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Novel pharmacological actions of Trequinsin Hydrochloride improve human sperm cell motility and function
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Authors’ Roles
All authors were involved in the design of the study. S.M.d.S. obtained funding for the library compound high-throughput screening and identification of Trequinsin as well as the recruitment and consent of patients. S.M.d.S., A.G.H., D.W.G., C.L.R.B., S.G.B. contributed to the study design. R.C.M. performed the majority of the experiments and analysis. S.G.B. conducted the flow cytometry and patch-clamp experiments and data analysis. All authors contributed to the construction, writing and editing of the manuscript. The initial and interim manuscript was drafted by R.C.M., S.G.B., S.M.d.S. and S.G.B and J.F. obtained the funding for the studentship for R.C.M. All Authors approved the final manuscript.

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Conflict of Interest Statement
The authors declare no conflict of interest.
Declaration of transparency and scientific rigour
This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Bullet Point Summary
What is already known?
- There is an unmet clinical need for compounds to treat asthenozoospermia (poor sperm motility).

What this study adds
- Trequinsin Hydrochloride elevates intracellular calcium and cyclic GMP in human sperm and improves motility.

Clinical significance
- Trequinsin hydrochloride has clinically relevant positive effects on human sperm motility and has potential to be a novel treatment for male infertility.
Abstract

Background and purpose
Asthenozoospermia is a leading cause of male infertility, but the development of pharmaceuticals to improve sperm motility has been hindered by the lack of effective screening platforms and knowledge of suitable molecular targets. We have demonstrated that a high throughput screening (HTS) strategy in conjunction with established in vitro tests can identify and characterise the action of compounds that improve sperm motility. The study aimed to apply HTS to identify new compounds from a novel small molecule library that increase intracellular calcium, $[\text{Ca}^{2+}]$, promote human sperm cell motility and systematically determine the mechanism of action.

Experimental approach
A validated HTS fluorometric $[\text{Ca}^{2+}]$ assay was used to screen an in-house library of compounds. Trequinsin hydrochloride (a phosphodiesterase 3 inhibitor) was selected for detailed molecular (plate reader assays, electrophysiology and cyclic nucleotide measurement) and functional (motility and acrosome reaction) testing in sperm from healthy volunteer donors and, where possible, patients.

Key results
The fluorometric analysis identified Trequinsin as an efficacious agonist of $[\text{Ca}^{2+}]$, although less potent than progesterone (P4). Functionally, Trequinsin significantly increased cell hyperactivation and penetration into viscous medium in all donor sperm samples and cell hyperactivation in 22/25 (88%) patient sperm samples. The Trequinsin-induced $[\text{Ca}^{2+}]$ response was cross-desensitised consistently by prostaglandin E1 but not with P4. Whole-cell patch clamp electrophysiology confirmed that Trequinsin activates CatSper and partially inhibits potassium channel activity. Trequinsin also increases intracellular cGMP.

Conclusion and Implications
Trequinsin exhibits a novel pharmacological profile in human sperm and may be a suitable lead compound for the development of new pharmaceuticals to improve patient sperm function and fertilisation potential.
Introduction

Asthenozoospermia (low sperm motility) has been reported as the leading cause of male infertility (Kumar & Singh, 2015). Intra Cytoplasmic Sperm Injection (ICSI) is the most common and successful treatment for male infertility. While it is a pragmatic solution, it involves invasive treatment of the female partner and bypasses all natural sperm selection processes. There are concerns that ICSI may be associated with long-term health issues for the children born, particularly in cases where the spermatozoa are predominately immotile and do not have the capacity to fertilise under natural conditions (Esteves & Roque et al., 2018; Hanevik & Hessen et al., 2016). Therefore, the development of novel direct treatments for male infertility is desirable, although this represents a significant challenge due to the limited understanding of the regulation of normal and dysfunctional sperm (Barratt & Björndahl et al., 2017).

Intracellular calcium ([Ca^{2+}]_{i}) is an established regulator of sperm function and a wealth of evidence suggests that the principle cation channel in sperm (CatSper) influences sperm function and fertilisation potential through regulation of extracellular calcium influx (Singh & Rajender, 2015; Strünker & Goodwin et al., 2011; Tamburrino & Marchiani et al., 2014; Williams & Mansell et al., 2015). CatSper is confined to the principle piece of the flagellum is modulated by intracellular pH (pHi) and membrane potential. It is sensitive to progesterone (P4) (Lishko & Botchkina et al., 2011; Strünker & Goodwin et al., 2011), which stimulates cell penetration into a viscous medium (used as an in vitro model for regions of the female reproductive tract) (Alasmari & Costello et al., 2013; Barratt & Publicover, 2012). [Ca^{2+}]_{i} also plays a significant role in the regulation of soluble cyclases that drive the production of cyclic nucleotides. These key secondary messengers have been shown to be fundamental for human sperm cell motility, cell capacitation and acrosome reaction. Cyclic nucleotides are actively degraded by enzymatic phosphodiesterases (PDE), and PDE-inhibitors can positively impact on sperm cell motility and function (Maréchal & Guillemette et al., 2017; Tardif & Madamidola et al., 2014; Willipinski-Stapelfeldt & Lübberstedt et al., 2004).

Identifying CatSper agonists to improve sperm motility and function is a logical approach to drug discovery for male infertility. We have previously described the development of a high throughput screening (HTS) system to identify compounds that increase [Ca^{2+}]_{i} concentration and thereafter assessed the functional consequence of in vitro application of 2 compounds (Martins da Silva & Brown et al., 2017). However, sperm motility is multiform, adaptive and not every patient sample responded to treatment in vitro. As such, there remains a clear need
to continue to identify potential therapeutic compounds. In this study, we hypothesised that novel CatSper agonists could be identified by screening a library of small molecules with defined molecular targets (chemogenomic library). This library was assembled from well characterised, commercially available ligands (Tocris) for a range of validated drug targets including enzymes, receptors and transporters. We demonstrate that Trequinsin hydrochloride, a phosphodiesterase 3-inhibitor (PDE3i) (Lal & Dohadwalla et al., 1984; Degerman & Belfrage et al., 1997) is highly effective at inducing an increase in [Ca^{2+}i], which corresponded with improved sperm motility. Detailed characterisation of the mechanism of action of Trequinsin suggests that these effects are achieved through complex and novel pharmacological activity in human spermatozoa.

**Experimental Design**
The study aimed to investigate hit compounds from a chemogenomic drug library screen for effects on sperm motility and to determine the mechanism responsible. This was achieved in three phases. Phase 1 employed HTS of compounds for their ability to increase sperm [Ca^{2+}i], relative to a saturating concentration of P4. Phase 2 involved detailed sperm function tests, and Phase 3 involved molecular analysis of Trequinsin hydrochloride, which was selected due to its high efficacy in Phase 1 and its purported PDE3i activity. A schematic outlining the experimental approach is shown in Figure 1.

