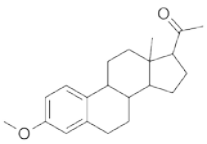
Supplementary information

Synthesis of RU1968

All reactions were performed under an inert atmosphere of argon or nitrogen, unless otherwise stated. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance DPX 400 or DPX 500 spectrometer (Bruker Daltonics, Bremen, Germany) at 298 K; chemical shifts are expressed in parts per million (ppm)with respect to the solvent signals (^{1}H NMR: $CDCl_3$ 7.27; DMSO-d_6 2.50; MeOD 3.31; ^{13}C NMR: $CDCl_3$ 77.16; MeOD 49.00). Analytical HPLC was performed on a Shimadzu Prominence system (Shimadzu, Tokyo, Japan) using a Nucleodur Isis column (4 × 150 mm, 5 µm Macherey&Nagel, Düren, Germany) with a binary gradient of H_2O and Acetonitrile (each containing 0.1% (v/v) trifluoroacetic acid (TFA)) at a flow rate of 1 ml/min. Preparative HPLC was performed on a Shimadzu LC-8A system using a Nucleodur Isis column (20 ml/min, 21 × 250 mm, 5 µm) with gradients of H_2O and Acetonitrile (each containing 0.1% (v/v) TFA) at a flow rate of 20 ml/min. High-resolution mass spectra (HRMS) were obtained on a MAT 95 XL or MAT 90 mass spectrometer (Thermo Finnigan, San Jose, USA). All reagents were obtained from Sigma–Aldrich (Munich, Germany) and were used as received. (±) Estrone methylether was synthesized as previously described (Ananchenko et al., 1962, Ananchenko and Torgov, 1963). Nitrile **1** was obtained from (±) estrone metyhlether following a published procedure (Van Leusen and Van Leusen, 2004).

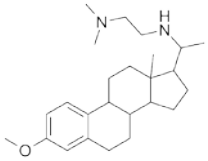
1-(3-methoxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl) ethanone (**2**): To a 0 °C solution of nitrile **1** (3.973 g; 13.45 mmol) in dry Et_2O (67 ml) was added MeLi (1.6 M in Et_2O, 67.3 ml; 107.6 mmol) over 20 minutes. After stirring for further 10 minutes at 0 °C, the reaction was quenched by careful addition of H_2O (20 ml) and stirred for another 30 minutes. The mixture was acidified with conc. HCl (pH = 1) and the Et_2O evaporated *in vacuo*. The resulting slurry was taken up in DCM (200 ml), the aqueous layer separated and extracted with another portion of DCM. Organic layers were combined, washed with sat. $NaHCO_3$ (aq), dried (brine, $MgSO_4$), filtered, and the solvent evaporated *in vacuo*. The crude material was purified by flash chromatography using a silica gel column with a gradient of pentane/ethylacetate (98/2 to 92/8) to obtain **2** as an off-white solid (3.34 g, 79%, 1:1 mixture of diastereomers).



1H-NMR (400 MHz, $CDCl_3$) δ = 7.21 d (1H, J = 8.6 Hz), 7.18 d (1H, J = 8.6 Hz), 6.72 (m, 2H), 6.64 (m, 2H), 3.79 (s, 3H), 3.78 (s, 3H), 2.87 (m, 3H), 2.63 (t, 1H, J = 9.1 Hz), 2.40 – 2.15 (m, 6H), 2.18 (s, 3H), 2.17 (s, 3H), 2.03 – 1.27 (m, 22H), 0.96 (s, 3H), 0.67 (s, 3H).
13C-NMR (100 MHz, $CDCl_3$) δ = 212.98 (CO), 209.59 (CO), 157.62 (C), 157.57 (C), 138.15 (C), 138.04 (C), 132.63 (C), 132.49 (C), 126.36 (CH), 113.91 (CH), 113.87 (CH), 111.64 (CH), 111.59 (CH), 63.98 (CH), 61.47 (CH), 55.79 (CH), 55.30 (CH_3), 49.45 (CH), 46.13 (C), 44.53 (C), 43.79 (CH), 43.26 (CH), 39.14 (CH), 39.12 (CH_2), 38.87 (CH), 35.40 (CH_2), 33.03 (CH_3), 31.62 (CH_3), 30.02 (CH_2), 29.94 (CH_2), 28.13 (CH_2), 27.80 (CH_2), 26.78 (CH_2), 26.71 (CH_2), 25.72 (CH_2), 24.25 (CH_2), 22.99 (CH_2), 21.04 (CH_3), 13.53 (CH_3).

HRMS: Calc. for $C_{21}H_{28}O_2H+$ (M + H) 313.2168, found 313.2176.

