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A novel cross-species inhibitor to study the function of CatSper Ca^{2+} channels in sperm

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Background and Purpose

Sperm from many species share the sperm-specific Ca^{2+} channel CatSper (cation channel of sperm) that controls the intracellular Ca^{2+} concentration and, thereby, the swimming behaviour. A growing body of evidence suggests that the mechanisms controlling CatSper activity and the role of the channel during fertilization differ among species. However, a lack of suitable pharmacological tools has hampered the elucidation of the function of CatSper. Known CatSper inhibitors exhibit considerable side effects and inhibit also Slo3, the K^+ channel in mammalian sperm.

Experimental Approach

The drug RU1968 was reported to suppress Ca^{2+} signaling in human sperm by an unknown mechanism. We resynthesized the drug and revisited its mechanism of action in sperm form humans, mice, and sea urchins.

Key Results

We show by Ca^{2+} fluorimetry, single-cell Ca^{2+} imaging, electrophysiology, opto-chemistry, and motility analysis that RU1968 inhibits CatSper in sperm from invertebrates and mammals. The drug lacks toxic side effects in human sperm, does not affect mouse Slo3, and inhibits human Slo3 with about 15-fold lower potency than CatSper. Moreover, in human sperm, the inhibitor mimics CatSper dysfunction and suppresses motility responses evoked by progesterone, an oviductal steroid that activates CatSper. Finally, we show that the drug abolishes CatSper-mediated chemotactic navigation in sea urchin sperm.

Conclusion and Implications

We propose RU1968 as a novel tool to elucidate the function of CatSper in sperm across species.

Non-standard abbreviations

2-AG	2-arachidonoylglycerol (2-AG)
ABHD2	alpha/beta hydrolase domain-containing protein 2
ASW	artificial sea water
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
CASA	computer-assisted sperm analysis
CatSper	cation channel of sperm
CI	confidence interval
F	fluorescence
HC	HC-056456
HSA	human serum albumin
HTF	human tubal fluid
LED	light-emitting diode
MDL	MDL 12330A
NNC	NNC 0936
PGE1	prostaglandin E1
pH_i	intracellular pH
RT	room temperature
sEBSS	supplemented Earle´s balanced salt solution
Slo3	slowpoke channel isoform 3
TYH	Toyoda, Yokoyama and Hosi´s medium
UV	ultraviolet light
VAP	velocity average path
V_m	membrane potential

Introduction

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) modulates the beat of the sperm flagellum and, thereby, the swimming behavior (Alvarez et al., 2014, Publicover et al., 2008). In many but not all species, $[Ca^{2+}]_i$ is controlled by the sperm-specific CatSper Ca^{2+} channel (Lishko et al., 2010, Ren et al., 2001, Kirichok et al., 2006, Quill et al., 2001, Seifert et al., 2015, Loux et al., 2013). CatSper appeared early in evolution, before the branching of eukaryotes into unikonts and bikonts (Cai and Clapham, 2008, Chung et al., 2017, Cai et al., 2014). So far, most of our knowledge about CatSper originates from physiological studies of native channels in mammalian and sea urchin sperm. In general, CatSper is activated by depolarization of the membrane potential (V_m) and by alkalization of the intracellular pH (pH_i) (Lishko et al., 2011, Lishko et al., 2010, Kirichok et al., 2006, Strünker et al., 2011, Seifert et al., 2015). In sea urchin sperm, the egg´s chemoattractant evokes rapid changes in V_m and pH_i and, thereby, stimulates Ca^{2+} influx via CatSper (Seifert et al., 2015, Espinal-Enriquez et al., 2017); the chemoattractant-evoked Ca^{2+} influx controls chemotactic steering. Targeted ablation of genes encoding CatSper subunits provided insight into the function of CatSper in mouse sperm (Chung et al., 2017, Chung et al., 2011, Wang et al., 2009, Liu et al., 2007, Carlson et al., 2003, Ren et al., 2001, Quill et al., 2001, Carlson et al., 2005, Qi et al., 2007, Zeng et al., 2013): mouse CatSper$^{-/-}$ sperm suffer from impaired motility (Qi et al., 2007, Ren et al., 2001, Miki and Clapham, 2013), fail to traverse the oviduct (Ho et al., 2009, Chung et al., 2014, Miki and Clapham, 2013), and are unable to penetrate the egg coat (Ren et al., 2001), resulting in male infertility (Qi et al., 2007, Quill et al., 2001, Ren et al., 2001). CatSper is essential for fertilization also in humans: mutations in *CATSPER* genes (Avenarius et al., 2009, Hildebrand et al., 2010) and CatSper dysfunction (Williams et al., 2015) are associated with male infertility. However, mouse and human CatSper have distinct properties, indicating that the channel might serve various different functions (Alvarez, 2017, Kaupp and Strünker, 2016). For example, in human but not in mouse sperm, CatSper serves as a polymodal sensor that integrates diverse chemical cues (Brenker et al., 2018, Schiffer et al., 2014, Brenker et al., 2012): human CatSper is activated by progesterone and prostaglandins (Brenker et al., 2012, Lishko et al., 2011, Strünker et al., 2011), two hormones present in the oviductal fluid (Schuetz and Dubin, 1981). The ensuing Ca^{2+} influx controls the swimming behaviour and promotes the penetration of the egg coat (Schaefer et al., 1998, Harper et al., 2003, Publicover et al., 2008, Oren-Benaroya et al., 2008, Baldi et al., 2009, Tamburrino et al., 2015, Tamburrino et al., 2014, Alasmari et al., 2013a, Kilic et al., 2009, Schiffer et al., 2014). Moreover, progesterone facilitates the migration of human sperm in viscous medium

encountered by the sperm during their voyage across the female genital tract (Alasmari et al., 2013b). However, in humans, neither the role of CatSper nor that of progesterone and prostaglandins during fertilization has been fully established. The function of CatSper in species other than sea urchin, mouse, and human is largely unknown. To address these questions, we rely on pharmacological tools that allow manipulating CatSper function.

Several drugs have been identified that suppress CatSper activity, for example NNC-0396 (NNC) (Lishko et al., 2011, Strünker et al., 2011), Mibefradil (Strünker et al., 2011), MDL12330A (MDL) (Brenker et al., 2012), and HC-056456 (HC) (Carlson et al., 2009). In patch-clamp experiments, NNC, Mibefradil, and MDL abolish CatSper currents (Brenker et al., 2012, Lishko et al., 2011, Strünker et al., 2011); HC attenuates CatSper currents (Carlson et al., 2009), but it is unknown whether the drug inhibits the channel completely. Of note, none of these drugs is selective for CatSper: the drugs also inhibit the sperm-specific K^+ channel Slo3 (Carlson et al., 2009, Brenker et al., 2014, Navarro et al., 2007, Mansell et al., 2014) - the principal K^+ channel in mouse (Zeng et al., 2011, Santi et al., 2010) and human sperm (Brenker et al., 2014). Notably, each drug inhibits CatSper and Slo3 with similar potency. Moreover, NNC, Mibefradil, and MDL exhibit serious adverse actions in human sperm: at high micromolar concentrations required to abolish Ca^{2+} influx via CatSper, NNC and Mibefradil evoke a sizeable and sustained increase of $[Ca^{2+}]_i$ and pH_i (Strünker et al., 2011, Brenker et al., 2012, Chavez et al., 2017) and stimulate acrosomal exocytosis (Chavez et al., 2017) (Figure S3). Similarly, MDL at high micromolar concentrations also evokes a sustained $[Ca^{2+}]_i$ increase in human sperm (Brenker et al., 2012). Finally, the drugs affect the vitality and overall motility of sperm (Tamburrino et al., 2014) (Figure S3). HC has not been further characterized in human sperm, because it is not commercially available. In conclusion, novel potent and selective CatSper inhibitors without toxic side effects are required.