**Methods**

**Ethical Approval**
Study Approval was in accordance with the Human Fertilisation and Embryology Authority (HFEA) Code of Practice (version 8) and local ethical approval (13/ES/0091) from East of Scotland Research Ethics Service (EoSRES) REC 1. Following informed consent, samples for research were obtained from patients undergoing investigation and treatment at the Assisted Conception Unit (ACU), Ninewells Hospital, Dundee, and that were surplus to clinical requirement. Samples from healthy volunteer research donors with normal sperm motility parameters in agreement with World Health Organisation (WHO) 2010 criteria (Cooper & Noonan et al., 2010) were used in this study under the same ethical approval. All obtained samples for research were analysed in line with suggested guidance for human semen studies where appropriate (Björndahl & Barratt et al., 2015).
Preparation of Donor and Patient Sperm Samples

All donors and patients adhered to an abstinence period of 2-5 days before sample collection by masturbation into a sterile plastic container. The sample was placed in a 37°C incubator for 30 min to allow liquefaction. Semen samples from patients were categorised according to WHO guidelines (Cooper & Noonan et al., 2010).

Donor and andrology semen samples were prepared by density gradient centrifugation (DGC) as described by Martins da Silva & Brown et al., (2017). Solutions for the manufacturing of Non-Capacitating media (NCM), Capacitating media (CM) and density gradient solutions can be found in Appendix 1 (supplementary materials). Preparation of capacitated patient sperm was carried out by the ACU using commercial media from PureSperm™ (Nidacon, Mölndal, Sweden) and Quinn’s Advantage Medium with HEPES (SAGE In-Vitro Fertilization; Pasadena, CA, USA) (Brown & Publicover et al., 2016).

Chemogenomics Library High-Throughput Screen

Dundee University Drug Discovery Unit in-house chemogenomics library was screened for compounds that increase [Ca$^{2+}$], in human sperm. The compound library is composed of a set of 223 commercially available small molecules and drugs (Tocris), each with a well-defined mechanism of action, potency at the primary target and selectivity. The compounds were selected as representative ligands for a diverse range of drug targets including enzymes, GPCRs, ion channels and transporters. The compound library was initially screened on a single 384 well assay plate, at a single concentration of 40 μM. HTS and data analysis were performed as previously described (Martins da Silva & Brown et al., 2017). In brief, spermatozoa from two to four different donors were pooled together after preparation by DGC, diluted to a density of 2.2 x 10$^7$/ml in Flexstation assay buffer (1 X Hanks Buffered Salt Solution (HBSS, Invitrogen), 20 mM HEPES, 0.5 mM probenecid, pH 7.4) and incubated for 60 min (37 °C) with 2 x Calcium 3 dye (Molecular Devices). Spermatozoa were washed following incubation, resuspended in Flexstation assay buffer and plated in 384 well clear bottom, black well assay plates (Greiner Bio One) at a density of 2.5 x 10$^5$ cells / 50 μl / well. [Ca$^{2+}$], was measured using a Flexstation 3 (Molecular Devices). Baseline calcium-dependent fluorescence (excitation wavelength = 485 nm, emission wavelength = 525 nm, cut-off = 515 nm) was measured for 18 secs. 12.5 μl of each test compound was transferred to the assay plate using an internal 16 channel robotic pipette head and the resulting change in fluorescence monitored for a further 82 secs. Follow up assays were performed to determine the potency of hit compounds. All assay plates in the screen were subject to quality control analysis.
Preliminary analysis of all HTS primary and potency raw data was performed using the area under the curve (AUC) function within the SoftMax Pro analysis software (SoftMax Pro Data Acquisition and Analysis Software, RRID: SCR_014240) to quantitate agonist-evoked fluorescence as previously described. Data was exported as a text file for further data processing and analysis in Activity Base version 7.3.1.4 (IDBS) and the percentage effect (PE) for each compound was normalised to the paired positive control (10 μM P4). Compounds were pragmatically classified on the basis of calcium fluorescence elicited and designated as low responder (blue 20 - 49%), mild responder (orange 50 – 89%) and high responder (green 90 – 120%) relative to P4 (Table 1).

**Motility Assessment: Computer Assisted Semen Analysis**

Prepared spermatozoa were incubated for 3 h at 37°C in CM or NCM as appropriate, then mixed with Dimethyl sulfoxide (DMSO; vehicle control, 1% final concentration) or Trequinsin (10 μM final concentration) (Tocris Bioscience, Abingdon, UK). Sperm cells were incubated for 20 min, then motility was assessed (using four chamber 20 μM deep slides) (Vitrolife, Sweden). At least 200 sperm cells were analysed per chamber per condition for each motility parameter (Tardif & Madamidola et al., 2014). Motility readings were recorded over a 2 h period (0, 20, 40, 60, 90 and 120 min) for donor and patient samples using computer-assisted sperm analysis (CASA) [CEROS machine (version 12), Hamilton Thorne Research, Beverly, MA, USA]. Parameters measured included progressive motility (PM), total motility (TM) and hyperactivated motility (HA). Proprietary algorithms on the CASA determined the percentage of cells displaying HA automatically. Specifically, a subpopulation sperm displaying curvilinear velocity (VCL) ≥ 150 μm/s, linearity (LIN) < 50% and amplitude of lateral head displacement (ALH) ≥ 7 μm algorithms were designated as hyperactive (Mortimer & Mortimer, 2013).

**Motility Assessment: Sperm Penetration Test**

Sperm penetration test was conducted using Kremer tubes (0.4 x 4 mm ID) (CM Scientific Ltd, New Jersey, USA) placed into eppendorfs containing approximately 1x10^5 spermatozoa in CM at 37°C, 5% CO_2 for 1 h (Martins da Silva & Brown et al., 2017). The number of spermatozoa was counted manually at 1 cm and 2 cm, and compared between control (1% DMSO), 10 μM Trequinsin, 3.6 μM P4, and 500 μM 3-isobutyl-1-methylxanthine (IBMX), a non-specific agonist for sperm motility used as a PDEi positive control (Strünker & Goodwin et al., 2011). Data were normalised to paired controls and expressed as a penetration index, i.e. number of
spermatozoa observed with treatment/number of spermatozoa without treatment (control) (Ivic & Onyeaka et al., 2002; Martins da Silva & Brown et al., 2017).

**Flow Cytometry Analysis**

Following 3 h incubation in capacitating conditions, two aliquots containing 2M sperm were centrifuged at 0.3g for 5 min. The supernatant was removed and the pellets were resuspended in a staining solution containing (final concentrations): 10 µg/ml Alexa Fluor™ 647 conjugated peanut agglutinin (PNA-647, Life Technologies Ltd, Paisley, UK) and 0.8 µg/ml propidium iodide (PI, Life Technologies Ltd, Paisley, UK) in sEBSS. Control (1% DMSO) and Trequinsin (10 µM) treated sperm were incubated at 37°C / 5% CO₂ for 20 min prior to flow cytometry analysis. Paired positive controls were conducted within each experiment using control cells treated with the calcium ionophore A21387 (10 µM) or Triton X-100 (0.1%) to induce the acrosome reaction and cell membrane damage respectively.

The effect of Trequinsin on acrosome reaction and membrane integrity was assessed using an Intellicyt iQue Screener equipped with a 488 nm laser. In accordance with Intellicyt guidelines, emission of fluorescence was detected using fluorescence detector 3 (670 nm LP filter) and 4 (675/25 nm) for PI and PNA-647, respectively. Forward scatter and side scatter fluorescence data were recorded from a minimum of 10000 events per condition. Threshold levels were selected to exclude cellular debris and the gates to discriminate between live/dead and acrosome-reacted/non-reacted were set using the positive control samples. Data were analysed using Intellicyt’s proprietary Forecyt software.

