1 l acetonitrile + 0.5 ml formic acid (50% in water); gradient: 0.0 min 90% A $\rightarrow$ 2.5 min 30% A $\rightarrow$ 3.0 min 5% A $\rightarrow$ 4.5 min 5% A; flow: 0.0 min 1 ml/min, 2.5 min/3.0 min/4.5 min 2 ml/min; oven: 50°C; UV detection: 210 nm.
2-Methyl-8-[(1E)-prop-1-en-1-yl]-4H-chromen-4-one (3)
To a solution of 1-{2-hydroxy-3-[(1E)-prop-1-en-1-yl]phenyl}ethanone (1) (211 g, 1.2 mol) in 3.2 L ethyl acetate was added a solution of lithium hexamethyldisilazide in THF (1M, 3.6 L, 3.6 mol) while keeping the temperature below 20 °C. The resulting mixture was refluxed for 4 h. The mixture was cooled down to room temperature using an ice bath and hydrolysed with hydrochloric acid (3M, 2.7 L) keeping the temperature below 27 °C. The resulting phases were separated and the aqueous phase was extracted with 2.0 L ethyl acetate. The combined organic phases were washed with 3.0 L of water and dried with sodium sulfate. The solvent was removed in vacuo. The resulting residue (325 g) was dissolved in 1.65 L cyclohexane and allowed to crystallize overnight. The crystals were separated by filtration and dried in vacuo to yield 124.7 g (48%) of 1-{2-hydroxy-3-[(1E)-prop-1-en-1-yl]phenyl}butane-1,3-dione (2) which was sufficiently pure (>90%, analysed by HPLC/MS) to be used without further purification. The batch of 2 as obtained (85.6 g, 392 mmol) was dissolved in acetic acid (385 mL). Subsequently, hydrochloric acid (37%, 120 mL) was added and the mixture was stirred at 80 °C for 30 min. The product precipitates while cooling the reaction mixture to 15 °C by use of an ice bath. The product was collected by suction filtration and was washed with acetonitrile to yield 73.6 g (94 %, based on 1) of the desired compound 3. HPLC (method 1): $R_t = 2.19$ min (purity: 90% based on HPLC area-%); MS (Elpos): $m/z = 201$ [M+H]$^+$; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ [ppm] = 1.99 (dd, $J$ = 6.6, 1.0 Hz, 3H), 2.43 (s, 3H), 6.19 (s, 1H), 6.30 - 6.52 (m, 1H), 6.41 (dd, $J$ = 15.9, 6.8 Hz, 1H), 6.86 (d, $J$ = 16.1 Hz, 1H), 7.31 (t, $J$ = 7.8 Hz, 1H), 7.74 (s,1H), 8.06 (s,1H). $^{13}$C NMR (125 MHz, [D$_6$]DMSO): $\delta$ = 18.8, 19.9, 109.6, 122.8, 123.1, 123.2, 124.7, 127.0, 129.7, 129.9, 152.2, 166.5, 176.7. HRMS calcd for C$_{13}$H$_{13}$O$_2$ + [H$^+$]: 201.0910; found: 201.0909.