N1-(1-(3-methoxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)ethyl)-N2,N2-dimethylethane-1,2-diamine (3): To a solution of **2** (1.3 g, 4.16 mmol) in 1,2-dichloroethane (25 ml) were added AcOH (50 mmol, 2.87 ml), N,N-dimethylethylenediamine (41.6 mmol, 4.5 ml), activated molsieves (4 Å; ~ 1 g), and $NaBH(OAc)_3$ (12.5 mmol, 2.66 g). The mixture was stirred for 18 h at RT, filtered and evaporated. The residue was taken up in Et_2O and washed with 1M NaOH, followed by sat. NaCl



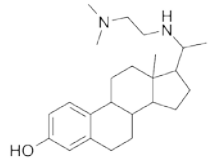
(aq). The organic layer was dried ($MgSO_4$), filtered and evaporated to obtain a crude oil that was purified by flash chromatography. Careful elution with DCM/MeOH (97/3 to 9/1) afforded **3a** (582 mg, 36%); **3b** (330 mg, 25%) was eluted with DCM/MeOH 6/4.

3a: 1H-NMR (400 MHz, $CDCl_3$) δ = 7.19 (m, 2H), 6.70 (m, 2H), 6.62 (m, 2H), 3.76 (s, 6H), 2.95-2.75 (m, 8H), 2.69-2.48 (m, 6H), 2.42-2.10 (m, 10H), 2.25 (s, 6H), 2.22 (s, 6H), 1.95-1.12 (m, 18H), 1.09 (d, 6H, J=6.2 Hz), 0.80 (s, 3H), 0.73 (s, 3H).
13C-NMR (100 MHz, $CDCl_3$) δ = 157.28 (C), 137.99 (C), 137.82 (C), 132.79 (C), 132.67 (C), 126.15 (CH), 113.62 (CH), 111.33 (CH), 111.32 (CH), 58.57 (CH_2), 58.04 (CH_2), 56.55 (CH), 55.82 (CH), 55.07 (CH_3), 54.88 (CH), 54.37 (CH), 54.12 (CH), 52.07 (CH), 45.33 (CH_3), 45.14 (CH_3), 44.97 (CH_2), 44.34 (CH_2), 43.65 (CH), 43.54 (C), 43.45 (CH), 42.36 (C), 39.78 (CH_2), 39.15 (CH), 38.61 (CH), 33.89 (CH_2), 29.85 (CH_2), 29.74 (CH_2), 28.25 (CH_2), 27.53 (CH_2), 26.73 (CH_2), 26.70 (CH_2), 26.62 (CH_2), 25.64 (CH_2), 23.75 (CH_2), 22.58 (CH_2), 22.00 (CH_3), 19.95 (CH_3), 18.55 (CH_3), 12.31 (CH_3).

HRMS: Calc. for $C_{25}H_{40}N_2OH+$ (M + H) 385.3219, found 371.3221.

3b: **1H-NMR** (400 MHz, $CDCl_3$) δ = 7.20 (m, 2H), 6.70 (m, 2H), 6.62 (m, 2H), 3.77 (s, 6H), 2.94-2.72 (m, 6H), 2.68-2.40 (m, 8H), 2.40-1.20 (m, 28H), 2.24 (s, 6H), 2.23 (s, 6H), 1.15 (d, 3H, J=6.5 Hz), 1.04 (d, 3H, J=6.2 Hz), 0.85 (s, 3H), 0.72 (s, 3H).
13C-NMR (100 MHz, $CDCl_3$) δ = 155.15 (C), 138.32 (C), 131.78 (C), 126.74 (CH), 115.73 (CH), 113.31 (CH), 58.33 (CH_2), 55.54 (CH), 51.38 (CH), 45.11 (CH_3), 44.12 (CH_2), 43.58 (CH), 41.23 (C), 39.76 (CH), 39.31 (CH_2), 30.11 (CH_2), 28.19 (CH_2), 27.07 (CH_2), 26.91 (CH_2), 24.42 (CH_2), 16.91 (CH_3), 12.43 (CH_3).
HRMS: Calc. for $C_{25}H_{40}N_2OH+$ (M + H) 385.3219, found 371.3228.

17-(1-(2-(dimethylamino)ethylamino)ethyl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-ol (RU1968F1 + RU1968F2): A suspension of **3a** (52 mg, 0.135 mmol) in 48% HBr (aq) (1.8 ml) was heated at 100°C in a sealed vial for 6 h, cooled to RT and diluted with ethyl acetate and water. The resulting mixture was neutralized by incremental addition of Na_2CO_3 (s), the aqueous layer separated, and extracted with ethyl acetate. Organic layers were combined, washed with brine, dried ($MgSO_4$), filtered, and the solvent evaporated *in vacuo*. The resulting material was purified by flash chromatography on a silica gel column (DCM/MeOH 96/4 + 0.4% NH_4OH (28% aq)) to obtain 38 mg (76%) brown foam. Further purification on preparative HPLC (Gradient: 20% Acetonitrile to 25% over 60 minutes) afforded **RU1968F1 * 2 TFA** (25 mg, 31%) and **RU1968F2 * 2 TFA** (5 mg, 6%) as white solids. **RU1968F3 + F4**: **3b** (48 mg, 0.125 mmol) was treated as described above to obtain **RU1968F3 * 2TFA** (4 mg, 5%) and **RU1968F4 * 2TFA** (19 mg, 25%) as white solids.