**[Ca²⁺]i Fluorescence Measurements**

After incubation for 3 h in CM or NCM, approximately 3M/mL spermatozoa were incubated with 4.5 µM of FLUO-4 AM, (Thermo Fisher Scientific, Oregon. USA) for 20 min at 37°C, 5% CO₂ before centrifugation at 500g for 3 min. The supernatant was removed, and the pellet resuspended in supplemented Earls Buffered Salt Solution (sEBSS) (supplementary materials). Fluorescence measurements were carried out on a FLUOstar Omega reader (BMG Labtech, Offenburg, Germany) at 37°C. 3x10⁵ cells were imaged per well (Martins da Silva & Brown et al., 2017; Tardif & Madamidola et al., 2014). To construct the Trequinsin dose-response curve the Trequinsin data were normalised to paired [Ca²⁺], response evoked by 3.4 µM P4 (to control for unwanted sources of variation).
Desensitisation experiments were carried in accordance with an established methodology (Brenker & Schiffer et al., 2018; Schaefer & Hofmann et al., 1998; Strünker & Goodwin et al., 2011). The first compound addition was added after 1 min recording of baseline fluorescence, followed by addition of the second compound after 5 min. Control experiments were conducted to demonstrate that P4 and PGE1 do not cross-desensitise. Control experiments to demonstrate desensitisation, involved either additional of P4 followed by 17α-hydroxyprogesterone (17-OH-P4) or addition of PGE1 followed by PGE2. The protocol used to assess the mode of action of Trequinsin was similar. Cells were first challenged with either P4 or PGE1 followed, after 5 min, by Trequinsin. Readings from an additional time control well (baseline) were taken as were readings from a well that was exposed to a single agonist at the time point that matched the time point of addition of the second agonist in the desensitisation experiments. All compounds we used at a final concentration of 10 µM.

**Measurement of Intracellular pH (pHi)**

After 3 h in CM, spermatozoa (4M/mL) were incubated with 2 µM ‘2’,7’-bis (2-carboxyethyl) – 5,6 – carboxyfluorescein, (BCECF; ThermoFisher, Paisley, UK) for 30 min at 37°C. The cells were centrifuged for 3 min at 500g, the supernatant removed, and the cells were then resuspended in sEBSS. A FLUOstar Omega reader (BMG Labtech, Offenburg, Germany) was used to detect the emitted fluorescence (excitation wavelength ratio of 440/490 nm and emission wavelength of 530 nm). Cell calibration was achieved following cell lysis by the addition of 1% Triton X-100, a reading was taken from each well, and a calibration curve was constructed using 1 M HCl and 1 M NaOH. Fluorescence measurements for control (cells + 1% DMSO) and Trequinsin 10 µM were recorded, as well as ammonium chloride (NH₄Cl), which was used as a positive control (10 mM final concentration).

**Electrophysiology**

The effect of Trequinsin on individual sperm plasma membrane ion channels was investigated using whole cell patch clamp electrophysiology (Brown & Publicover et al., 2016). Sperm were allowed to settle on a glass coverslip prior to being placed in the recording chamber that was perfused with standard extracellular solution (supplementary solutions). Gigaseals were achieved between sperm midpiece and high resistance (8-12 Mohm) borosilicate glass pipettes filled with either quasi-physiological standard intracellular solution or Cs⁺-based divalent-free intracellular solution to study membrane slope conductance (Gm) that is predominantly carried by K⁺ ions (Brown & Publicover et al., 2016) and CatSper channels respectively (supplementary materials). Transition to whole-cell configuration was achieved by applying
To study outward membrane conductance, a depolarising ramp protocol was imposed (−92 to 68 mV) over 2500 ms, and membrane potential was held at −92 mV between test pulses. The effect of Trequinsin on reversal potential (Erev) and membrane slope conductance of outward currents was assessed by regression analysis over the voltage range where membrane current crosses the x-axis (I = 0) and outward current from 20 to 68 mV respectively (Brown & Publicover et al., 2016).

After achieving the whole-cell configuration, monovalent CatSper currents were recorded by superfusing sperm with Cs+-based divalent-free bath solution (supplementary solutions). Currents were evoked by a ramp protocol (−80 to 80 mV over 1 s). Membrane potential was held at 0 mV between ramps. Data were sampled at 2 kHz and filtered at 1 kHz (PClamp 10 software, Axon Instruments, USA). The post-recording analysis was conducted as described previously to adjust for liquid junction potential and normalise for cell size (Brown & Publicover et al., 2016).

Detection of Cyclic Nucleotides by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

HPLC Sample Preparation
After 3 h incubation in CM, 9M/mL spermatozoa were treated with 1% DMSO (vehicle control), 10 µM Trequinsin or 500 µM IBMX (positive control) and incubated for a further 20 min. The samples were centrifuged (5 min, 300g), the supernatant disposed of and the pellet re-suspended in 0.5 mL of 100 mM sodium acetate (pH 4), sonicated for 1 min in a water bath, briefly vortexed and centrifuged again (5 min, 3000g). The supernatant was removed and placed in a fresh Eppendorf, snap frozen in liquid nitrogen and stored on dry ice until solid phase extraction (SPE).

Solid Phase Extraction
Cyclic nucleotides were extracted using Strata™- X-AW (Phenomenex, Cheshire, UK) 33 µM polymeric weak anion SPE cartridges. Cartridges were pre-treatment with 1:1 100 mM sodium acetate and water (final pH 4), conditioned with 0.5 mL 100% methanol and equilibrated with 0.5 mL 100 mM sodium acetate (final pH 4). The supernatant was then loaded into the cartridge, washed with 0.5 mL of sodium acetate (final pH 4), followed by 0.5 mL of 100% methanol; dried for 5 min under full vacuum before 0.5 mL of 28-30% weight solution of ammonium hydroxide was added to methanol (95:5) in order to elute the cyclic nucleotides into ice-cold 1.5 mL centrifuge tubes. This solution was dried under nitrogen for 30 min and

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suspended in 0.5 mL of mobile phase (20 mM potassium phosphate in 100% ultrapure water, 0.1% TFA and 0.1% ACN, pH 2.8 adjusted with 2.5% phosphoric acid).

**Standards and Stock Solutions**

Stock solutions of cyclic nucleotides (cAMP and cGMP) were prepared to 3 mol/L in mobile phase (see HPLC set-up). From the stock solutions, five 10-fold serial dilutions were produced to achieve a 6-point standard curve (peak area under the curve). This was used for quantification of cyclic nucleotides in sperm samples.

**HPLC Set-up**

The HPLC system: Waters 1525 binary HPLC pump, 2487 dual λ Absorbance detector, 717 plus autosampler and a Synergi™ 4 µm fusion – RP80A (Phenomenex, Cheshire, UK) C18 analytical column (150 mm × 4.6 mm Internal Diameter (I.D), 4 µm particle size). Mobile Phase: Isocratic elution of 100% 20 mM potassium phosphate in ultrapure, filtered and degassed water, with 0.1% TFA and 0.1 ACN. pH 2.8 with 2.5% phosphoric acid. Chromatographic Conditions: Flow Rate: 1 mL/min. Injection Volume: 200 µl. Detection Wavelengths: 255 nm (cGMP) and 256 nm (cAMP).