2-Methyl-4-oxo-4H-chromene-8-carbaldehyde (4)
To a solution of 2-methyl-8-[(1E)-prop-1-en-1-yl]-4H-chromen-4-one (3) (60.0 g, 299 mmol) in water/THF (4/1, 600 mL) was added benzyltriethylammonium chloride (2.0 g, 8.8 mmol) and osmium tetroxide (4% solution in water, 2.0 mL, 0.315 mmol). Subsequently, sodium periodate (128.2 g, 600 mmol) was added in small portions. During the addition, the temperature is monitored and kept between 30 and 35 °C. The reaction mixture was allowed to stir for additional 30 min and then 1.0 L water was added. The precipitate formed was collected by suction filtration, washed with acetonitrile to yield 33.0 g (59%, based on 1) of the desired compound 4. HPLC (method 1): $R_t = 1.47$ min (purity: 97% based on HPLC area-%); MS (Elpos): $m/z = 189$ [M+H]$^+$; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ [ppm] = 2.48 (s, 3H), 6.27 (s, 1H), 7.51 (t, $J$ = 7.6 Hz, 1H), 8.21 (dd, $J$ = 7.3 Hz, $J$ = 1.5 Hz, 1H), 8.45 (dd, $J$ = 7.8 Hz, $J$ = 1.5 Hz, 1H), 10.67 (s, 1H). $^{13}$C NMR (125 MHz, [D$_6$]DMSO): $\delta$ = 19.8, 110.3, 123.6, 124.6, 125.0, 131.1, 133.0, 156.0, 166.8, 175.8, 187.9. HRMS calcd for C$_{11}$H$_9$O$_3$ + [H$^+$]: 189.0546; found: 189.0543.

Ethyl 5-acetyl-2,6-dimethyl-4-(2-methyl-4-oxo-4H-chromene-8-yl)-1,4-dihydropyridine-3-carboxylate (4(R)-5)
To a solution of 2-methyl-4-oxo-4H-chromene-8-carbaldehyde (4) (24 g, 128 mmol), ethyl 3-aminobut-2-enoate (8.04 g, 134 mmol) and pentane-2,4-dione (22.3 g, 223 mmol) in 2-propanol (720 mL) was added acetic acid (7.67 mL, 134 mmol). The reaction mixture was stirred overnight at reflux temperature and cooled to room temperature. The solvent was evaporated under reduced pressure. The desired compound was isolated by column
chromatography (silica gel, ethyl acetate) to yield 19.1 g (39 %) of racemic material. HPLC (method 2): R<sub>t</sub> = 1.67 min; MS (EIpos): m/z = 382 [M+H]<sup>+</sup>. ¹H NMR (400 MHz, [D<sub>6</sub>]DMSO): 1.02 (t, J = 7.1, 3H), 2.16 (s, 3H), 2.24 (s, 3H), 2.28 (s, 3H), 2.40 (s, 3H), 3.93 (m, 2H), 5.44 (s, 1H), 6.23 (s, 1H), 7.33 (t, J = 7.8, 1H), 7.58 (dd, J = 7.3, J = 1.5, 1H), 7.79 (dd, J = 7.8, J = 1.5, 1H), 8.98 (s, 1H). ¹³C NMR (125 MHz, [D<sub>6</sub>]DMSO): δ = 13.9, 18.0, 18.9, 20.4, 29.9, 39.0, 59.0, 100.8, 109.5, 110.8, 122.8, 122.9, 124.5, 134.4, 136.5, 144.3, 145.7, 153.0, 166.1, 166.6, 176.8, 196.6. HRMS calcd for C<sub>22</sub>H<sub>23</sub>NO<sub>5</sub> + [H<sup>+</sup>]: 382.1649; found: 382.1645. Subsequent chiral chromatography (column: Daicel Chiralpak AD-H, eluent: iso-hexane/2-propanol = 75:25) delivered 7.70 g of the title compound that turned out to be the enantiomer having the longer retention time. Analytical characterization by HPLC revealed an enantiomeric excess of 98.9% and a specific optical rotation of -6.0° (CHCl<sub>3</sub>, 589 nm, 20.5 °C, c = 0.51000 g / 100 mL). R-configuration at C4 was confirmed by X-Ray crystallography.