RU1968F1: **1H-NMR** (400 MHz, MeOD) δ = 7.06 (d, 1H, J=8.5 Hz), 6.58 (dd, 1H, J_1=8.5 Hz, J_2=2.6 Hz), 6.51 (d, 1H, J=2.6 Hz), 3.20–3.06 (m, 2H), 3.02-2.92 (m, 1H), 2.86-2.63 (m, 4H), 2.35 (s, 6H), 2.25 (m, 1H), 2.16 (m, 1H), 2.09-1.95 (m, 2H), 1.91-1.78 (m, 2H), 1.70-1.26 (m, 11H), 0.75 (s, 3H).
13C-NMR (100 MHz, MeOD) δ = 155.15 (C), 138.32 (C), 131.78 (C), 126.74 (C), 132.71 (C), 126.23 (CH), 126.19 (CH), 113.68 (CH), 111.38 (CH), 111.35 (CH), 59.20 (CH_2), 58.91 (CH_2), 56.90 (CH), 56.33 (CH), 55.24 (CH), 55.12 (CH_3), 54.47 (CH), 53.66 CH), 51.35 (CH), 45.45 (CH_3), 45.35 (CH_3), 44.91 (CH_2), 44.14 (CH_2), 43.62 (CH), 43.54c(CH), 43.46 (C), 42.35 (C), 39.39 (CH_2), 39.08 (CH), 38.55 (CH), 34.23 (CH_2), 29.90 (CH_2), 29.81 (CH_2), 28.20 (CH_2), 27.61 (CH_2), 27.07 (CH_2), 26.78 (CH_2), 26.55 (CH_2), 25.56, (CH_2) 23.86 (CH_2), 23.82 (CH_2), 22.02 (CH_3), 19.16 (CH_3), 18.21 (CH_3), 12.31 (CH_3).
HRMS: Calc. for $C_{24}H_{38}N_2OH+$ (M + H) 371.3062, found 371.3049.

RU1968F2: **1H-NMR** (400 MHz, DMSO-d6) δ = 9.03 (s, 1H), 7.04 (d, 1H, J=8.5 Hz), 6.51 (dd, 1H, J_1=8.5 Hz, J_2=2.7 Hz), 6.43 (d, 1H, J=2.7 Hz), 3.34 (m, 4H), 2.85 (s, 6H), 2.70 (m, 2H), 2.30 (m, 1H), 2.19-2.06 (m, 2H), 1.89-1.58 (m, 6H), 1.52-1.26 (m, 6H), 1.23 (d, 3H, J=6.4 Hz), 0.84 (s, 3H).
HRMS: Calc. for $C_{24}H_{38}N_2OH+$ (M + H) 371.3062, found 371.3060.

RU1968F3: 1H-NMR (400 MHz, DMSO-d6) 8.96 (s, 1H), 7.09 (d, 1H, J=8.3 Hz), 6.57 (dd, 1H, J_1=8.3 Hz, J_2=2.7 Hz), 6.51 (d, 1H, J=2.7 Hz), 2.30-2.22 (m, 2H), 2.13 (s, 6H), 1.86-1.50 (m, 9H), 1.41-1.15 (m, 6H), 0.96 (d, 3H, J=6.2 Hz), 0.74 (s, 3H).

HRMS: Calc. for $C_{24}H_{38}N_2OH+$ (M + H) 371.3062, found 371.3055.

RU1968F4: 1H-NMR (400 MHz, DMSO-d6) 9.00 (s, 1H), 7.04 (d, 1H, J=8.7 Hz), 6.50 (dd, 1H, J_1=8.7 Hz, J_2=2.5 Hz), 6.43 (d, 1H, J=2.5 Hz), 3.30 (m, 4H, below HDO), 2.82 (s, 6H, broad), 2.71 (m, 2H), 2.25 (m, 1H), 2.11 (m, 1H), 1.96 (m, 1H), 1.84-1.65 (m, 6H), 1.51 (m, 2H), 1.39-1.15 (m, 9H), 0.68 (s, 3H).

HRMS: Calc. for $C_{24}H_{38}N_2OH+$ (M + H) 371.3062, found 371.3067.