**Compliance with design and statistical analysis requirements**

This research did not include the use of animals. Statistical power analysis was conducted to ensure the group size was sufficient to measure an effect for each experiment using R pwr package ([R Project for Statistical Computing, RRID: SCR_001905](https://www.r-project.org/)) (pwr.2p.test) and Cohen’s effect size analysis (Control vs treatment, Sig level = 0.05, power = 0.8). N numbers refer to data from independent samples. Donor samples were allocated randomly by the technical team, and patient samples were provided by the ACU based on consent and recruitment on the day of treatment. In cases of analysis of responses from individual patients, replicates were not possible. Therefore, the analysis was conducted, as indicated in the “Data and Statistical Analysis” section. Blinding of the operator and the data analysis was not undertaken; however, multiple investigators were utilised throughout the study to ensure the consistency of observed effects.

**Data and Statistical Analysis**

Data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology ([Curtis & Bond et al., 2015](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4424213/)). A total of 28 donors and 25 patients were included in this study. Statistical comparisons for the effect of Trequinsin vs control conditions used paired t-tests, unpaired t-tests or 2-way ANOVA and Sidak’s multiple
comparison analysis as appropriate using the statistical package GraphPad Prism 7 (La Jolla, CA, USA) (GraphPad Prism, RRID: SCR_002798) unless stated otherwise. P < 0.05 as represented by * is considered significant. [Ca^{2+}]_i and pH_i studies were recorded as the percentage change in fluorescence from baseline conditions. For the analysis of individual patient sperm motility results, statistical significance was recorded when the ± SD did not overlap for control and treatment conditions (Tardif & Madamidola et al., 2014). HPLC data was extracted using Breeze 2 software and analysed using Microsoft Excel. Results are expressed as picomoles per million cells.

**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 & Sharman et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander & Kelly et al., 2017).

**Results**

**Phase 1: Drug library screen**

Dundee University Drug Discovery Unit chemogenomic library compounds were screened for their ability to evoke an increase in [Ca^{2+}]_i in capacitated sperm relative to a saturating dose of P4 (3.6 µM) using a fluorometric HTS assay and Flexstation 3 microplate reader. When tested at a single concentration of 40 µM, we identified 27 putative hits eliciting >23% effect (12.1% hit rate), 23 putative hits with >50% effect (10.3% hit rate) and 4 putative hits with >90% effect (1.8% hit rate; Table 1). Trequinsin hydrochloride was notable amongst the compounds eliciting greatest [Ca^{2+}]_i response, as it is a phosphodiesterase enzyme inhibitor (PDEi) and we have previously shown that similar compounds provide clinically relevant enhancement of sperm motility (Tardif & Madamidola et al., 2014). Trequinsin caused a concentration-dependent increase in [Ca^{2+}]_i (EC_{50} = 6.4 µM (95% CI: 4.1 µM to 9.9 µM; Supplementary Fig 1). The functional and molecular profile of Trequinsin was studied in further detail and is presented in this report. The other 3 compounds eliciting >90% increase in [Ca^{2+}]_i did not promote motility (as assessed by CASA, data not shown) and were therefore not studied further.
Phase 2: Functional Effects of Trequinsin

Donor Sperm Assessment

It is well accepted that activation of CatSper and elevation of cyclic nucleotides are fundamental for sperm motility and function (Ahmad & Murata et al., 2015; Alasmari & Costello et al., 2013; Lefèvre & de Lamirande et al., 2002). Motility and kinematic parameters of capacitated spermatozoa from healthy volunteer donors (80% DGC fraction) after 20 min exposure to Trequinsin was studied using CASA. Trequinsin (0.1 – 100 µM) had no significant effect on TM or PM (supplementary Fig 2). However, a bell-shaped dose-response curve was obtained for HA (supplementary Fig 2). As ≥ 30 µM had no effect on HA, 10 µM Trequinsin was used in subsequent experiments. Capacitated sperm from the 80% DGC fraction exposed to 10 µM Trequinsin showed no change in TM or PM (Supplementary Fig 3 A and B) over a 2 h period. However, the percentage of HA cells sperm was significantly increased (Fig 2). We also assessed the ability of Trequinsin to stimulate penetration into viscous medium (Kremer test) as a measure of functional motility in the same spermatozoa population (80%, Capacitated). Trequinsin, P4 and IBMX all significantly and similarly increased cell penetration into viscous medium at 1cm. However, Trequinsin and P4 were significantly better than IBMX at stimulating penetration at 2cm (Fig 3). Trequinsin did not induce premature acrosome reaction in capacitated cells (Supplementary Fig 4 B). In contrast, Trequinsin had no effect on the motility parameters of cells from the 80% fraction in non-capacitating conditions (Supplementary Fig 5 A-C).

Cells with poor motility isolated from 40% fraction after DGC preparation of ejaculates from healthy volunteer sperm donors were initially used as a surrogate for patient sperm, as previously described (Tardif & Madamidola et al., 2014). In contrast to donor 80% fraction cells incubated in capacitating conditions, 40% fraction capacitated cells showed a Trequinsin-induced significant increase in PM 40 min after initial exposure, which was maintained for the duration of the assay period (Fig 4 A). Although there was no effect on TM (Supplementary Fig 6 A), hyperactivation was also significantly increased, similar to donor 80% fraction cells (Fig 4 B). Under non-capacitating conditions, Trequinsin significantly improved PM of 40% fraction sperm for the entire experimental period (Fig 4 C) but had no effect on TM or hyperactivation (Supplementary Fig 6 B and C). The significant changes in motility seen in sperm from the 40% DGC fraction in capacitating conditions provided proof of concept that Trequinsin may similarly boost sperm motility in poorly motile sperm from patients.
investigate this further, we assessed patient sperm motility over a 2 h period in response to treatment with Trequinsin exposed to capacitating conditions.

**Patient Sperm Assessment**

A total of 25 patients attending the ACU for routine andrology assessment, IVF, ICSI and sperm study (SS) patients (Table 2) consented their sample for research. Trequinsin increased the percentage of hyperactivated cells in the majority (88%) of capacitated patient sperm samples (22/25). Total motility and progressive motility were unaffected by treatment with Trequinsin in the majority of samples (18/25 for TM and 18/25 samples for PM). Of note, Trequinsin did not alter motility parameters in two samples (R2946 and R2947) and negatively affected all motility parameters in only one sample (R2117).

**Phase 3: Molecular Actions of Trequinsin**

Elevated hyperactivation was the most consistent effect induced by Trequinsin. Therefore, we subsequently explored the molecular actions of sperm incubated in capacitating conditions. We first analysed the Trequinsin-induced \([\text{Ca}^{2+}]_i\) increase from capacitated donor cell populations (80% DGC) normalised to a saturating concentration of P4 (3.6 \(\mu\)M) (to control for unwanted sources of variation). Trequinsin induced a concentration-dependent increase in \([\text{Ca}^{2+}]_i\) (Trequinsin-induced peak \(\text{EC}_{50} = 3.43\ \mu\)M, 95% CI: 2.19 \(\mu\)M to 5.82 \(\mu\)M) (Fig 5 A and B), with an agonist profile analogous to P4. Interestingly, although the potency of P4 is reported to be higher (P4 peak = EC\(_{50}\) 33 nM) (Strünker et al., 2011), the efficacy of 10 \(\mu\)M Trequinsin was equivalent to P4 (Fig 5 C).

