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RECEIVED 12 May 2023

ACCEPTED 19 June 2023

PUBLISHED 20 July 2023

CITATION

Wehrli L, Galdadas I, Voirol L, Smieško M,
Cambet Y, Jaquet V, Guerrier S,
Gervasio FL, Nef S and Rahban R (2023),
The action of physiological and synthetic
steroids on the calcium channel CatSper
in human sperm.
Front. Cell Dev. Biol. 11:1221578.
doi: 10.3389/fcell.2023.1221578

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The action of physiological and synthetic steroids on the calcium channel CatSper in human sperm

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The sperm-specific channel CatSper (cation channel of sperm) controls the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and plays an essential role in sperm function. It is mainly activated by the steroid progesterone (P4) but is also promiscuously activated by a wide range of synthetic and physiological compounds. These compounds include diverse steroids whose action on the channel is so far still controversial. To investigate the effect of these compounds on CatSper and sperm function, we developed a high-throughput screening (HTS) assay to measure changes in $[\text{Ca}^{2+}]_i$ in human sperm and screened 1,280 approved and off-patent drugs including 90 steroids from the Prestwick chemical library. More than half of the steroids tested (53%) induced an increase in $[\text{Ca}^{2+}]_i$ and reduced the P4-induced Ca^{2+} influx in human sperm in a dose-dependent manner. Ten of the most potent steroids (activating and P4-inhibiting) were selected for a detailed analysis of their action on CatSper and their ability to act on sperm acrosome reaction (AR) and penetration in viscous media. We found that these steroids show an inhibitory effect on P4 but not on prostaglandin E1-induced CatSper activation, suggesting that they compete for the same binding site as P4. Pregnenolone, dydrogesterone, epiandrosterone, nandrolone, and dehydroepiandrosterone acetate (DHEA) were found to activate CatSper at physiologically relevant concentrations within the nanomolar range. Like P4, most tested steroids did not significantly affect the AR while stanozolol and estropipate slightly increased sperm penetration into viscous medium. Furthermore, using a hybrid approach integrating pharmacophore analysis and statistical modelling, we were able to screen *in silico* for steroids that can activate the channel and define the physicochemical and structural properties required for a steroid to exhibit agonist activity against CatSper. Overall, our results indicate that not only physiological but also synthetic steroids can modulate the activity of CatSper with varying potency and if

bound to CatSper prior to P4, could impair the timely CatSper activation necessary for proper fertilization to occur.

KEYWORDS

CatSper, steroids, human sperm, high-throughput screening, calcium signaling, pharmacophore modelling, acrosome reaction

Introduction

After being deposited in the female reproductive tract, spermatozoa undergo multiple complex processes before reaching and fertilizing the oocyte. Although motile upon ejaculation, sperm undergo a priming step referred to as capacitation which promotes their response to chemical cues (such as progesterone) and helps them locate the oocyte by chemotaxis. The sperm must then acquire a specific type of motility pattern characterized by an asymmetric flagellar beat movement known as hyperactivation, allowing them to break through the protective egg vestment and release digestive enzymes to penetrate the egg through the acrosome reaction (reviewed by [Tosti and Menezo, 2016](#)). These diverse processes are known to be partly controlled by the intracellular calcium concentration ($[Ca^{2+}]_i$) which is regulated almost exclusively by the principal sperm-specific Cationic channel of Sperm (CatSper) (reviewed by [Kaupp and Strünker, 2017](#); [Rahban and Nef, 2020](#); [Wang et al., 2021](#)). Indeed, a suboptimal calcium influx is associated with reduced male fertility, and men with mutations in CATSPER are infertile ([Avidan et al., 2003](#); [Avenarius et al., 2009](#); [Hildebrand et al., 2010](#); [Smith et al., 2013](#); [Williams et al., 2015](#); [Brown et al., 2018](#); [Luo et al., 2019](#); [Schiffer et al., 2020](#)).