Supplementary Figures

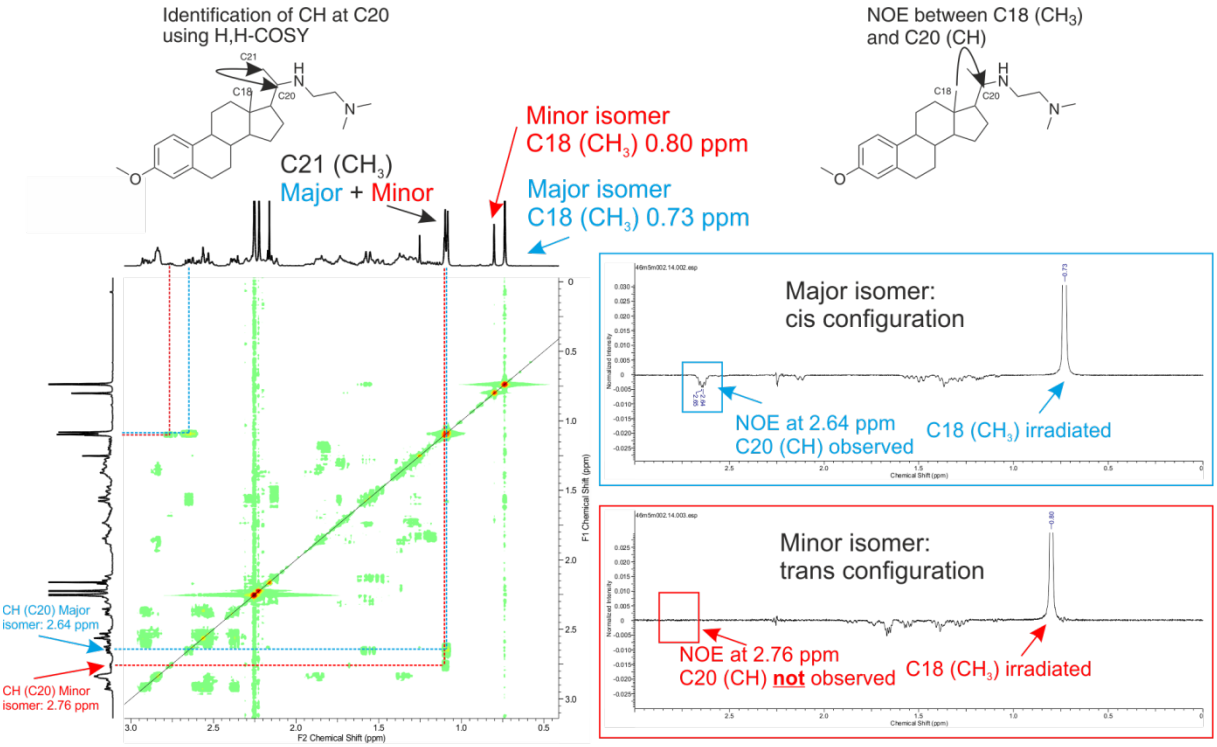


Figure S1: **Relative stereochemistry at C18 and C20 in 3a**. Right panel: H-H COESY spectrum of 3a. C18-CH_3 groups were identified as singlets at δ = 0.73 ppm (blue, major isomer) and δ = 0.80 ppm (red, minor isomer). CH_3 groups at C21 were identified as overlapping doublets (δ = 1.09 ppm major isomer and δ = 1.10 ppm minor isomer), each of which could be cross-referenced to a multiplet at δ > 2.5 ppm, which consequently was assigned to the CH-group at C20. (Major isomer, dashed blue lines, δ = 1.09 ppm → δ = 2.64 ppm; minor isomer, dashed red lines, δ = 1.10 ppm → δ = 2.76 ppm). Left panel: NOE experiments to assign relative stereochemistry between C18 and C20. Blue box: major isomer; irradiation of the C18-CH_3 singlet (δ = 0.73 ppm) results in a strong negative NOE resonance at δ = 2.64 ppm, indicating spatial proximity of C18- and C20-protons. Cis configuration was assigned for this isomer. Red box: minor isomer; irradiation of the C18-CH_3 singlet (δ = 0.80 ppm) results in no observable NOE resonance at δ = 2.76 ppm, indicating no spatial proximity of C18- and C20-protons. Trans configuration was assigned for this isomer.