CatSper is a ligand-activated, pH sensitivity and voltage-sensitive channel. Therefore, to investigate the mechanism by which Trequinsin causes an increase in \([\text{Ca}^{2+}]_i\), we utilised whole-cell patch clamp electrophysiology to examine the drug’s ability to modulate ion channel function directly and monitored changes in pH using the ratiometric dye BCECF. Predictably, Trequinsin significantly potentiated inward and outward CatSper currents, to a degree not significantly different from P4 (Fig 6 A and B). However, Trequinsin also had inhibitory activity on potassium channel function as 10 \(\mu\)M Trequinsin also significantly suppressed the membrane slope conductance of outward current (Fig 6 E and F) causing a significant shift in \(E_{\text{rev}}\) (Control = -29.7 ± 6.6mV, + 10 \(\mu\)M Trequinsin = -17.4 ± 6.7mV, Fig 6 E). Trequinsin had no direct effect on intracellular pH (Supplementary Fig 7). PGE1 and P4 activate CatSper through mechanisms that exhibit limited cross-desensitisation (Brenker & Schiffer et al., 2018; Lishko & Botchkina et al., 2011; Miller & Mannowitz et al., 2016; Schaefer & Hofmann et al.,
1998; Shimizu & Yorimitsu et al., 1998; Strünker & Goodwin et al., 2011). Therefore, we exploited this phenomenon to investigate the mechanism of the Trequinsin induced increase in [Ca\(^{2+}\)]. Pre-treatment with P4 caused desensitisation of the response to 17-OH-P4 but not PGE1 or Trequinsin (Fig 7). Fittingly, pre-treatment with PGE1 caused desensitisation of the Trequinsin, but not P4 response (Fig 7). Trequinsin is a potent PDE3-Inhibitor (Tinsley & Bernard et al., 2009). PDE enzymes control the hydrolysis of cyclic nucleotides, specifically cAMP and cGMP, both of which are substrates for PDE3 (Lefèvre & de Lamirande et al., 2002). In contrast to the non-specific PDE-inhibitor IBMX, Trequinsin did not significantly induce elevation of cAMP (Fig 8 A). However, it induced a significant ~4 fold increase of cGMP in capacitated cells (Fig 8 B).

**Patient Intracellular Calcium Profile in Response to Trequinsin**

Given that poor motility and impaired fertilisation potential are associated with impaired CatSper function (Kelly & Brown et al., 2018), it is important to determine, where feasible, the functionality of CatSper in patient sperm. Sufficient spermatozoa were available in 9/25 patient samples to examine the ability of Trequinsin to increase [Ca\(^{2+}\)]. 10 µM Trequinsin increased [Ca\(^{2+}\)] as efficaciously as P4 (Supplementary Fig 8), indicating no fundamental abnormality in calcium signalling in these samples. Interestingly, Trequinsin did not alter motility parameters of sperm from patient R2947 despite a robust increase (>50% ∆F in [Ca\(^{2+}\)]\(_i\)) (Supplementary Fig 10).

**Discussion**

Male infertility is a significant health challenge that is estimated to affect one in ten men (Datta & Palmer et al., 2016). In up to 40% of these cases, the cause may be due to reduced sperm motility (asthenozoospermia) (van der Steeg & Steures et al., 2011). However, as there are currently no licensed pharmaceuticals to treat infertile men, ICSI remains the only viable treatment option to ensure oocyte-spermatozoon interaction. A fundamental reason for the shortfall in progression in the field of male fertility therapeutics has been the lack of knowledge regarding a suitable molecular target in sperm, thereby limiting the opportunity for implementing drug discovery strategies (Barratt & De Jonge et al., 2018; Hughes & Rees et al., 2011). However, a wealth of studies now demonstrate that CatSper is a key determinant of sperm motility and fertilisation competence (Alasmari & Costello et al., 2013; Brown & Miller et al., 2018; Kelly & Brown et al., 2018; Ren & Navarro et al., 2001; Smith & Syritsyna et al., 2013; Strünker & Goodwin et al., 2011; Williams & Mansell et al., 2015) and therefore represents a plausible target for the development of novel therapeutics for male infertility. We

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have previously described a high-throughput drug screening methodology in conjunction with relevant in vitro tests to identify compounds that increase functional sperm motility (Martins da Silva & Brown et al., 2017). While this study validated our drug discovery strategy, there continues to be a significant unmet clinical need to identify efficacious compounds that influence different forms of sperm motility and function. In this study, we utilised a HTS strategy to screen an in-house drug discovery library and identified Trequinsin hydrochloride, a putative selective PDE3 inhibitor, which significantly increased $[\text{Ca}^{2+}]_i$.

CatSper is the primary calcium-conducting plasma membrane ion channel in sperm that is activated by intracellular alkalisation, membrane depolarisation and physiological ligands such as P4 and PGE1 (Singh & Rajender, 2015; Strünker & Goodwin et al., 2011; Tamburrino & Marchiani et al., 2014). It can also be manipulated by compounds, including endocrine disrupting chemicals, that may compromise sperm function (Schiffer & Muller et al., 2014; Tavares & Silva et al., 2013). Detailed analysis of the Trequinsin-induced $[\text{Ca}^{2+}]_i$ increase in cell populations showed that the kinetics of the response mirrored that of P4. However, while whole-cell patch clamp electrophysiology confirmed the ability of Trequinsin to potentiate CatSper currents to a degree not significantly different from P4, it was also able to suppress outward $G_m$. P4 is proposed to activate CatSper indirectly through stimulation of a plasma membrane lipid hydrolase ABHD2 which metabolises endogenous inhibitory endocannabinoids to cause channel opening. In contrast, PGE1 activates the channel directly (Miller & Mannowetz et al., 2016). We exploited observations that these mechanisms exhibit limited cross-desensitisation (Brenker & Schiffer et al., 2018; Lishko & Botchkina et al., 2011; Miller & Mannowetz et al., 2016; Schaefer & Hofmann et al., 1998; Shimizu & Yorimitsu et al., 1998; Strünker & Goodwin et al., 2011) to show that the Trequinsin cross-desensitisation profile is indistinguishable from that of PGE1. As Trequinsin did not alter pH$i$, we conclude that Trequinsin increases $[\text{Ca}^{2+}]_i$ by a combination of direct activation of CatSper as well as by membrane potential depolarisation through a partial blocking effect on the sperm potassium channel. However, we cannot rule out additional direct actions on pathways that regulate intracellular stores (Correia, Michelangeli, & Publicover, 2015) or extracellular calcium entry (De Blas & Darszon et al., 2009; De Toni & Garolla et al., 2016; Kumar & Majhi et al., 2016).