CatSper is one of the most complex channels ever described ([Zhao et al., 2022](#)). Located specifically in the membrane of the flagellum, CatSper is a heterotetrameric channel composed of at least 10 subunits with four pore-forming α subunits (CatSper 1–4) and six auxiliary subunits, out of which four are transmembrane non-pore forming auxiliary subunits (β , γ , δ , and ϵ) and two are cytosolic (CatSper ζ and EFCAB9) ([Quill et al., 2001](#); [Carlson et al., 2003](#); [Ren and Xia, 2010](#); [Darszon et al., 2011](#); [Chung et al., 2017](#); [Hwang et al., 2019](#)). Expression and association of the pore-forming and the transmembrane auxiliary CatSper subunits are required for channel function and fertilization. The cytosolic subunits EFCAB9 and the ζ are not required to form a functional channel but confer pH- and calcium-sensitivity to CatSper, respectively ([Hwang et al., 2019](#)). Two recent studies reported a novel C2 membrane-associated domain-containing protein encoded by *C3cd6* named CatSperT, which plays an important role in the spatiotemporal trafficking of the channel to the flagellar membrane ([Hwang et al., 2022](#); [Yang et al., 2022](#)). Several previously uncharacterized transmembrane proteins in the CatSper structure, which have also been recently identified further complicate the mechanism of action, such as the testis-specific organic anion transporter SLC06C1, located adjacent to CatSper 2 and ϵ , and three small transmembrane proteins (TMEM 262, TMEM 249, and an unassigned single transmembrane protein), which are located on the side of CatSper 4 and β ([Zhao et al., 2022](#)). How these proteins come together to orchestrate the function of CatSper and the physiological significance of the new CatSper subunits remain to be defined ([Hwang and Chung, 2022](#)).

CatSper activation triggers a calcium influx and release from internal stores, resulting in oscillations in the $[Ca^{2+}]_i$ that propagate from the flagellum to the midpiece and head within seconds ([Torrezan-Nitao et al., 2021](#)). To enable rapid propagation of calcium signals along the flagellum, the CatSper channels form a complex of long zigzag lines resulting in four linear threads within the principal piece ([Chung et al., 2014](#); [Chung et al., 2017](#); [Zhao et al., 2022](#)).

CatSper is activated by alkalization of intracellular pH (pH_i), membrane depolarization ([Kirichok et al., 2006](#)), and by natural ligands such as the steroid progesterone (P4) and the arachidonic acid derivative prostaglandins, mainly prostaglandin E1 (PGE1) ([Lishko et al., 2011](#); [Strünker et al., 2011](#)). These ligands have distinct binding sites to activate the channel ([Strünker et al., 2011](#)), but the mechanism through which they activate CatSper is poorly understood and highly controversial. It has been suggested that P4 does not directly activate CatSper but rather binds to an upstream lipid hydrolase called the α/β hydrolase domain-containing protein 2 (ABHD2) ([Miller et al., 2016](#)). Upon P4 binding, ABHD2 is suggested to degrade the endocannabinoid 2-arachidonoylglycerol (2-AG), which blocks the channel and thereby releases CatSper from inhibition ([Miller et al., 2016](#)).