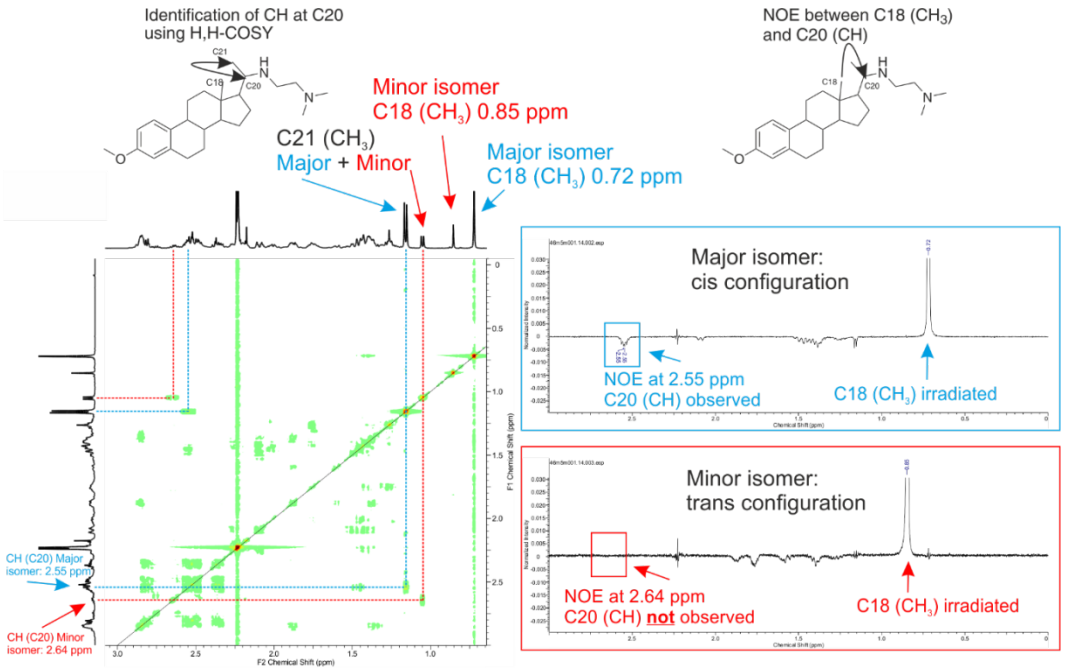


Figure S2: **Relative stereochemistry at C18 and C20 in 3b.** Right panel: H-H COESY spectrum of 3b. C18-CH_3 groups are easily identified as singlets at δ = 0.72 ppm (blue, major isomer) and δ = 0.85 ppm (red, minor isomer). CH_3 groups at C21 are identified as doublets (δ = 1.16 ppm major isomer and δ = 1.05 ppm minor isomer), each of which could be cross referenced to a multiplet at δ > 2.5 ppm, which consequently was assigned to the CH-group at C20. (Major isomer, dashed blue lines, δ = 1.16 ppm → δ = 2.55 ppm; minor isomer, dashed red lines, δ = 1.05 ppm → δ = 2.64 ppm). Left panel: NOE experiments to assign relative stereochemistry between C18 and C20. Blue box: major isomer; irradiation of the C18-CH_3 singlet (δ = 0.72 ppm) results in a strong negative NOE resonance at δ = 2.55 ppm, indicating spatial proximity of C18- and C20-protons. Cis configuration was assigned for this isomer. Red box: minor isomer; irradiation of the C18-CH_3 singlet (δ = 0.85 ppm) results in no observable NOE resonance at δ = 2.64 ppm, indicating no spatial proximity of C18- and C20-protons. Trans configuration was assigned for this isomer.