Before the discovery of CatSper, the steroidal sigma-receptor ligand RU1968 was reported to suppress progesterone- and prostaglandin-induced Ca^{2+} signals in human sperm (Schaefer et al., 2000). The mechanism of RU1968 action in sperm has remained unclear, except that it does not involve the activation of sigma receptors (Schaefer et al., 2000). We wondered whether RU1968 might inhibit CatSper and revisited the drug´s action in sperm. We show that RU1968 potently abolishes CatSper-mediated Ca^{2+} signals in mouse, human, and sea urchin sperm. Patch-clamp recordings from mouse and human sperm corroborated that RU1968 inhibits CatSper. The drug does not affect mouse Slo3 and inhibits human Slo3 with about 15-fold lower potency than human CatSper. When present during the capacitation process, RU1968 suppresses hyperactivation in human sperm. The drug also inhibits

progesterone-evoked motility responses, showing that these involve Ca^{2+} influx via CatSper. Finally, we demonstrate that RU1968 abolishes chemotaxis of sea urchin sperm. In summary, RU1968 is a potent cross-species CatSper inhibitor that is selective for CatSper over Slo3. The drug seems well-suited to study CatSper function in sperm from invertebrates to mammals.

Material and Methods

Sperm preparation

The studies involving human sperm were performed in agreement with the standards set by the Declaration of Helsinki. Samples of human semen were obtained from volunteers with their prior written consent. Approval of the institutional ethics committees of the medical association Westfalen-Lippe and the Medical Faculty of the University of Münster: 4INie; approval of the ethical committee of the University of Birmingham Life and Health Sciences: ERN12-0570R**.** For Ca^{2+} fluorimetry in sperm populations, single-cell Ca^{2+} imaging, patch-clamp recordings, single-cell motility studies, and assays for acrosomal exocytosis and viability, sperm were purified by the swim-up procedure in human tubal fluid (HTF) medium containing (in mM): 93.8 NaCl, 4.69 KCl, 0.2 $MgSO_4$, 0.37 KH_2PO_4, 2.04 $CaCl_2$, 0.33 Na-pyruvate, 21.4 lactic acid, 2.78 glucose, 21 HEPES, and 4 $NaHCO_3$, pH 7.35 (adjusted with NaOH). Sperm were washed and re-suspended in HTF containing 3 mg/ml human serum albumin (HSA, Irvine Scientific, Santa Ana, CA, USA). For Kremer test, sperm were purified by the swim-up procedure in Supplemented Earle´s Balanced Salt Solution (sEBSS), containing (in mM): 98.5 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5.5 glucose, 25 $NaHCO_3$, 2.5 Na-pyruvate, 19 Na-lactate, 0.81 $MgSO_4$, 15 HEPES, and 0.3% bovine serum albumin, pH 7.4 (adjusted with NaOH). Before experiments, sperm were incubated for at least 300 min at 37°C and 5% CO_2 atmosphere. For computer-assisted sperm analysis (CASA), sperm were purified by the swim-up procedure in HTF lacking $NaHCO_3$ and HSA. Sperm were washed and re-suspended in this medium (non-capacitating conditions) or in HTF fortified with 25 mM NaHCO3 and 3 mg/ml HSA (capacitating conditions). Before experiments, sperm were incubated for at least 300 minutes at 37°C and 5% CO_2 atmosphere.

C57BL/6N wildtype and C57BL/6N CatSper1$^{-/-}$ mice were kept specific pathogen-free in ventilated cages (Greenline, Tecniplast). Maximally five mice were housed per cage and handled and sacrificed in accordance with the guideline set by the Animal Center of Nanchang University (Approval: SYXK2010-0002) and in accordance with the German

Animal Welfare Act and the district veterinary office under approval by the LANUV (AZ.02.05.50.16.011 and AZ.84-02.04.2012.A192). Mouse epididymides were obtained from at least 15 weeks old male mice that were anaesthetized with CO_2 or isoflurane (Abbvie Deutschland, Ludwigshafen, Germany) and sacrificed by cervical dislocation. For patch-clamp recordings, sperm were isolated from the cauda epididymis by swim-out in HS solution containing (in mM): 135 NaCl, 5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 20 HEPES, 5 glucose, 10 lactic acid, 1 Na-pyruvate, pH 7.4 (adjusted with NaOH). After 20 min swim-out at 37 °C and 10% CO_2, the supernatant was collected. Sperm were washed twice and re-suspended in HS solution. For Ca^{2+} fluorimetry, sperm were isolated by swim-out in TYH-medium containing (in mM): 138 NaCl, 4.8 KCl, 2 $CaCl_2$, 1.2 KH_2PO_4, 1 $MgSO_4$, 5.6 glucose, 0.5 Na-pyruvate, 10 Na-lactate, 10 HEPES, pH 7.4 (adjusted with NaOH). After 15 min swim-out at 37 °C and 5% CO_2, sperm were counted and capacitated in TYH-medium supplemented with 25 mM $NaHCO_3$ and 3 mg/ml BSA.

Sperm from the sea urchin *Arbacia punctulata* were obtained by injecting 0.5 M KCl into the body cavity or electrical stimulation of the animal. The ejaculate ("dry sperm") was diluted in artificial sea water (ASW) containing (in mM): 423 NaCl, 9.27 $CaCl_2$, 9 KCl, 22.94 $MgCl_2$, 25.5 $MgSO_4$, 0.1 EDTA, 10 HEPES, pH 7.8 (adjusted with NaOH).

Measurement of changes in intracellular Ca^{2+}

In human sperm populations, changes in $[Ca^{2+}]_i$ and pH_i were measured with the fluorescent Ca^{2+} indicator Fluo4 and BCECF (Thermo Fisher, Waltham, MA, USA), respectively, in 384 multi-well plates in a fluorescence plate reader (Fluostar Omega, BMG Labtech, Ortenberg, Germany) at 29°C, or in a rapid-mixing device in the stopped-flow mode (SFM400, Bio-Logic, Grenoble, France) at 37°C. Sperm were loaded with Fluo4-AM (10 µM) in the presence of Pluronic F-127 (0.05% w/v) at 37°C for 45 min or with BCECF-AM (10 µM) at 37°C for 15 min. After incubation, excess dye was removed by centrifugation (700 x *g*, 10 min, room temperature (RT)). Sperm concentration was adjusted to 5 x 10^6 cells /ml in HTF and equilibrated for 5 min at 29°C (Fluostar) or 37°C (stopped-flow).

In plate-reader experiments, wells were filled with 50 µl of the sperm suspension; the fluorescence was excited at 480 nm (Fluo-4) or 440 nm and 480 nm (dual excitation, BCECF) and fluorescence emission was recorded at 520 nm. Fluorescence was monitored before and after injection of 25 µl (1:3 dilution) RU1968F1, followed after 5 min by injection of stimuli (1:10 dilution). The solutions were injected into the wells with an electronic multichannel pipette. Changes in Fluo-4 fluorescence are depicted as ΔF/F (%), i.e. the change in

fluorescence (ΔF) relative to the mean basal fluorescence (F) before application of buffer or stimuli, to correct for intra- and inter-experimental variations in basal fluorescence among individual wells. Changes in BCECF-fluorescence ratio (R, 480/440 nm) are depicted as ΔR/R (%), i.e. the change in ratio (ΔR) relative to the mean basal ratio (R) before application of buffer or stimuli, to correct for intra- and inter-experimental variations in the basal fluorescence ratio among individual wells. In stopped-flow experiments, the sperm suspension was rapidly mixed (1:1; flow rate = 1 ml/s) with HTF containing RU1968 and other stimuli, or with K8.6-, KCl-, or pH_o8.6-HTF containing RU1968. Fluorescence was excited with a SpectraX Light Engine modulated at 10 kHz (Lumencor, Beaverton OR, USA) and passed through a 494/20 nm excitation filter (Semrock, Buffalo NY, USA). Emission was passed through a 536/40 nm filter (Semrock) and recorded with a photomultiplier (H9656-20; Hamamatsu Photonics, Hamamatsu, Japan). Signals were amplified with a lock-in amplifier (7230 DSP, Signal Recovery, Oak Ridge TN, USA) and recorded with a data acquisition pad (PCI-6221; National Instruments, Germany) and BioKine software v. 4.49 (Bio-Logic). K8.6-HTF (in mM): 98.5 KCl, 0.2 $MgSO_4$, 0.37 KH_2PO_4, 2.04 $CaCl_2$, 0.33 Na-pyruvate, 21.4 lactic acid, 2.78 glucose, 21 TAPS, and 4 $KHCO_3$, pH 8.6 (adjusted with KOH). KCl-HTF (in mM): 98.5 KCl, 0.2 $MgSO_4$, 0.37 KH_2PO_4, 2.04 $CaCl_2$, 0.33 Na-pyruvate, 21.4 lactic acid, 2.78 glucose, 21 HEPES, and 4 $KHCO_3$, pH 7.35 (adjusted with KOH). pH_o8.6-HTF (in mM): 93.8 NaCl, 4.69 KCl, 0.2 $MgSO_4$, 0.37 KH_2PO_4, 2.04 $CaCl_2$, 0.33 Na-pyruvate, 21.4 lactic acid, 2.78 glucose, 21 TAPS, and 4 $NaHCO_3$, pH 8.6 (adjusted with NaOH). Changes in Fluo-4 fluorescence are depicted as ΔF/F (%), i.e. the change in fluorescence (ΔF) relative to the fluorescence (F) right after the mixing, to correct for intra- and inter-experimental variations in basal fluorescence. For single-cell Ca^{2+} imaging, sperm were incubated in the wells of PLL-coated Greiner Cellview glass slides with Fluo-4-AM (5 µM) for 30 min at 37°C, followed by another 15 min at room temperature to allow settling of sperm on the glass surface. Afterwards, the buffer was replaced twice with 90 µl of "fresh" HTF to remove excess extracellular dye. Progesterone and RU1968F1 were injected in a 1:10 dilution (10 µl) into the well and the ensuing changes in $[Ca^{2+}]_i$ were observed under an Olympus IX73 inverted microscope, equipped with a 20x/0.75 objective (U Plan S Apo, Olympus, Germany), coupled to an Andor Zyla 4.2 sCMOS camera (Andor Technology, Belfast, UK). Images were captured at 1 Hz. Changes in $[Ca^{2+}]_i$ were determined from a region of interest around the head and neck of single sperm. Signals are displayed as $F\text{-}F_0/F_{max}\text{-}F_0$; F_0 is the mean fluorescence of ≥ 5 images before injection of RU1968F1 or progesterone, whereas F_{max} is the peak fluorescence signal evoked by a subsequent injection of ionomycin. This procedure