Trequinsin is a potent (subnanomolar IC$_{50}$) inhibitor of recombinant PDE3 (Tinsley & Bernard et al., 2009). As cyclic nucleotides are essential second messengers for sperm motility (Balbach & Beckert et al., 2018; Jansen & Alvarez et al., 2015; Mukherjee & Jansen et al., 2016) we utilised HPLC to measure cAMP and cGMP changes in sperm exposed to Trequinsin and
demonstrated that only cGMP was significantly increased. Given that PDE3 enzymes metabolise cAMP and cGMP (Ahmad & Degerman et al., 2012), this result is surprising because pharmacological and immunological evidence supports the presence of PDE3 in human sperm, localised to the postacrosomal region of the head (Lefèvre & de Lamirande et al., 2002). In contrast, PDE3 isoforms were not among the seven phosphodiesterase enzymes identified in a study analysing the human sperm proteome (Wang & Guo et al., 2013). Therefore, our data may reflect the inhibitory activity of cGMP-phosphodiesterase PDE6D.

The notion of non-selective PDE-inhibitory activity of Trequinsin is supported by the relatively high concentration that is required to increase HA. In fact, 10 µM Trequinsin is above the IC50 at the cAMP-specific PDE2A and 4 and cGMP-specific PDE5A (Souness & Rao, 1997; Tinsley & Bernard et al., 2009; Wunder & Gnoth et al., 2009). However, proteomic data does not support their expression in human sperm (Wang & Guo et al., 2013). It is notable that micromolar concentrations of PDE-I are generally required to induce improvements in human sperm motility (Alasmari & Barratt et al., 2002.; Lefèvre & de Lamirande et al., 2002; Maréchal & Guillemette et al., 2017; Tardif & Madamidola et al., 2014) but the reason for this is unknown.

Given that nitric oxide donor compounds can modify sperm kinematic parameters, including VCL and VSL, it is entirely plausible that an effect of Trequinsin on cGMP levels may contribute to the changes seen in VCL and VSL (Supplementary Fig 11) (Miraglia & De Angelis et al., 2011). In further support for this mode of action, Trequinsin boosted the percentage of sperm exhibiting hyperactivated motility and penetration into viscous medium under capacitating conditions in all donor samples. Reassuringly, premature acrosome reaction was not induced in these samples; implying sperm-zona pellucida binding would not be hindered.

As expected, the increase in hyperactivation was dependent upon cell capacitation status. Hyperactivation was unaltered in cells maintained in non-capacitating conditions, despite Trequinsin giving a robust [Ca²⁺]i increase in these cells (Supplementary Fig 9). Although Trequinsin was highly effective at increasing hyperactivation in patient sperm samples incubated in capacitating conditions (22/25), two were unresponsive, and all motility parameters were reduced in one. The reason for this profile is unknown, but we could demonstrate that one unresponsive case (R2947) was not due to defective [Ca²⁺]i signalling (Supplementary Fig 10). Biological variability is certainly seen within human sperm.
populations. Indeed, not all patients respond to drugs, and this finding may not be uncommon (Alvarez & Castilla et al., 2003; Moohan & Winston et al., 1993).

Consequently, the same level of exposure to a drug, for example, Trequinsin, may result in different levels of biologic effects in individual patients. This is the key concept encompassed by the term ‘individualised medicine’. Determining the reasons for the biological variability seen in this and other studies (Martins da Silva & Brown et al., 2017) is an important consideration for future drug development and is dependent upon robust screening strategies and phenotypic assays to identify and treat specific molecular and functional impairment. Additionally, the development of multi-target compounds could be advantageous. For example, it would be interesting to determine if Trequinsin could restore the fertilising potential of sperm affected by CatSper and sperm potassium channel dysfunction (Brown & Publicover et al., 2016; Kelly & Brown et al., 2018; Williams & Mansell et al., 2015).

In Summary, we have shown that Trequinsin hydrochloride is an efficacious CatSper agonist, that suppresses sperm potassium channel activity, elevates cGMP (but not cAMP) and induces similar kinetics of [Ca^{2+}]_i increase as progesterone through a mechanism that cross-desensitises with PGE1. This novel pharmacological profile results in a phenotype of increased hyperactivation and penetration into viscous medium, which is relevant to sperm function required for natural conception. We conclude that the pharmacological profile of Trequinsin in human sperm is unique in terms of effect on multiple key intracellular mediators that influence sperm function (Esposito & Jaiswal et al., 2004; Hess & Jones et al., 2005; Martins da Silva & Brown et al., 2017; Tardif & Madamidola et al., 2014; Williams & Mansell et al., 2015) and holds promise as a novel agent to treat male infertility.
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Figure 1: Experimental Plan. Systematic functional and mechanistic screening strategy for the identification of the molecular and functional effects of Trequinsin.
Figure 2: Effect of Trequinsin on Capacitated Donor Sperm Cell Hyperactivation.

Trequinsin significantly increased HA in all donor cells from the 80% DGC fraction exposed to capacitating conditions (2-way ANOVA Sidak’s multiple comparison analysis). The increase in hyperactivation was sustained for a 2hr period after initial exposure. A minimum of 200 cells were counted at each time point. Hyperactivation classified by CASA parameters: VCL >150 µM/s, LIN <50% and ALH >7 µM. In the same sample set %TM and %PM were unaffected by the addition of Trequinsin (Supplementary Fig 3).
Figure 3: Sperm Penetration Assay. The ability of Trequinsin to stimulate sperm penetration into viscous medium was assessed using capacitated sperm from the 80% DGC fraction (n = 5). A significant increase in cell penetration was observed in the presence of Trequinsin in comparison to control, but not in comparison to cells stimulated with P4. Cell penetration at 1 cm was not significantly different between Trequinsin, or IBMX treated cells. However, Trequinsin stimulated a significantly greater cell number to penetrate at 2 cm compared to IBMX. Statistical significance shown on the graph is relative to control.
Figure 4: Effect of Trequinsin on 40% DGC Fraction (poor motility) Donor Sperm Motility. Trequinsin significantly increased the percentage of progressively motile sperm in capacitating (A, n = 5) and non-capacitating (B, n = 6) conditions. Hyperactivation was also significantly increased when sperm were incubated in capacitating conditions (C, n = 8). Corresponding motility data can be found in Supplementary Fig 6.
Figure 5: Effect of Trequinsin on [Ca$^{2+}$] in Capacitated Donor Cells. (A) Mean dose response traces for Trequinsin (0.1 – 30 µM). (B) Dose-response curve for Trequinsin induced peak of [Ca$^{2+}$], relative to P4 (3.6 µM) (EC$_{50}$ = 3.43 µM, 95% Cl: 2.19 µM to 5.82 µM) (n =5) in 80% DGC fraction capacitated donor sperm. (C) Mean data set (n = 6) trace of 10 µM Trequinsin and 3.6 µM P4 [Ca$^{2+}$], recording (↓ represents the addition of compounds, O highlights the peak and I represents the sustained (between 180 -200 s) fluorescent measured). Peak and sustained responses for P4 and Trequinsin are not significantly different.
Figure 6: Patch Clamp Electrophysiology. (A, B) Inward CatSper-mediated Cs$^+$ currents ($n = 6$) in response to Trequinsin (10 µM) were not significantly different to P4 (C, D). (E) I-V relationship showing the shift in Erev and Gm inhibitory effect caused by 10 µM Trequinsin ($n = 7$). (F) Dose-response curve for showing the partial inhibitory effect of Trequinsin on Gm ($n = 5$). Patch-clamp solution configurations are shown in insets. Donor sperm were from the 80% DGC fraction incubated in capacitating conditions.
Figure 7: Examination of agonist cross-desensitisation. Population average [Ca$^{2+}$]i trace using capacitated donor sperm from the 80% DGC fraction (n = 5) showing initial agonist addition of either a saturating concentration of 10 µM P4 (A, C and E) or 10 µM PGE1 (B, D and F), followed by the 2nd agonist addition. A baseline control shown in Blue was included in each experiment and a blank (sEEBS, represented as “B”) followed by the addition of the second agonist green. Cross-Desensitisation experiments are shown in red. (G) Bar chart showing cell exposed to 10 µM P4 did not produce a significant Ca$^{2+}$ response compared to that of PGE (10 µM) and Trequinsin (10 µM). PGE1 and Trequinsin were not significantly different. (H) Cells pre-exposed to 10 µM PGE1 had significantly lower Ca$^{2+}$ responses (<2%) compared to p4 exposure. PGE2 and Trequinsin were not significantly different.
Figure 8: Measurement of Cyclic Nucleotides levels in capacitated 80% DGC fraction donor sperm using RP-HPLC. (A) Trequinsin did not alter intracellular cAMP in comparison to control (Cells + 1% DMSO) (n = 11). (B) Trequinsin significantly increased intracellular cGMP (n = 11). IBMX, a non-specific PDEi was used as a positive control. IBMX significantly increased both cAMP and cGMP (n = 11).
Table 1: Summary of $[Ca^{2+}]_i$ elevating Compounds Identified in Screen of the Chemogenomics library. 27 US Food and Drug Administration approved active compounds were identified from the DDU Chemogenomics library screen following Flexstation assay testing and categorised based on their ability to increase $[Ca^{2+}]_i$ (low to high percentage increase relative to 10 µM P4 (positive control). Trequinsin Hydrochloride was selected for this study as it was highly efficacious and a phosphodiesterase inhibitor (The compound library screen of all 223 commercially available small molecules and drugs $[Ca^{2+}]_i$ is shown in Supplementary Fig 12.)