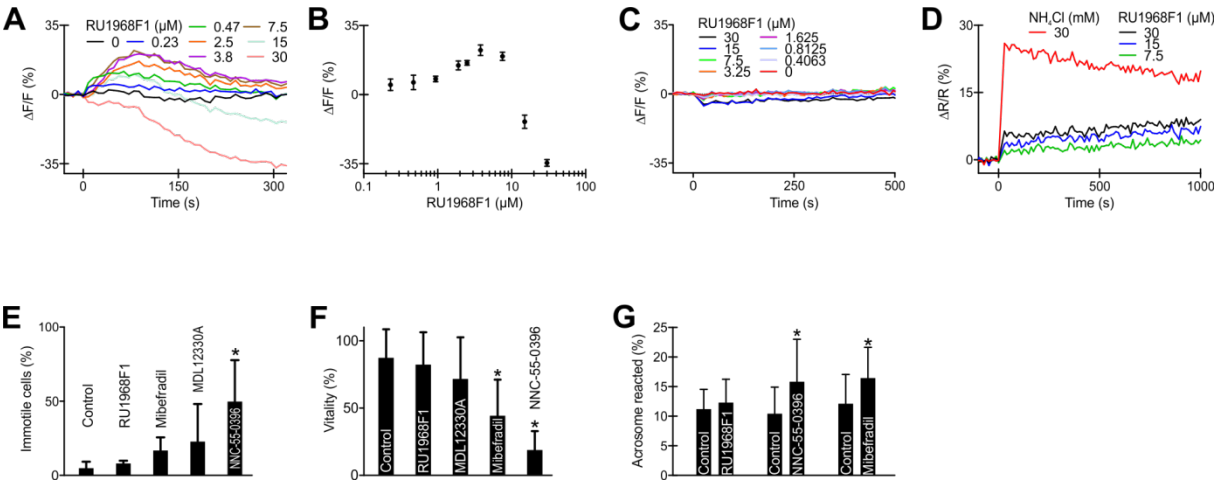


Figure S3: The action of RU1968F1 itself in human sperm. (A) RU1968F1-induced Ca^{2+} signals in human sperm. (B) Dose-response relation of the RU1968F1-evoked signal amplitudes (n = 3). Error bars indicate SD. (C) RU1968F1-induced Ca^{2+} signals in human sperm at 10 nM extracellular Ca^{2+}. (D) Changes in pH_i evoked by RU1968F1 or NH_4Cl in sperm loaded with the pH indicator BCECF. The signals were recorded in the FluoStar. (E) Fraction of immotile sperm in the absence (n= 9) and presence of the RU1968F1 (30 μM), MDL12330A (100 μM), Mibefradil (60 μM), or NNC-55-0396 (30 μM) (n = 5). Error bars indicate SD. *P < 0.05 versus control. (F) Fraction of vital cells in the absence and presence of the inhibitors (n = 5). Vitality was evaluated by

the Eosin test, performed according to the WHO manual. Error bars indicate SD. *P < 0.05 versus control (G) Fold increase in acrosome-reacted sperm after treatment with RU1968F1 (30 µM), Mibefradil (40 µM), or NNC-55-0396 (20 µM) (n = 10). Error bars indicate SD. *P < 0.05 versus control

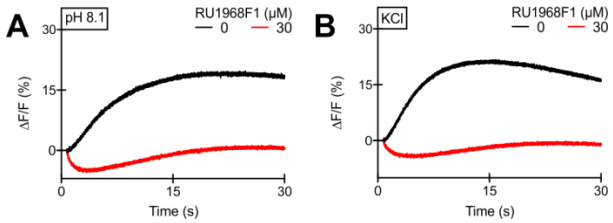


Figure S4: RU1968F1 inhibits depolarization- and alkaline-evoked Ca^{2+} signals in human sperm. (A) Ca^{2+} signals evoked by mixing of sperm with pH8.6-HTF and RU1968F1 in a stopped-flow apparatus. The final pH after mixing was 8.1. (B) Ca^{2+} signals evoked by mixing of sperm with K^+-HTF and RU1968F1 in a stopped-flow apparatus. The final K^+ concentration after mixing was 51.25 mM.

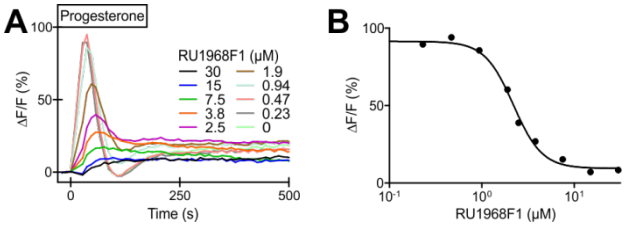


Figure S5: **RU1968F1 inhibits progesterone responses in sperm bathed in NH_4Cl**. (A) Progesterone-induced Ca^{2+} signals (500 nM) in human sperm bathed for 20 minutes in 30 mM NH_4Cl. (B) Dose-response relation of signals from (A). Mean IC_{50}: 3.1 ± 1.2 (n = 5).