corrects for intra- and inter-experimental variations in resting $[Ca^{2+}]_i$ and dye loading among individual sperm.

In mouse sperm populations, changes in $[Ca^{2+}]_i$ were measured in sperm loaded with Cal520-AM (5 μM) (ATT Bioquest, USA) in the presence of Pluronic F-127 (0.02% w/v) for 45 min at 37°C in TYH buffer. After loading, excess dye was removed by three centrifugations (700 x g, 7 min, RT). Recordings were performed using the stopped-flow apparatus as described above, but with mixing at a flow rate of 0.5 ml/s. K8.6-TYH (in mM): 4.8 NaCl, 138 KCl, 2 $CaCl_2$, 1.2 KH_2PO_4, 1 $MgSO_4$, 5.6 glucose, 0.5 Na-pyruvate, 10 lactic acid, 10 TAPS, pH 8.6 (adjusted with KOH).

In sea urchin sperm populations, changes in $[Ca^{2+}]_i$ were recorded in Fluo4-loaded sperm. To this end, dry sperm (diluted 1:6 (v/v)) were loaded with Fluo4-AM (10 μM) in the presence of Pluronic F-127 (0.02% w/v) for 45 min at 18°C in ASW. After loading, sperm were diluted 1:20 (v/v) in ASW and allowed to equilibrate for 5 min. Recordings were performed using the stopped-flow apparatus with a flow rate of 1 ml/s. Fluorescence was excited, recorded, and processed as described above.

KCl-ASW (in mM): 216 KCl, 216 NaCl, 9.27 $CaCl_2$, 22.94 $MgCl_2$, 25.5 $MgSO_4$, 0.1 EDTA, 10 HEPES, pH 7.8, (adjusted with NaOH).

Patch-clamp recordings

Patch-clamp recordings from human sperm were performed in the whole-cell configuration, as previously described (Strünker et al., 2011). Seals between pipette and sperm were formed either at the cytoplasmic droplet or the neck region in standard extracellular solution (HS) containing (in mM): 135 NaCl, 5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 5 glucose, 1 Na-pyruvate, 10 lactic acid, and 20 HEPES, pH 7.4 (adjusted with NaOH). CatSper currents were recorded in divalent-free solutions containing (in mM): 140 CsCl, 40 HEPES, 1 EGTA, pH 7.4 (adjusted with CsOH); the pipette solution contained (in mM): 130 Cs-aspartate, 50 HEPES, 5 EGTA, 5 CsCl, pH 7.3 (adjusted with CsOH). Slo3 currents were recorded in HS with a pipette solution containing (in mM): 140 K-aspartate, 50 HEPES, 10 NaCl, 5 KCl, 0.5 $CaCl_2$, pH 7.3 (adjusted with KOH). Hv1 currents were recorded in a bath and pipette solution containing (in mM): 120 NMDG, 100 MES, 5 TEA-Cl, 2 EGTA, pH 6 (adjusted with methanesulfonic acid). To depict mean changes in CatSper currents, CatSper current amplitudes were normalized to that of the monovalent currents in the absence of any progesterone, NH_4Cl, or RU1068F1. This procedure corrects for variations in amplitudes among individual sperm to ease and to improve the clarity of the graphical illustration. Patch-

clamp recordings from mouse sperm were performed in the whole-cell configuration, as previously described (Kirichok et al., 2006, Zeng et al., 2011). Seals between pipette and sperm were formed at the cytoplasmic droplet. For Slo3 recordings, the extracellular solution contained (in mM): 160 KOH, 10 HEPES, 150 MES, and 2 $Ca(MES)_2$, adjusted to pH 7.4 with MES; the pipette solution contained (in mM): 155 KOH, 5 KCl, 10 BAPTA, 20 HEPES, 115 MES, pH 8.0 (adjusted with KOH). CatSper currents were recorded in divalent-free solutions containing (in mM): 150 NaCl, 20 HEPES, 5 EDTA, pH 7.4 (adjusted with NaOH); and with a pipette solution containing (in mM) 135 Cs-MES, 10 HEPES, 10 EGTA, and 5 CsCl, pH 7.2 (adjusted with CsOH). Current amplitudes were normalized to that of the monovalent currents in the absence of NH_4Cl or RU1968F1 (control) to correct for variations in amplitudes among individual sperm.

Human T-type ($Ca_v3.2$) and L-type ($Ca_V1.2$+β2b+α2δ1) Ca^{2+} channels were studied in HEK293T cells (The European Collection of Cell Cultures, Porton Down, UK) that were cultured according to the supplier's protocol in the presence of penicillin G (100 U/ml) and streptomycin (10 mg/ml). Cells were transfected at 40% confluency with pcDNA3.1-CACNA1C, pcDNA3.1-CaVb2b, and pIRES-dsRed-CaVa2d1 in a ratio of 2:1:1 µg, or with 2 µg of pCMV-Entry-CACNA1H, using the calcium-phosphate precipitation method. Patch-clamp recordings from HEK293T cells were performed in the whole-cell configuration, using a HEKA EPC 10 amplifier with PatchMaster software (both HEKA Elektronik, Lambrecht, Germany). The extracellular solution contained (in mM): 125 TEA-Cl, 15 glucose, 10 HEPES, 5 $CaCl_2$, pH 7.4, (adjusted with CsOH); the pipette solution contained (in mM): 100 CsCl, 10 EGTA, 10 HEPES, 5 TEA-Cl, 5 MgATP, 0.2 NaGTP, pH 7.4 (adjusted with CsOH). RU1968F1 was applied via a gravity-driven perfusion system.

Analysis of sperm motility

To evaluate the acute action of RU1968F1 on motility parameters, sperm from a particular sample were incubated side-by-side in HTF lacking $NaHCO_3$ and HSA (non-capacitating medium) and capacitating medium (25 mM $NaHCO_3$ / 3 mg/ml HSA). After 3 hours of incubation at 37°C, sperm kinematic parameters were analyzed by a CASA system (CEROS, Hamilton Thorn Research, Beverly, MA, USA) before and after application of RU1968F1. Sperm were bathed in RU1968F1 for 5 min prior to the experiment. To evaluate the long-term action of RU1968F1 on sperm motility parameters, sperm were re-suspended in capacitating medium (HTF containing 25 mM $NaHCO_3$ and 3 mg/ml HSA) with or without RU1968F1. After 3 hours of incubation at 37°C, sperm kinematic parameters were analyzed

by CASA. To evaluate the action of RU1968F1 on progesterone-induced hyperactivation, capacitated sperm were incubated for 5 min in the absence (control) and presence of progesterone, RU1968F1, and progesterone plus RU1968F1, and the motility was analyzed by CASA. The following parameters were determined by CASA: curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), linearity of progression (LIN, %), percentage of total, progressive, and rapid motility as well as percentage of motile, hyperactivated sperm. The threshold values for hyperactivation were manually set (VCL >150 mm/s, ALH>7 mm, LIN < 50% (Mortimer et al., 1998; Tamburrino et al, 2014). A minimum of 100 cells and 5 fields of view were analysed for each aliquot. The experiments were performed at 37°C.