**Synthesis of [³H]1 ([³H]BR-4628)**

A mixture of 6.1 mg (16 μmol) compound 5 and 6.1 mg (1,5-cyclooctadien)-bis-(methyldiphenylphosphine)-iridium(I)-hexafluorophosphate, dissolved in 1.0 mL dichloromethane, was stirred with tritium gas at a partial pressure of about 90 kPa (900 mbar) for 2 hours in a reaction vessel which was connected to a tritium labelling apparatus. After freezing of the reaction mixture with liquid nitrogen the non-reacted tritium and the solvent were adsorbed in a trap filled with platinum oxide and charcoal at the temperature of liquid nitrogen. To remove the labile tritium the dry residue was dissolved in a mixture of 1.0 mL ethanol/acetonitrile (3 + 1) and the solvent was evaporated in the vacuum. This process was repeated five times. The dry residue was dissolved with 1.0 mL of a mixture of acetonitrile/0.2 % trifluoroacetic acid 50 : 50 (v + v) and purified in 4 equal portions. According to the labelling degree, the specific activity was calculated as 2.87 GBq/mg (29.7 Ci/mmol). The combined HPLC fractions were evaporated to dryness and from the residue two stock solutions were prepared using ethanol with concentrations of 1.04 mCi/mL and 2.74 mCi/mL. The total radioactivity in both solutions was determined as 10.1 GBq (274 mCi).

**Protein modelling and ligand docking**

The X-ray structure of the wild type MR LBD complexed with deoxycorticosterone (PDB ID 2ABI (1)) served as model for docking of BR-4628. In contrast to other X-ray structures it is missing the C808S mutation leading slightly more natural side chain arrangements. The structure was complemented for not resolved loops by superimposing it with 2A3I (4) and transferring loops Arg904-Gln919 and Gly753-Asp760. Helix 12 (Pro957-His982) was deleted. The resulting protein structure including crystallographic water molecules was protonated. Likely protonation states and hydrogen bonding networks were predicted using the “protein preparation workflow” in Maestro, Schrödinger LLC. Initial docking was carried out by manually placing the small molecule x-ray derived conformation of BR-4628 into the binding site and varying the relative orientation of the ligand. The conformation of Met852, Ser811 and Thr945 side chains were altered in order to enlarge the volume of the binding pocket. The program Glide SP Version 3.5 was used to dock BR-4628 into the binding cleft. The complex with the most probable binding pose was energy minimized using the OPLS2005 force field (dielectric constant 1.0, constant dielectric, solvent water, Polak-Ribier Conjuague Gradient, convergence threshold 0.5) in MacroModel, Schrödinger LLC.
Molecular dynamics simulation

All simulation systems were set up using Desmond, placing the nuclear receptor in the center of the simulation box and filling the voided space with 11522 “simple point charge” (SPC) water molecules (5). The dimensions of the simulation boxes were chosen so that no protein atom was within 10 Å of the edge. Two Na+ ions were added to obtain a neutral total charge for the system. All systems were energy minimized using 4000 steps of steepest descent minimization. The systems were relaxed by a short (12 ps) NVT simulation at 10 K followed by 3 NPT simulations (24 ps) with decreasing barostat coupling in order to adjust the volume of the system. All molecular dynamics simulations were performed by using the parallel MD program Desmond (6). The standard OPLS-AA parameters for ions (7), and the OPLS-AA/L force field for proteins (8,9) were used. Long-range electrostatic interactions were computed by the smooth particle-mesh Ewald method. The real-space part of the electrostatic and Lennard–Jones interactions was cut off at 9 Å. The M-SHAKE algorithm was used to constrain the lengths of bonds containing hydrogen. All MD simulations were at constant pressure (1 bar, NPT), maintained using a Martyna-Tobias-Klein barostat with isotropic coupling and 2 ps relaxation time, and constant temperature (300 K) maintained using Nose-Hover thermostat with 1 ps relaxation time. The pressure and temperature control used a relaxation time of 1.0 ps. All simulations used a RESPA integrator (10) with a 2.0-fs time step for the bonded, van der Waals and short-range Coulomb interactions, and a 6-fs time step for the long-range Coulomb interactions. All c-alpha backbone atoms were constrained with a force constant of 5 kcal/mol/Å². By introducing an orientational potential, the overall orientation of the protein was restrained with its principal axes held approximately parallel to the edges of the simulation box throughout the simulation.

Supplemental References

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