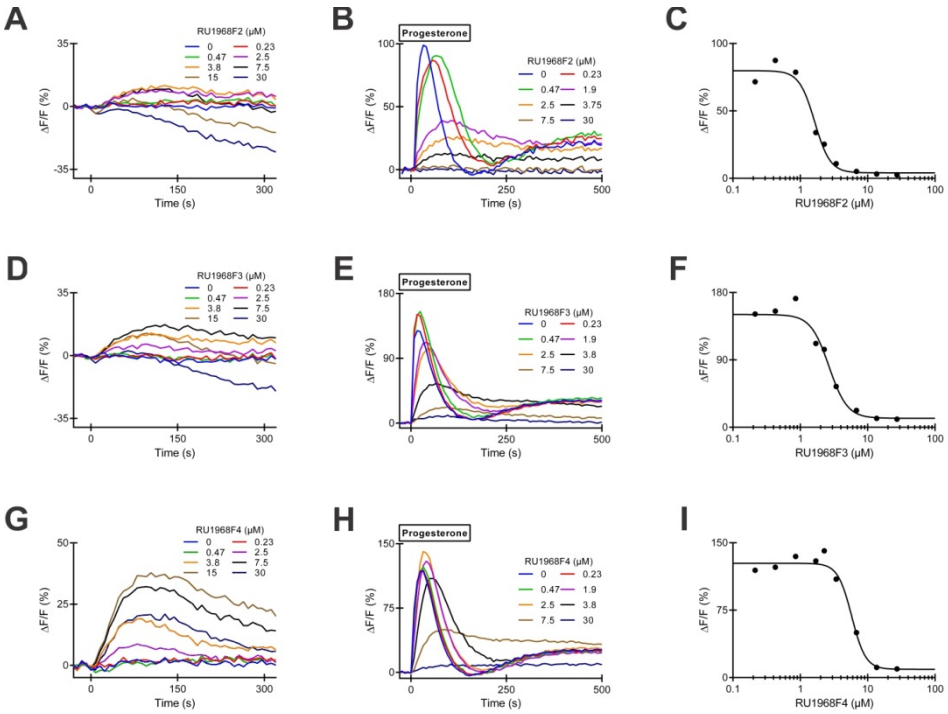


Figure S6: Action of RU1968F2-F4 on their own and on CatSper-mediated Ca^{2+} signals in human sperm populations. (A) RU1968F2-induced Ca^{2+} signals in human sperm. (B) Progesterone (500 nM)-induced Ca^{2+} signals in human sperm in the presence of RU1968F2; (C) Dose-response relation of the signal amplitudes from (B) (IC_{50} = 1.6 µM). (D) RU1968F3-induced Ca^{2+} signals in human sperm. (E) Progesterone-induced Ca^{2+} signals in the presence of RU1968F3. (F) Dose-response relation of the signal amplitudes from (E) (IC_{50} = 2.7

μM). (G) RU1968F4-induced Ca^{2+} signals in human sperm. (H) Progesterone-induced Ca^{2+} signals in human sperm in the presence of RU1968F4. (I) Dose-response relation of the signal amplitudes from (H) (IC_{50} = 5.8 μM)

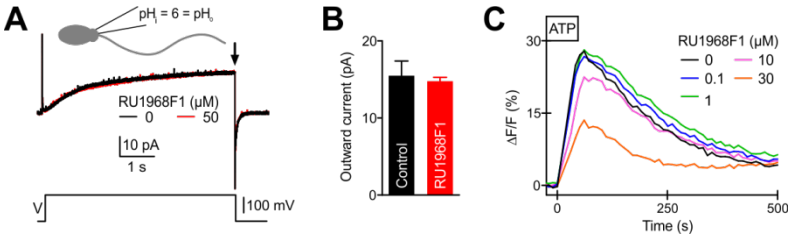


Figure S7: Action of **RU1968F1 on Hv1 currents in human sperm and ATP-evoked Ca^{2+} responses in mouse sperm**. **(A)** Voltage-gated proton currents recorded from a human sperm cell before (blue) and after superfusion with RU1968F1 (red). (B) Mean outward current before and after application of RU1968F1 (n = 3). Arrow in panel (A) indicates the time point at which outward currents were determined. Error bars indicate SD. (C) ATP-evoked Ca^{2+} signals in CatSper1$^{-/-}$ mouse sperm in the absence and presence of RU1968F1. The Ca^{2+} responses were recorded in the FluoStar. ATP was injected at t = 0 s.

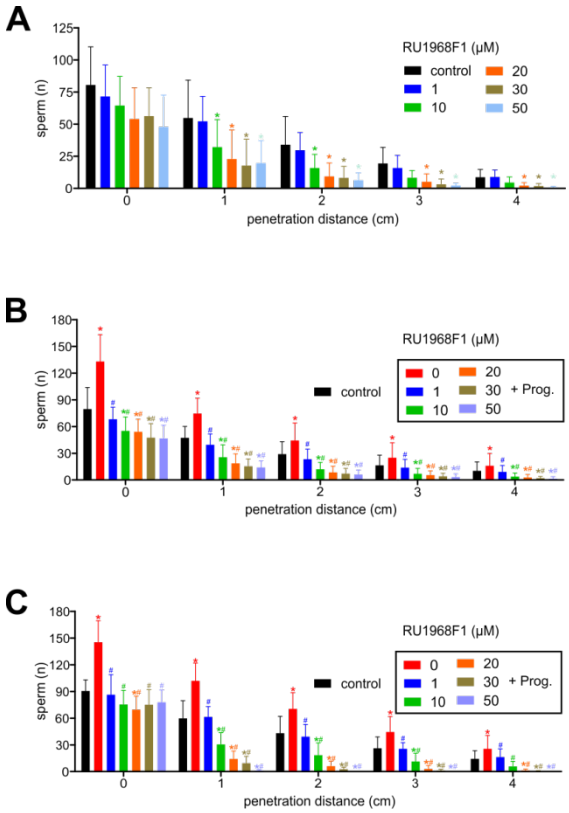


Figure S8: RU1968F1 suppresses penetration of sperm into viscous media. (A) Number of sperm at penetration distances of 0-4 cm in a modified Kremer's sperm-mucus penetration test. Sperm were incubated in buffer (0) or RU1968F1 (n = 21). Error bars indicate SD. *P < 0.05 versus control (0, without RU1968F1). $^{\#}$P < 0.05 versus progesterone without RU1968F1 (B) Number of sperm after incubation in buffer (0), progesterone, or progesterone plus RU1968F1 (n = 21). Error bars indicate SD. *P < 0.05 versus control, $^{\#}$P < 0.05 versus progesterone without RU1968F1 (0, without progesterone). (C) Number of sperm bathed in buffer (control) or progesterone when the capillary included buffer (0) or RU1968F1 (n = 6). Error bars indicate SD. *P < 0.05 versus control (0, without progesterone), $^{\#}$P < 0.05 versus progesterone without RU1968F1 (0, without progesterone)