Motility in human sperm evoked by uncaging of progesterone were studied in an observation chamber (100 µm depth) under an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan), equipped with a 4x microscope objective (0.13 NA, UPLFLN-PH, Olympus) under dark-field illumination (red LED, M660L3-C1, Thorlabs). Movies were recorded at a total magnification of 6.4x with a high-speed CMOS camera (Dimax HD, PCO, Kelheim, Germany) at 150 Hz. Photolysis of caged progesterone (1 µM) (Kilic et al., 2009) was achieved using a 200 ms light flash delivered by a 365 nm LED (M365L2-C, Thorlabs, Munich, Germany). Movies were processed and analyzed using a customized CASA (computer-assisted sperm analysis) plugin for ImageJ. Changes in the average path velocity (VAP) are depicted as VAP (%), i.e. the change in VAP relative to the VAP right before the UV flash, to correct for intra- and inter-experimental variations and for the different resting VAP in the absence and presence of the inhibitor. This procedure eases and improves the clarity of the graphical illustration.

Kremer penetration assays were performed in sEBSS supplemented with methylcellulose (1% w/v) and 0.3% BSA, equilibrated overnight at 4°C (penetration medium). The penetration medium, with or without RU1968F1, was filled into flattened glass capillary tubes (dimensions: 1.2 x 4.8 x 50 mm, 400 µm depth; CM scientific, UK); one end of the tubes was sealed with CristaSeal wax (Hawksley, UK). The open ends of the tubes were submersed in a sperm suspension (3 x 10^6 /ml) with or without stimuli and/or RU19868F1. Penetration was assessed after 1h (37°C, 5.5% CO_2) by counting sperm at 2 cm using a phase contrast microscope at a 200x magnification. 3 fields of view were chosen and in each field, three focal planes were counted, yielding 9 fields altogether.

Human sperm viability and motility as shown in Figure S3 was tested following incubation of sperm for 5 min at room temperature with RU1968F1, NNC-55-0396,

Mibefradil, or the vehicle (DMSO). The fraction of immotile and dead sperm was assessed by counting and by an eosin vitality test, respectively, at 200x magnification under a phase-contrast microscope (Axiostar, Carl Zeiss), in accordance with the WHO guidelines for semen analysis (WHO, 2010). For the eosin staining, 5 µl of the sperm suspension was mixed with 5 µl of eosin staining solution (0.5 % (w/v) eosin Y dissolved in a 0.9 % NaCl solution) on a microscope slide, covered with a 22 x 22 mm coverslip, and incubated for 30 s at room temperature. Eosin-positive (dead) vs. eosin-negative (live) sperm were counted. To determine the fraction of immotile and viable sperm, a total number of 400 sperm was assessed.

Sea urchin sperm chemotaxis was studied as described (Seifert et al., 2015). In brief, sperm (~ 10^8 cells/ml) were observed in a recording chamber (150 µm depth) under an IX71 microscope (Olympus), equipped with a 10x microscope objective (UPlanSApo; NA 0.4; Olympus), with stroboscopic (500 Hz) dark-field illumination (white LED; K2 star; Luxeon). Movies were recorded with an EMCCD camera (DU-897D; Andor) at 20 Hz through a bandpass filter (HQ520/40; Chroma). Photolysis of caged resact was achieved using a 200 ms pulse from a 365 nm LED (M365L2-C, Thorlabs). The relative dispersion was calculated as described before (Seifert et al., 2015).

Acrosomal exocytosis

Human sperm, capacitated for at least 300 min, were incubated with either 0.1% DMSO (vehicle control), RU1968F1 (10 µM), progesterone (10 µM), or a mixture of both (10 µM each) for 1 h at 37°C. Afterwards, sperm were washed by centrifugation and re-suspended in 0.5 ml of hypo-osmotic swelling medium (WHO, 2010). After 1 h at 37°C, sperm were washed again and fixed in 50 µl ice-cold methanol. The sperm were layered on a slide, air-dried, and stored at -20°C. For acrosome staining, sperm were incubated for 20 min in the dark with 1 mg/ml FITC-labeled *Arachis hypogaea* (peanut) lectin (PNA-FITC, Sigma Aldrich) in PBS. Slides were analyzed using an Axiolab A1 FL microscope (Carl Zeiss, Jena, Germany). For each condition, 200 curled-tail (viable) cells were analyzed for their acrosomal status, as previously described (Tamburrino et al., 2014).

Data analysis and statistical evaluation

The data analysis complies with the recommendations on experimental design and analysis in pharmacology (Curtis et al, 2015). All data are presented as mean ± standard deviation. Statistical analysis and fitting of dose-response relations were performed using

GraphPad Prism 5 (Prism, La Jolla, USA). Half-maximal inhibitory concentrations (IC_{50}) were derived by nonlinear regression analysis, using a four parameter fit:

$$Y = \text{bottom} + \frac{(\text{top}-bottom)}{(1+10^{(logIC50-x)n})}.$$

Y = signal amplitude; bottom and top = plateaus in the units of Y; x = log(concentration of inhibitor); IC_{50} = concentration of agonist that gives the response half way between bottom and top; n = Hill coefficient.

Most of the experiments were performed in a randomized block design, i.e. for each experimental replicate, sperm prepared from one particular semen sample were subjected in parallel to all treatment conditions. If the experiment involved two conditions (control and treatment), we used the paired t-test. If the experiment involved ≥ 3 conditions, we used one-way randomized block ANOVA, assuming sphericity. When ANOVA´s F-test and the test for matching efficacy achieved $P < 0.05$, means were compared to the control´s mean by Dunnett's multiple comparisons post-hoc test, unless otherwise indicated. If experiments were not performed in a randomized block design, we used unpaired t-test or one-way ANOVA; when ANOVA´s F-test achieved $P < 0.05$ and Bartlett's test yielded no significant variance inhomogeneity, means were compared to the control´s mean or to each other by Dunnett's or Bonferron´s multiple comparisons post-hoc test, respectively. In Figure 6E, J, and Figure 8I, for the ease of illustration and for clarity, we show data normalized to the control. Yet, we normalized the data only after the statistical analysis using one-way ANOVA, because normalization makes any data set violate the ANOVA.

Randomization and blinding

Experiments and data analysis were performed without randomization and blinding, except for the Eosin test and the manual counting of motile/immotile sperm. Otherwise, non-treated and treated conditions were measured and analyzed side-by-side by the same experimenter, using objective measures and analysis methods.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017a, b).

Results

Synthesis of RU1968

We synthesized RU1968 from (±) estrone methyl ether (Figure 1A, SI) that is readily accessible via the Torgov route (Ananchenko et al., 1962, Ananchenko and Torgov, 1963). Van Leusen reaction yielded the nitrile (1) (Van Leusen and Van Leusen, 2004), followed by addition of methyllithium to yield the ketone (2). Reductive amination with *N, N*-dimethylethylenediamine established the aza side chain, yielding a mixture of four diastereomers (3) (at C-17 and C-20; Figure 1A, 3). We separated two diastereomers (Figure 1A, compounds 3a and 3b), each a mixture of a *cis* and a *trans* isomer. Relative configurations (C-18 (CH_3) and C-20 (CH)) were assigned by NMR spectroscopy (H,H-COSY and NOE, Figure S1 and S2); the *cis* isomers were the dominant species (*cis/trans* ratio for compound 3a was 3:1; and for 3b it was 4:1). Finally, cleavage of the phenolic methyl ether yielded a diastereomeric mixture of RU1968. The diastereomers eluted from a preparative HPLC in four fractions called RU1968F1-4; RU1968F1 and 2 are derived from 3a *cis* and *trans*, respectively, whereas RU1968F3 and 4 are derived from 3b *trans* and *cis*, respectively (Figure 1C). Because the actions of RU1968F2-4 in sperm were similar to that of RU1968F1 (see Figure 2, S3, and S6), we chose RU1968F1 to characterize its action in sperm. We first examined the action of RU1968F1 in populations of human sperm loaded with a fluorescent pH_i or Ca^{2+} indicator (Figure S3A-D). The drug evoked negligible changes in pH_i. At concentrations ≤ 7.5 μM, RU1968F1 caused a small, transient Ca^{2+} increase; $[Ca^{2+}]_i$ peaked and returned to basal levels within about 250 s. At concentrations > 7.5 μM, RU1968F1 evoked a slow decrease of $[Ca^{2+}]_i$. The mechanism(s) underlying the drug-evoked changes in $[Ca^{2+}]_i$ are unclear; yet, in the absence of extracellular Ca^{2+}, the drug did not change $[Ca^{2+}]_i$, indicating that Ca^{2+} release from internal stores is not involved (Figure S3C). Most importantly, even at high micromolar concentrations (30 μM), the drug did not affect overall human sperm motility and viability and did not evoke acrosomal exocytosis (Figure S3E-G). Thus, RU1968F1 lacks the toxic and adverse actions of NNC, Mibefradil, and MDL in human sperm.