| Compound name       | Primary Action (Tocris)                                                                 | Percentage increase in fluorescence in sperm |
|---------------------|---------------------------------------------------------------------------------------|---------------------------------------------|
| Zaprinast           | PDE5/6/9/11 inhibitor                                                                  | 23                                          |
| SB 218078           | Inhibitor of checkpoint kinase 1 (Chk1)                                               | 25                                          |
| RO-3                | Selective P2X3 and P2X2/3 antagonist                                                   | 26                                          |
| GP 1a               | Highly selective CB2 agonist                                                           | 32                                          |
| NNC 55-0396         | Highly selective Ca$^{2+}$ channel blocker (T-type)                                    | 32                                          |
| EHT 1864            | Potent inhibitor of Rac family GTPases                                                 | 32                                          |
| SD 208              | Potent ATP-competitive TGFRI inhibitor                                                 | 34                                          |
| SANT-2              | Inhibitor of hedgehog (Hh) signalling; antagonizes smoothened activity                 | 36                                          |
| Repaglinide         | KATP channel blocker                                                                  | 38                                          |
| EO 1428             | Selective inhibitor of p38α and p38 α2                                                | 39                                          |
| BI 78D3             | Selective, competitive JNK inhibitor                                                   | 45                                          |
| IKK 16              | Selective inhibitor of IKK                                                             | 47                                          |
| BRL 50481           | Selective PDE7 inhibitor                                                               | 49                                          |
| Calcipotriol        | Vitamin D3 analog                                                                     | 51                                          |
| ID            | Name                                    | Description                                                                 | Page |
|---------------|-----------------------------------------|------------------------------------------------------------------------------|------|
| AS 1949490    | SH2 domain-containing inositol 5'-phosphatase 2 (SHIP2) inhibitor | 54                                                                           |      |
| U 89843A      | Positive allosteric modulator of GABA\textsubscript{A} receptors      | 57                                                                           |      |
| SANT-1        | Inhibitor of hedgehog (Hh) signalling; antagonizes smoothened activity | 57                                                                           |      |
|               | **Ciglitazone**                        | Selective PPAR agonist                                                     | 64   |
|               | **UK 78282 hydrochloride**              | Blocker of KV1.3 and KV1.4 channels                                         | 66   |
|               | **GW 9508**                            | Potent and selective FFA1 (GPR40) agonant                                   | 67   |
|               | **FPL 64176**                          | Potent activator of Ca\textsuperscript{2+} channels (L-type)                | 75   |
|               | **NVP 231**                            | Potent, selective and reversible CerK inhibitor                              | 77   |
|               | **Y 29794 oxalate**                    | Prolyl endopeptidase inhibitor                                              | 88   |
|               | **Trequinsin hydrochloride**            | Ultrapotent inhibitor of PDE3                                                | 91   |
|               | **Lylamine hydrochloride**              | CB1 agonist                                                                 | 108  |
|               | **PHA 665752**                         | Potent and selective MET inhibitor                                           | 111  |
|               | **JX 401**                             | Potent, reversible p38\textalpha inhibitor                                 | 115  |
Table 2: Effect of Trequinsin on Patient Sperm Motility. Summary of motility changes in patient samples (IVF, ICSI and Andrology) treated with 10 µM Trequinsin. The motility of 25 patient samples was assessed using CASA over a 2 h period at regular intervals (see methods), and an average for each parameter was taken overall. A minimum of 200 cells were counted at each time point. ↑, significant increase; —, no change; ↓, significant decrease. Significant means SD (control vs treatment at each time point) do not or do overlap for increase and decrease, respectively (TM: total motility, PM: progressive motility and HA: Hyperactivated motility). Patient samples are categorised based on semen WHO parameters (see methods). ✓ represents a WHO guideline criterion met, × represents a criterion not meeting WHO guidelines.

| Patient ID | Initial WHO Semen Criteria assessment | Effect of Trequinsin on patient sperm motility |
|------------|--------------------------------------|-----------------------------------------------|
|            | Conc. (M/mL) | PM (%) | Classification | TM (%) | PM (%) | HA (%) |
| R2117      | ✓          | ✓      | Normal         | ↓       | ↓       | ↓      |
| R2926      | ✓          | ✓      | Normal         | —       | —       | ↑      |
| R2929      | ✓          | ✓      | Normal         | —       | —       | ↑      |
| R2937      | ✓          | ✓      | Normal         | —       | ↓       | ↑      |
| R2939      | ✓          | ✓      | Normal         | —       | —       | ↑      |
| R2945      | ✓          | ✓      | Normal         | —       | ↓       | ↑      |
| R2946      | ✓          | ✓      | Normal         | —       | —       | —      |
| R2947      | ✓          | ✓      | Normal         | —       | —       | —      |
| R2949      | ✓          | ✓      | Normal         | ↑       | ↑       | ↑      |
| R2951      | ✓          | ✓      | Normal         | —       | —       | ↑      |
| R2952      | ✓          | ✓      | Normal         | —       | —       | ↑      |
| R2919      | ✓          | ✓      | Normal         | —       | —       | ↑      |
| R2927      | ✓          | ×      | Borderline     | ↑       | —       | ↑      |
| R2792      | ✓          | ×      | Borderline     | —       | —       | ↑      |
| R2931      | ✓          | ×      | Borderline     | ↓       | —       | ↑      |
| R2935      | ✓          | ×      | Borderline     | —       | —       | ↑      |
| R2943      | ×          | ✓      | Borderline     | —       | —       | ↑      |
| R2950      | ✓          | ×      | Borderline     | ↑       | ↑       | ↑      |
| R2953      | ✓          | ×      | Borderline     | —       | —       | ↑      |
| R2971      | ✓          | ×      | Borderline     | —       | —       | ↑      |
| R2974      | ✓          | ×      | Borderline     | —       | —       | ↑      |
| R2976      | ×          | ×      | Low            | ↑       | ↑       | ↑      |
| R2340      | ×          | ×      | Low            | —       | —       | ↑      |
| R2730      | ×          | ×      | Low            | ↑       | ↑       | ↑      |
| R2938      | ×          | ×      | Low            | —       | —       | ↑      |
Abbreviations