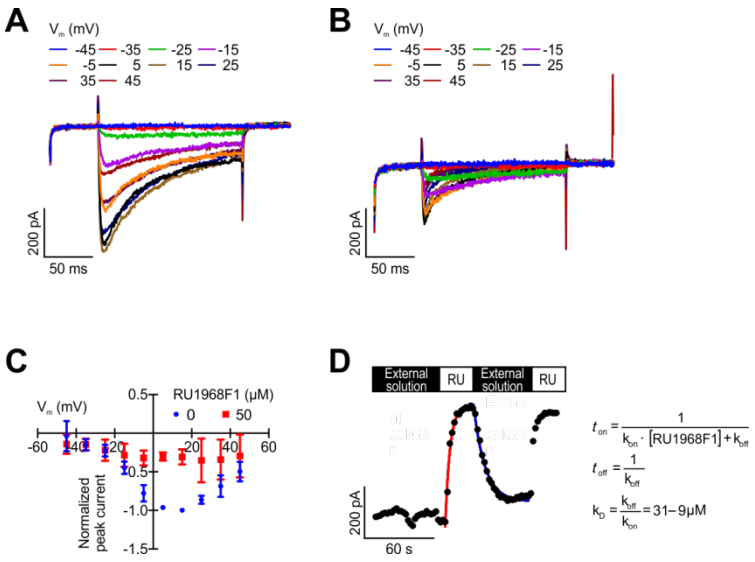


Figure S9: **RU1968F1 inhibits $Ca_V1.2$ channels**. Representative whole-cell current recorded from a HEK293T cell expressing human $Ca_V1.2+\beta_{2b}+\alpha_2\delta_1$ before (A) and after perfusion of the cell with 50 µM RU1968F1 (B). (C) Plotting the mean peak current versus voltage demonstrates the incomplete block of $Ca_V1.2$ by RU1968F1 (n = 5). (D) Peak currents at +20 mV recorded every 2 s while the cell was perfused with external solution or solution containing 50 µM RU1968F1. The reduction and recovery of the current during the wash-in (red) and wash-out (blue) of RU1968F1, respectively, was fitted with an exponential function. The time constants were used to calculate an approximate affinity (k_D) of 31 ± 9 µM (n = 4).

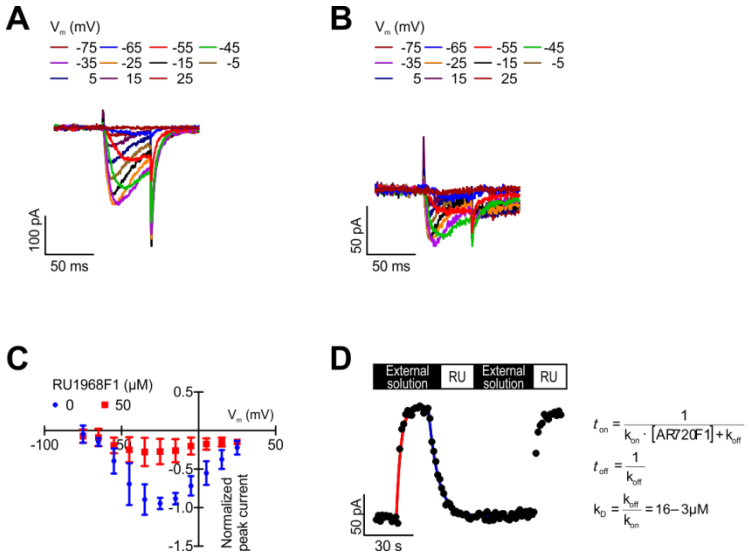


Figure S10: **RU1968F1 inhibits $Ca_V3.2$ channels.** Representative whole-cell current recorded from a HEK293T cell expressing $Ca_V3.2$ before (A) and after perfusion of the cell with RU1968F1 (B). (C) Plotting the mean peak current versus voltage demonstrates the incomplete block of $Ca_V3.2$ by RU1968F1 (n = 5). (D) Peak currents at +20 mV recorded every 2 s while the cell was perfused with external solution or solution containing 50 µM RU1968F1. The reduction and recovery of the current during the wash-in (red) and wash-out (blue) of RU1968F1, respectively, was fitted with an exponential function. The time constants were used to calculate an approximate affinity (k_D) of 16 ± 3 µM (n = 4).