RU1968F1 is a potent cross-species CatSper inhibitor

To investigate whether RU1968F1 inhibits human CatSper, we studied progesterone- and PGE1-evoked Ca^{2+} signals in human sperm bathed in the drug. RU1968F1 slowed down and completely suppressed the Ca^{2+} signals in a dose-dependent fashion; the IC_{50} values of 4 ± 2 μM (progesterone) and 3.8 ± 0.5 μM (PGE1) (n = 7, mean ± SD) were similar to those reported previously (Schaefer et al., 2000) (Figure 2A-D). The drug was effective within a

range of extracellular pH (pH_o) values: at pH_o 6.8 and 7.8, the IC_{50} value of the progesterone-evoked Ca^{2+} signal was 4.4 ± 1.4 µM and 2.2 ± 0.6 µM, respectively (n = 6) (Figure 2B). The actions of RU1968F2-4 alone and on progesterone-induced Ca^{2+} signals were similar to those of RU1968F1 (see Figure 2, S3, and S6).

Furthermore, we studied whether RU1968F1 also inhibits Ca^{2+} signals evoked by intracellular alkalization via weak bases, e.g. NH_4Cl (Figure 2E-G). The drug slowed down and almost completely suppressed Ca^{2+} signals evoked by $NH_4Cl \leq 3$ mM (Figure 2E, F); the IC_{50} value for 3 mM NH_4Cl was 4.0 ± 2.8 µM (n = 5) (Figure 2F). Ca^{2+} signals evoked by 10 mM NH_4Cl were only slightly attenuated, whereas for 30 mM NH_4Cl, the signal was rather similar in the absence and presence of RU1968F1. We conclude that RU1968F1 inhibits also ΔpH_i-evoked Ca^{2+} responses; its potency seems to decrease with increasing ΔpH_i. Alternatively, the presence of NH_4^+ or NH_3 might impair binding of RU1968F1 to its blocking site. Arguing against that notion, the drug readily suppressed progesterone responses in sperm that were bathed for about 20 min in NH_4Cl (30 mM) (Figure S5; IC_{50} = 3.5 ± 1.4 µM, n = 3); with time, the pH_i slowly recovers from the NH_4Cl-evoked alkalization (Strünker et al. 2011).

Influx of Ca^{2+} via CatSper can also be evoked by simultaneous extracellular alkalization (which increases pH_i) and depolarization by K^+ (K8.6 buffer) (e.g. Carlson et al., 2003). RU1968F1 suppressed Ca^{2+} signals evoked by simultaneous alkalization/depolarization (Figure 2K, L; IC_{50} = 1.2 ± 0.6 µM, n = 3) and by alkalization or depolarization alone (Figure S4). Finally, when sperm were mixed simultaneously with progesterone and RU1968F1 in a stopped-flow apparatus, the inhibition of Ca^{2+} responses was similar to that under pre-incubation conditions (Figure 2I, J) (IC_{50} = 3.0 ± 1.1 µM; n = 4). This result suggests that RU1968F1 rapidly reaches its blocking site.

Next, we studied the action of RU1968F1 in single human sperm by Ca^{2+} imaging. At concentrations ≥ 10 µM, RU1969F1 evoked a slow decrease of $[Ca^{2+}]_i$ (Figure 3A); For low micromolar RU1969F1 concentrations, we did not observe Ca^{2+} transients, which might reflect differences in sensitivity of population *versus* single-cell fluorimetry. In the presence of RU1968F1, progesterone-induced Ca^{2+} responses were suppressed in a dose-dependent fashion (Figure 3B-D) with an IC_{50} of 4.8 ± 1.2 µM (standard error of the fit). Altogether, the action of the RU1868F1 itself on $[Ca^{2+}]_i$ and on progesterone-evoked Ca^{2+} responses is similar when investigated in sperm populations and in single sperm.

We further tested whether the drug also inhibits Ca^{2+} influx via CatSper in mouse sperm. Mouse CatSper is insensitive to progesterone and prostaglandins (Lishko et al., 2011). Therefore, we activated CatSper via simultaneous alkalization/depolarization or via 8-Br-

cAMP, which activates mouse (Ren et al., 2001) and human (Brenker et al., 2012) CatSper at high concentrations. RU1968F1 suppressed Ca^{2+} responses evoked by alkalization/depolarization or 8-Br-cAMP with an IC_{50} of 0.83 ± 0.07 µM and 0.84 ± 0.03 µM, respectively (n = 3) (Figure 4A-D).

Finally, we investigated the action of RU1968F1 on CatSper in sperm of the sea urchin *Arbacia punctulata*. To this end, we studied CatSper-mediated Ca^{2+} responses evoked either by the chemoattractant resact, depolarization of V_m, or by NH_4Cl. Irrespective of the stimulus, RU1986F1 suppressed the Ca^{2+} responses with IC_{50} values of 1.3 ± 0.1, 1.1 ± 0.4, and 4 ± 2, respectively (n = 3) (Figure 5A-F). Altogether, these results suggest that RU1968F1 is a potent cross-species CatSper inhibitor.

To scrutinize this conclusion by an independent technique, we recorded by whole-cell patch-clamping CatSper currents in human and mouse sperm. In human sperm, monovalent CatSper currents were evoked by stepping the membrane voltage from –100 mV to +150 mV in increments of 10 mV from a holding potential of 0 mV. RU1968F1 completely suppressed the currents with an IC_{50} of 0.4 ± 0.3 µM (n = 5) (Figure 6A, B). Superfusion with progesterone or NH_4Cl enhanced the current amplitudes (Fig, 6C, D). The progesterone- and NH_4Cl-evoked currents were either completely suppressed or strongly attenuated by RU1968F1 (Figure 6C-E). In mouse sperm, monovalent CatSper currents were evoked by ramping the membrane voltage between -100 and +100 mV from a holding potential of 0 mV. Superfusion with RU1968F1 completely suppressed the currents with an IC_{50} of 10 ± 1 µM (Figure 6F, G). CatSper currents evoked at pH_i 8 and by NH_4Cl were strongly attenuated by the drug (Figure 6H-J). Thus, RU1968F1 inhibits human and mouse CatSper at rest and upon activation by ligands and ΔpH_i. Similar to the results obtained by Ca^{2+} fluorimetry, the potency of the drug seems to decrease with increasing pH_i. We did not test whether higher RU1968F1 concentrations completely suppress the currents evoked by NH_4Cl and at pH_i 8.

RU1968F1 inhibits human but not mouse Slo3

We studied the interaction of RU1968F1 with sperm ion channels other than CatSper. In mouse sperm, currents carried by the K^+ channel Slo3 were similar in the absence and presence of RU1968F1 (Figure 7A, B). By contrast, in human sperm, the Slo3 current was inhibited with an IC_{50} of 7 ± 6 µM (n = 4) (Figure 7C, D). Thus, although not perfectly selective for CatSper, about 15-fold higher RU1968F1 concentrations are required to block human Slo3 channels. At concentrations up to 10 µM, the drug does not inhibit the voltage-gated proton channel Hv1 and the ATP-gated P_2X channel (Figure S7), which are expressed

in human and mouse sperm, respectively (Navarro et al., 2011, Lishko et al., 2010). We conclude that in mouse sperm, RU1968F1 acts rather selectively on CatSper. In human sperm, the drug inhibits also Slo3, yet, with about 15-fold lower potency. Finally, RU1968F1 inhibited heterologously expressed L- and T-type Ca^{2+} channels with IC_{50} values of about 20 and 10 µM, respectively (Figs. S9, S10), indicating that the drug acts with lower potency also on classic voltage-gated Ca^{2+} channels of somatic cells.