[Ca\textsuperscript{2+}]_i Intracellular Calcium Concentration
A23187 Calcium Ionophore
ACU Assisted Conception Unit
ALH Amplitude of lateral head displacement
AR Acrosome reaction
ART Assisted Reproductive Treatment
BCECF ‘2’,7’-bis (2-carboxyethyl) – 5,6 – carboxyfluorescein
CASA Computer-assisted sperm analysis
CM Capacitating media
DGC Density gradient centrifugation
EoSRES East of Scotland Research Ethics Service
Erev Reversal potential
FITC Fluorescein-isothiocyanate
Gm Membrane conductance
HA Hyperactivated Motility
HFEA Human Fertilisation and Embryology Authority
HPLC High-performance liquid chromatography
HTS High throughput screening
ICSI Intracytoplasmic sperm injection
IVF In Vitro Fertilisation
NCM Non-capacitating media
P4 Progesterone
PDE3i Phosphodiesterase 3-inhibitor
PDEs Phosphodiesterases
PDE Phosphodiesterase
PE Percentage Effect
PM Progressive Motility
pHi Intracellular pH
PSA-FITC Fluorescein-isothiocyanate conjugated
Pisum sativum lectin
RP: Reversed-phase
SA: Semen Andrology
sEBSS: Supplemented Earls Buffered Salt Solution
SPE: Solid phase extraction
SS: Sperm Studies
TM: Total Motility
VCL: Curvilinear velocity
Vm: Membrane potential
VSL: Straight line velocity
WHO: World Health Organisation
Appendix

Supplementary Methods

Density Gradient Centrifugation for Donor and Andrology Samples
Sperm cells were isolated using a 40:80% discontinuous density gradient procedure (Alasmari et al., 2013; Tardif et al., 2014). Briefly, after 30 min of liquefaction at 37°C, up to 2 mL of semen was loaded on top of a colloidal silica suspension (Percoll, Sigma Aldrich, UK) made of 80 and 40% layers (1.5 mL each). The density gradient was then centrifuged at 300 g for 20 min. Cells were washed in NCM (300g, 10 min) and suspended in either NCM or CM, depending on experimental requirements. Those Cells in CM were left to capacitate at 37°C, 5% CO₂ for 3 h prior to experimentation. Cells in NCM were left at 37°C for 3 h prior to experimentation.

Density Gradient Centrifugation for Patient (IVF/ICSI/SS) Samples
In the Assisted Conception Unit, commercially available media was used for sperm preparation. The spermatozoa were separated from semen by density gradient centrifugation (40:80%) using PureSpermTM (Nidacon, Molndal, Sweden) diluted with Quinns Advantage SWM, a HEPES-buffered solution (Cooper Surgical Inc., USA). After centrifugation, the pellet was washed by centrifugation at 500g for 10 min in 4 mL of SWM. The supernatant was discarded and the pellet resuspended in Quinns Advantage Fertilization Media (Cooper Surgical Inc.). For ICSI, once the cells were washed, they were incubated in SWM at 37°C. Cells surplus to requirement were made available for research.

Supplementary Solutions
All chemicals were purchased from Sigma-Aldrich UK unless specified.

Non- Capacitating media
1.8 mM CaCl₂, 5.4 mM KCl, 0.8 mM MgSO₄·7H₂O, 116.3 mM NaCl, 1.0 mM NaH₂PO₄, 5.55 mM C₆H₁₂O₆, 2.73 mM C₃H₅NaO₃, 41.75 mM C₃H₅NaO₃, 25 mM C₈H₁₈N₂O₄S and supplemented with 0.3% (W/V) fatty acid free BSA, pH 7.4.

Capacitating Media (synthetic tubal fluid)
1.8 mM CaCl₂, 5.4 mM KCl, 0.8 mM MgSO₄·7H₂O, 116.3 mM NaCl, 1.0 mM NaH₂PO₄, 5.55 mM C₆H₁₂O₆, 2.73 mM C₃H₅NaO₃, 25 mM C₃H₅NaO₃, 26 mM NaHCO₃ and supplemented with 0.3% (W/V) fatty acid free BSA, pH 7.4.
Density Gradient Media
Density gradient fractions 40% and 80% were manufactured using a 90% Percoll® solution (ratio of 9:1 100% Percoll® to 10x NCM) and NCM media for donor/andrology samples.

sEBSS salt solution
1.02 mM NaH₂PO₄, 5.4 mM KCl, 0.81 mM MgSO₄·7H₂O, 5.5 mM C₆H₁₂O₆, 2.5 mM C₃H₆NaO₃, 19 mM CH₃CH(OH)COONa, 25 mM NaHCO₃, 15 mM C₆H₁₈N₂O₄S, 1.8 mM CaCl₂·2H₂O, 118.4 mM NaCl, pH 7.4 ± 0.2. Supplemented with 0.3% (W/V) fatty acid free BSA.

HPLC Reagents
Acetonitrile (ACN) & trifluoroacetic acid (TFA) (LC-MS grade), NH₄Cl, cAMP, cGMP, CH₃OH, C₂H₃NaO₂. IBMX and Trequinsin were purchased from Tocris®. Ultrapure water (18.2 MΩ cm) was produced using the Milli-Q Advantage A10 system from Millipore®.

Standard Extracellular Solution
135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 20 mM HEPES, 5 mM Glucose, 1 mM Na pyruvate, 10 mM Lactic acid, pH adjusted to 7.4 with NaOH which brought [Na⁺] to 154 mM.

Standard Pipette Solution
10 mM NaCl, 18 mM KCl, 92 mM K gluconate, 0.5 mM MgCl₂, 0.6 mM CaCl₂, 1 mM EGTA, 10 mM HEPES, pH adjusted to 7.4 using KOH which brought [K⁺] to 114 mM and [Ca²⁺] to 0.1 µM.

Cs⁺-based Pipette Solution
130 mM Cs-methanesulphonate, 40 mM HEPES, 1 mM Tris–HCl, 3 mM EGTA, 2 mM EDTA and pH adjusted to 7.4 with CsOH.

Cs⁺-based Extracellular Solution
140 mM Cs-methane sulphonate, 40 mM HEPES, 3 mM EGTA, pH adjusted to 7.4 with CsOH.