RU19681F1 suppresses progesterone-evoked motility responses in human sperm

Next, we tested the action of RU1968F1 on the motility of human sperm using classical computer-assisted sperm analysis (CASA). A brief incubation (5 min) of non-capacitated or capacitated sperm with the drug did not impair overall motility (Figure 8A, B, black), whereas the fraction of progressively motile sperm decreased about twofold with increasing RU1968F1 concentrations (Figure 8A, B, blue). Whether this is due to the inhibition of CatSper or represents an adverse action of the drug is unclear.

Furthermore, the penetration of the egg coat requires hyperactivated motility, which is characterized by an asymmetric flagellar beat, lower beating frequency, wiggly swimming trajectory, and lower average path velocity (VAP) (Suarez, 2008). In mouse sperm, CatSper is required for hyperactivation (Ren et al., 2001), whereas the control of hyperactivation by CatSper in human sperm is debated (Tamburrino et al., 2014, Alasmari et al., 2013b). We studied whether RU1968F1 affects hyperactivation in human sperm. A brief incubation (5 min) of capacitated sperm with RU1968F1 did not suppress spontaneous hyperactivated swimming. In fact, RU1968F1 concentrations < 10 µM seem to slightly enhance hyperactivation, whereas higher drug concentrations had no effect (Figure 8B, green). Spontaneous hyperactivation develops during the capacitation process (compare Figure 8A and B, green). In sperm that were capacitated in the presence of RU1968F1 (10 µM), i.e. incubated for some hours under capacitating conditions, the drug suppressed spontaneous hyperactivation (Figure 8C); the fraction of progressively motile sperm or overall motility was not affected (Figure 8D, E). This result suggests that, in human sperm, CatSper is involved in the ability to undergo hyperactivation; though, the partial inhibition of Slo3 might contribute to this action of RU1968F1.

Finally, we studied the action of RU1968F1 on progesterone-induced changes in swimming behavior. Incubation of capacitated sperm with progesterone seemingly promoted hyperactivation, which was inhibited by RU1968F1 (Figure 8F); the effect of progesterone was, however, not statistically significant. Therefore, we studied the motility of human sperm

before and after rapid activation of CatSper using caged progesterone (Kilic et al., 2009). Uncaging of progesterone by a brief (200 ms) UV flash instantaneously evoked a wiggly swimming trajectory (Figure 8G) and a decrease of VAP (Figure 8G, I), reminiscent of hyperactivated motility. The VAP reached its minimum ~5 s after uncaging of progesterone and did not recover within the recording time of 10 s. In the presence of RU1968F1, the swimming trajectory and the swimming pattern and VAP remained unchanged upon uncaging of progesterone (Figure 8H, I). We conclude that progesterone-evoked hyperactivation requires Ca^{2+} influx via CatSper.

Furthermore, it is well established that progesterone facilitates the migration of human sperm into viscous medium (Alasmari et al., 2013b). In sperm from an infertile man lacking functional CatSper channels, this facilitation was abolished (Williams et al., 2015). Using a modified Kremer's sperm-mucus penetration test, we investigated whether CatSper inhibition by RU1968F1 recapitulates this phenotype. To this end, an open glass capillary, which contained medium fortified with methylcellulose, was submersed in a sperm suspension. The number of sperm at a penetration distance of 2 cm (Figure 9A-C) was determined; data for shorter or longer penetration distances are presented in Figure S8. Consistent with previous results (Alasmari et al., 2013b), bathing sperm in progesterone enhanced the number of sperm penetrating the viscous medium (Figure 9A, C; S8A, C). The progesterone action was abolished by 1 µM RU1968F1 (Figure 9A, S8A). Of note, at this concentration, the drug itself did not affect the number of penetrating cells (Figure 9B, S8B). The progesterone action was also abolished when RU1968F1 was added to the capillary medium instead of to the sperm suspension (Figure 9C, S8C). These results support the notion that progesterone acts via CatSper to promote swimming in high-viscosity media and shows that RU1968F1 mimics the lack of functional CatSper channels. Of note, at concentrations > 1 µM, RU1968F1 in a dose-dependent fashion lowered the number of penetrating sperm both in the absence and presence of progesterone (Figure 9A, B; S8A, B); this probably reflects the drug-related decrease of the fraction of progressively motile sperm.

Incubation of human sperm in high micromolar concentrations of progesterone evokes acrosomal exocytosis (Baldi et al., 2009) (Figure 9D), i.e. the release of proteolytic enzymes from a secretory vesicle in the sperm head. In the presence of RU1968F1, the progesterone action was attenuated (Figure 9D), whereas RU1968F1 itself did not evoke acrosomal exocytosis (Figure 9D, S3). These results suggest that the progesterone-induced acrosome reaction involves Ca^{2+} influx via CatSper and that RU1968F1 might allow unraveling the role of CatSper in this process in more detail. Altogether, we conclude that RU1968F1 can provide

important insight on the role of progesterone action on CatSper to control various sperm functions.

RU19681F1 inhibits chemotaxis of sea urchin sperm

Finally, we tested whether RU1968F1 affects CatSper-mediated chemotactic steering of sea urchin sperm. In a shallow observation chamber under a dark-field microscope, sperm were bathed in a caged derivative of the chemoattractant resact (Alvarez et al., 2012, Böhmer et al., 2005, Kaupp et al., 2003). A chemoattractant gradient was established by photolysis of caged resact via a UV flash in the center of the recording chamber (Figure 10A). After the flash, sperm accumulated in the irradiated area, indicated by a decrease of sperm dispersion in the field of view (Figure 10B); the accumulation was abolished by RU1968F1 (Figure 10), but the drug did not affect the overall motility of the sperm.

Discussion

Here, we introduce RU1968F1 as a new pharmacological tool to elucidate the presence and role of CatSper in sperm. RU1968F1 is superior to hitherto known inhibitors: the drug is rather selective for CatSper and lacks toxic side effects in human sperm. Yet, because the action of the drug is complex, we cannot exclude adverse actions in other sperm species. This cautious note has to be considered in future studies using RU1968F1.

What is the molecular mechanism underlying CatSper inhibition by RU1968F1? It has been proposed that progesterone acts via an endocannabinoid-signaling pathway, involving the receptor alpha/beta hydrolase domain-containing protein 2 (ABHD2) (Miller et al., 2016): at rest, CatSper is inhibited by the endocannabinoid 2-arachidonoylglycerol (2-AG) in the flagellar membrane. Upon progesterone binding, ABHD2 degrades 2-AG and, thereby, relieves CatSper from inhibition (Miller et al., 2016). Considering that RU1968F1 is a steroid, the drug might act as an antagonist at the steroid-binding site on ABHD2. However, CatSper activation by prostaglandins does not involve ABHD2 (Miller et al., 2016), and in mouse and sea urchin sperm, CatSper is not activated by progesterone or prostaglandins (Lishko et al., 2011, Seifert et al., 2015). Moreover, CatSper activation by alkaline pH_i and depolarization does probably not involve a ligand-binding site. Therefore, we suspect that RU1968F1 binds to residues in the pore region and, thereby, directly block ion flux. The drug´s inhibitory action on classical voltage-gated Ca^{2+} channels also supports this conclusion. Of note, the potency of RU1968F1 to inhibit activation of CatSper by alkalization seems to decrease with

610 increasing amplitude of ΔpH_i. This might reflect a pH sensitivity of the blocking mechanism
611 or pH-dependent distribution of the drug across membranes. The latter is rather unlikely: upon
612 rapid mixing, the drug blocks Ca^{2+} influx via CatSper without a measurable latency,
613 suggesting that the drug acts rather from the outside. However, the mechanism of CatSper
614 inhibition will be difficult to elucidate rigorously by structure-function analysis or site-
615 directed mutagenesis, because CatSper resists functional expression.

616 What is the nature of the blocking site in Slo3 in human sperm? Human, but not
617 mouse Slo3, is inhibited by micromolar concentrations of progesterone (Brenker et al., 2014).
618 This inhibition results from binding of progesterone either to a site on the channel itself or on
619 its accessory subunit LRRC52 (Brenker et al., 2014). RU1968F1 might act via this steroid-
620 binding site on human Slo3. To improve the inhibitor´s selectivity, a structure-activity
621 analysis is required to identify RU1968F1 derivatives that do not act on human Slo3, but
622 display a similar or even enhanced potency to inhibit CatSper. The fact that human Slo3 can
623 be functionally expressed in cultured cells (Brenker et al., 2014) facilitates this endeavor.

624 Although the make-up of Ca^{2+}-signaling pathways in sperm is quite diverse (Kaupp
625 and Strünker, 2016, Alvarez, 2017), the CatSper channel is a common component in many,
626 but not all species (Cai et al., 2014). Our finding that RU1968F1 inhibits CatSper across
627 species opens the possibility to use the drug in diverse experimental settings. First, teleost fish
628 seem to lack CatSper genes (Cai and Clapham, 2008), yet, the swimming behavior of
629 zebrafish is controlled by Ca^{2+} (Fechner et al., 2015). However, the absence of CatSper in fish
630 has been contested (Yanagimachi et al, 2017). RU1968F1 might help to solve this
631 controversy. Second, the genome of many marine species, including the saprophytic fungus
632 *Allomyces macrogynus*, the tunicate *Ciona intestinalis*, and the seastar *Asterias amurensis*
633 harbor CatSper genes (Cai and Clapham, 2008, Cai et al., 2014). Sperm from these species
634 also undergo chemotaxis (Matsumoto et al., 2003, Miller, 1975, Pommerville, 1978, Yoshida
635 et al., 2002). RU1968F1 might reveal whether chemotaxis involves CatSper. Third, the drug
636 might help to define the diverse CatSper functions among mammalian sperm. For example,
637 mouse sperm undergo rotational motion that governs rheotaxis in gradients of flow velocities
638 (Miki and Clapham, 2013). By contrast, $CatSper^{-/-}$ mouse sperm do not rotate and fail to
639 undergo rheotaxis, suggesting that Ca^{2+} influx via CatSper is required. However, another
640 study describes rheotaxis as a passive process that does not require Ca^{2+} influx (Zhang et al.,
641 2016). CatSper recruits several proteins into Ca^{2+}-signaling domains that form a quadrilateral
642 arrangement along the flagellar membrane (Chung et al., 2014, Chung et al., 2017). Targeted
643 deletion of CatSper subunits disrupts these signaling domains (Chung et al., 2014, Chung et

al., 2017). Therefore, the motility defects of CatSper$^{-/-}$ mouse sperm might be caused by the lack of Ca^{2+} influx via CatSper, by disruption of the supramolecular flagellar ultrastructure, or by a combination of both. Fourth, in human sperm, neither the role of oviductal CatSper ligands, nor the role of CatSper during fertilization has been fully established. This is due to the demanding challenge to mimic the complex chemical, hydrodynamic, and topographical environment of the oviduct in vitro (Xiao et al., 2017). We envision the use of RU1968F1 as a tool to study the role of CatSper and its ligands in human sperm navigating across artificial or explanted oviducts.

Finally, mutations in *CATSPER* genes (Avenarius et al., 2009, Hildebrand et al., 2010) and the lack of functional CatSper channels (Williams et al., 2015) are associated with male infertility. In human sperm, at least *in vitro*, RU1968F1 mimics the lack of CatSper, indicating that inhibition of CatSper *in vivo* might prevent fertilization. Thus, RU1968F1 could serve as a lead structure to develop new non-hormonal contraceptives. Drugs that specifically target CatSper should exhibit no side effects, because the expression of the channel is confined to sperm.

Author contributions

All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. AR and TS conceived and designed the study, coordinated the experiments, and wrote the manuscript. AR, CS, CB, DF, TEN, YMC, LT, MB, GS, TKB, MK, LA, DW, XHZ, EB, SP, UBK, and TS acquired, analyzed, and/or interpreted data and revised the manuscript critically for important intellectual content. All authors approved the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1: Synthesis of RU1968F1-F4 (A) Synthesis of RU1968. Carbon atoms referred to in the text are marked with circles. (B) Structure of RU1968. (C) HPLC-elution profile of the four diastereomers. Isomers are named according to their order of elution.

Figure 2: Action of RU1968F1 on CatSper-mediated Ca^{2+} signals in human sperm populations. (A) Progesterone-induced Ca^{2+} signals in human sperm in the absence and presence of RU1968F1. $\Delta F/F$ (%) indicates the percentage change in fluorescence (ΔF) with respect to the mean basal fluorescence (F) before application of progesterone (500 nM). (B) Dose-response relation for the maximal signal amplitudes of the data from (A) (pH 7.35) (IC_{50} = 5.5 µM) and of progesterone responses studied at an extracellular pH of 6.8 (IC_{50} = 4.2 µM) or 7.8 (IC_{50} = 2.6 µM). (C) PGE1-induced Ca^{2+} signals in human sperm in the absence and presence of RU1968F1; PGE1 = 500 nM. (D) Dose-response relation for the maximal signal amplitudes of the data from (C) (IC_{50} = 3.1 µM). (E) NH_4Cl-induced Ca^{2+} signals in the absence and presence of RU1968F1, NH_4Cl = 3 mM. (F) Dose-response relation for the maximal signal amplitude of the data from (E) (IC_{50} = 1.8 µM) (G) Ca^{2+} signals evoked by various NH_4Cl concentrations in the absence (control) and presence of RU1968F1 (30 µM). (H) Mean relative amplitude of Ca^{2+} signals evoked by various NH_4Cl concentrations in the presence of RU1968F1 (30µM) (n = 5); amplitude evoked in the absence of RU1968F1 = 1 (control). Error bars indicate SD. *P < 0.05 versus control. (I) Ca^{2+} signals evoked by simultaneous mixing of sperm with progesterone (500 nM) and RU1968F1 in a stopped-flow apparatus. $\Delta F/F$ (%) indicates the percentage change in fluorescence (ΔF) with respect to the fluorescence (F) immediately after mixing. (J) Dose-response relation of the data from (I) (IC_{50} = 2.4 µM). (K) Ca^{2+} signals evoked by mixing of sperm with K8.6-HTF and RU1968F1. The final K^+ concentration and pH after mixing was 51.25 mM and 8.1, respectively. (L) Dose-response relation of the data from (K) (IC_{50} = 1.7 µM).

Figure 3: Action of RU1968F1 on progesterone-evoked Ca^{2+} signals in single human sperm. (A) Changes in $[Ca^{2+}]_i$ evoked by RU1968F1 in immobilized sperm. Sperm were challenged with RU1968F1 at t = 0. Traces represent averages of 122 (0 RU1968F1), 344 (1), 42, (3), 165, (10), and 109 (30) sperm from 3 donors. Signals are displayed as $F\text{-}F_0/F_{max}\text{-}F_0$; F_0 is the mean fluorescence of ≥ 5 images before application of RU1968F1; F_{max} is the peak fluorescence signal evoked by ionomycin (not shown) to gauge the maximal response amplitude. (B) Progesterone-evoked Ca^{2+} responses (2 µM) in the absence and presence of RU1968F1 (30 µM); averages of 50 (control) and 109 (RU1968F1) sperm from 3 donors. Progesterone and RU1968F1 were applied at t = 0; following application of RU1968F1, progesterone was applied at the time point indicated by the arrow. (C) Amplitude of progesterone-evoked Ca^{2+} responses in the absence and presence of different RU1968F1 concentrations; averages of 122 (0 µm RU1968F1), 344 (1), 42 (3), 165 (10), 109 (30) sperm from 3 donors. (D) Mean amplitude of progesterone-evoked Ca^{2+} signals (2 µM) in the presence of RU1968F1; number of sperm: 310 (0 µM RU1968F1), 552 (1), 181 (3) 302 (10), 222 (30). Error bars indicate SD. Fitting of a dose-response curve to the data yielded an IC_{50} of 4.8 ± 1.2 µM (standard error of the fit).

Figure 4: Action of RU1968F1 on CatSper-mediated Ca^{2+} signals in mouse sperm populations. (A) Ca^{2+} signals evoked by simultaneous mixing of mouse sperm with K8.6-TYH and RU1968F1 in a stopped-flow apparatus. After mixing, the final K^+ concentration and pH was 69 mM and 8.1, respectively (B) Dose-response relation of the data from (A) (IC_{50} = 0.90 µM). (C) Ca^{2+} signals evoked by simultaneous mixing of mouse sperm with 8-Br-cAMP (20 mM) and RU1968F1. (D) Dose-response relation of the data from (C) (IC_{50} = 0.84 µM).

Figure 5: Action of RU1968F1 on CatSper-mediated Ca^{2+} responses in sea urchin sperm. (A) Resact-induced Ca^{2+} signals in sea urchin sperm evoked by simultaneous mixing of sperm with resact (20 pM) and RU1968F1 in a stopped-flow apparatus. (B) Dose-response relation of the data from (A) (IC_{50} = 0.7 µM). (C) Depolarization-induced Ca^{2+} signals evoked by mixing of sperm with KCl-ASW and RU1968F1. Final K^+ concentration after mixing was 108 mM. (D) Dose-response relation of the data from (C) (IC_{50} = 1.0 µM). (E) Alkaline-evoked Ca^{2+} signals in the presence of RU1968F1; the final NH_4Cl concentration after mixing was 30 mM. (F) Dose-response relation of the data from (E) (IC_{50} = 4.6 µM).

Figure 6: RU1968F1 inhibits monovalent CatSper currents in human and mouse sperm. (A) Representative current-voltage relationship of CatSper currents recorded from a human sperm cell in divalent-free extracellular and intracellular solution (pH 7.4) in the absence and presence of increasing RU1968F1 concentrations. Voltage was stepped from -100 mV to +150 mV in increments of 10 mV. Inset: Voltage protocol. (B) Dose-response relation for the inhibition of human CatSper currents by RU1968F1 at +100 mV (IC_{50} = 0.4 ± 0.3 µM; n = 5). (C) Representative monovalent CatSper currents recorded from a human sperm cell before (control) and after perfusion with progesterone (2µM) and progesterone plus RU1968F1 (3µM), evoked by the voltage protocol shown in (A). The dotted red line indicates the current at 0 mV. (D) Monovalent CatSper currents recorded from a human sperm cell before (control) and after perfusion with NH_4Cl (10 mM) and NH_4Cl plus RU1968F1 (3µM), evoked by the voltage protocol shown in (A). The dotted red line indicates the current at 0 mV. (E) Mean amplitudes of monovalent currents at +100 mV recorded in the presence of RU1968F1, progesterone (2 µM), progesterone plus RU1968F1, NH_4Cl (10 mM), and NH_4Cl plus RU1968F1. Amplitudes were normalized to that evoked in the absence of any drug (control, dashed line). Error bars indicate SD (n = 5). *P < 0.05 versus control. Data were normalized only after performing the statistical analysis using one-way ANOVA (see Methods for details and explanations). (F) Representative CatSper currents recorded from a mouse sperm cell in divalent-free extracellular and intracellular solution (pH 7.2) in the absence and presence of increasing RU1968F1 concentrations. Voltage was ramped between -100 and +100 mV from a holding potential of 0 mV. Inset: Voltage protocol. (G) Dose-response relation for the inhibition of mouse CatSper currents at +100 mV (IC_{50} = 10 ± 1 µM, n = 3). Error bars indicate SD. (H) Currents in the presence of extracellular divalent ions (HS) and monovalent currents in divalent-fee conditions (control) recoded form a mouse sperm cell evoked at a pH_i 8 before (control) and after perfusion with RU1968F1, using the voltage protocol shown in (F). (I) Currents recorded form a mouse sperm cell at pH_i 7.2 before (control) and after perfusion with NH_4Cl (30 mM) and NH_4Cl plus RU1968F1, using the voltage protocol shown in (F). (J) Mean amplitudes of monovalent currents at +100 mV recorded form mouse sperm in the presence of RU1968F1, NH_4Cl plus RU1968F1, and at pH_i 8 in the presence of RU1968F1, using the voltage protocol shown in (F). Amplitudes were normalized to the monovalent currents evoked in the absence of any drug (control, dashed line). Error bars indicate SD. (n = 5). *P < 0.05 versus control. Data were normalized only after performing the statistical analysis using one-way ANOVA (see Methods for details and explanations).

Figure 7: RU1968F1 inhibits human but not mouse Slo3. (A) Representative Slo3 currents in a mouse sperm cell, recorded in the presence of extracellular divalent ions at symmetric intra- and extracellular K^+ concentrations in the absence and presence of 50 µM RU1968F1. Inset: Voltage protocol. (B) Mean Slo3 currents in mouse sperm at +100 mV in the absence and presence of 50 µM RU1968F1 (n = 5). (C) Representative Slo3 currents recorded from a human sperm cell in the absence and presence of RU1968F1. Inset: Voltage protocol. (D) Dose-response relation for the inhibition of human Slo3 currents by RU1968F1 at +100 mV (IC_{50} = 7 ± 6 µM, n = 4). Error bars indicate SD.

Figure 8: RU1968F1 interferes with hyperactivation and abolishes progesterone-induced motility responses in human sperm. (A, B) Motility parameters of non-capacitated (A) and capacitated (B) human sperm in the absence and presence of RU1968F1 (n = 8); sperm were bathed in the drug for 300 s. Error bars indicate SD. *P < 0.05 versus control (absence of RU1968F1). (C-E) Fraction of hyperactivated (C), motile (D), and progressively swimming (E) sperm after capacitation in the absence and presence of RU1968F1 (n = 11). Error bars indicate SD. *P < 0.05 versus control (absence of RU1968F1). (F) Hyperactivation evoked by bathing sperm for 300 s in RU1968F1, progesterone, or progesterone plus RU1968F (n = 11). Error bars indicate SD. (G) Track of a single sperm cell recorded before (3 s, black), during (0.2 s, red), and after (2.8 s, blue) uncaging of progesterone. The arrow indicates the direction of movement. Inset: time course of the average path velocity (VAP); the red bar indicates the uncaging of progesterone. (H) Track of a single sperm cell recorded before (3 s, black), during (0.2 s, flash, red) and after (2.8 s, blue) uncaging of progesterone in the presence of RU1968F1 (30 µM). Inset: time course of VAP; the red bar indicates the uncaging of progesterone. (I) Mean relative changes in VAP averaged over 20-30 sperm in the field of view after uncaging of progesterone (n = 11). Error bars indicate SD. *P < 0.05 versus control (before flash, 0 s). Data were normalized only after performing the statistical analysis using one-way ANOVA (see Methods for details and explanations).

Figure 9: RU1968F1 suppresses penetration of sperm into viscous media. (A) Number of sperm at a penetration distance of 2 cm in a modified Kremer's sperm-mucus penetration test. The sperm were incubated in buffer (control), progesterone, or progesterone plus RU1968F1 (n = 21). Error bars indicate SD. *P < 0.05 versus control; $^{\#}$P < 0.05 versus progesterone without RU1968F1. (B) Number of sperm after incubation in buffer (control) or RU1968F1 (n = 21). Error bars indicate SD. *P < 0.05 versus control (n = 21). (C) Number of sperm when the sperm were bathed in buffer (control) or progesterone, in the absence (0) or presence of RU1968F1 in the capillary (n = 6). Error bars indicate SD. *P < 0.05 versus control; #P < 0.05 versus progesterone without RU1968F1. (D) Acrosome reaction evoked by RU1968F1, progesterone, or progesterone and RU1968F1 (n =10). Error bars indicate SD. *P < 0.05 versus control, $^{\#}$P < 0.05 versus progesterone.

Figure 10: RU1968F1 abolishes chemotaxis of sea urchin sperm. (A) Dark-field microscopy images of a sperm suspension before (top) and after (bottom) establishing a resact gradient by photolysis of caged resact (middle) in the absence (control, left panel) or presence of RU1968F1 (30 μM, right panel). RU1968F1 abolishes resact-induced sperm accumulation. (B) Relative change of the sperm dispersion in the field of view evoked by uncaging of resact (t = 0, flash) in the absence (control, red) or presence of RU1968F1 (black); a decrease of dispersion indicates sperm accumulation in the irradiated area (control, n = 5, RU1968F1, n = 6). Error bars (grey) indicate SD.