Studies over the last 30 years have shown that various endogenous and exogenous steroids are also able to evoke Ca^{2+} influx and motility responses in human sperm ([Blackmore et al., 1990](#); [Blackmore et al., 1996](#); [Luconi et al., 1999](#); [Luconi et al., 2004](#); [Rossato et al., 2005](#); [Brenker R. A. et al., 2018](#); [Brenker S. C. et al., 2018](#); [Rehfeld, 2020](#); [Jeschke et al., 2021](#)). Steroids such as 17-OH-progesterone, estradiol, dihydrotestosterone, androstenedione, and pregnenolone have been described to act via CatSper ([Lishko et al., 2011](#); [Strünker et al., 2011](#); [Brenker R. A. et al., 2018](#); [Brenker S. C. et al., 2018](#); [Rehfeld, 2020](#); [Jeschke et al., 2021](#)). However, the activity of several steroids on CatSper is still a matter of controversy. For example, testosterone, estradiol, and hydrocortisone have been reported to be both CatSper antagonists ([Mannowetz et al., 2017](#)) and agonists ([Brenker S. C. et al., 2018](#); [Rehfeld, 2020](#)). Furthermore, several studies demonstrated that CatSper is promiscuous and can be activated by a wide range of synthetic or natural compounds such as odorants ([Spehr et al., 2003](#)), endocannabinoids ([Miller et al., 2016](#)), and Endocrine Disrupting Chemicals (EDCs) ([Tavares et al., 2013](#); [Schiffer et al., 2014](#); [Rehfeld et al., 2016](#)). The list of compounds that can activate the channel was recently expanded to include commonly prescribed pharmaceutical drugs such as the selective serotonin reuptake inhibitors (SSRI) class of antidepressants ([Rahban et al., 2021](#)), the 5α reductase inhibitor finasteride ([Birch et al., 2021](#)), as well as the paracetamol metabolite N-arachidonoyl phenolamine (AM404) ([Rehfeld et al., 2022](#)). These results are consistent with the finding that human CatSper serves as a polymodal chemosensor that harbors promiscuous binding sites for structurally diverse ligands ([Brenker et al., 2012](#)). However, the

physiological effects on sperm cells and the molecular characteristics that allow multiple structurally diverse steroids, whether physiological or synthetic, to activate CatSper remain poorly understood and contradictory.

Using our novel high-throughput screening (HTS) assay, we have developed an accurate and rapid method to investigate the effects of a wide range of pharmaceutical drugs, including synthetic and physiological steroids, on CatSper. We studied in detail 10 steroids that can directly activate the channel and interfere with the P4 but not the PGE1-induced Ca^{2+} response. We also evaluated the effect of these 10 steroids on the ligand-independent activation of the channel through intracellular alkalization and their effects on acrosome reaction and penetration into viscous media. Finally, pharmacophore and statistical modelling were used to screen *in silico* for steroids that can activate the channel and better understand the molecular characteristics of the steroids that promote CatSper activation.

Materials and methods

Reagents

The Prestwick library composed of 1,280 compounds was purchased by the R.E.A.D.S. unit of the University of Geneva. The drugs were dissolved at a stock concentration of 10 mM in dimethyl sulfoxide (DMSO). P4 and PGE1 were purchased from Sigma-Aldrich (Buchs, Switzerland). The fluorescent Ca^{2+} indicator Fluo-4 AM was purchased from Invitrogen and dissolved in DMSO at a final concentration of 2.5 μ M (California, United States of America). The nuclear dye Propidium Iodide (PI) was purchased from Thermo Fisher Scientific (MA, United States of America), and diluted in water at a stock concentration of 1 mg/mL. Pisum sativum agglutinin (PSA) staining dye, was purchased from Sigma-Aldrich and dissolved in Phosphate Buffered Saline (PBS) at a stock concentration of 1 mg/mL. Hoechst-33342 was purchased from Sigma-Aldrich (Buchs, Switzerland), and dissolved in water at a stock concentration of 1.5 mg/mL (Buchs, Switzerland). Human serum albumin (HSA) was obtained from Polygon Diagnostics (Lucerne, Switzerland). MDL 12330A hydrochloride was purchased from Tocris Bioscience (Bristol, UK). The following steroids were purchased from Sigma-Aldrich (Buchs, Switzerland): P4, dehydroisoandrosterone 3-acetate (DHEA), estropipate, stanozolol, epiandrosterone, deoxycorticosterone, dydrogesterone, 3-alpha-Hydroxy-5-beta-androstan-17-one, pregnenolone, medrysone, equilin, estrone, norgestimate, canrenoic acid potassium salt, exemestane, finasteride, lithocholic acid, formestane, exemestane, fluorometholone, estradiol-17 beta, androsterone, medrysone, nandrolone, fluorometholone, oxymetholone, urosiol, oxandrolone, canrenone, fulvestrant, lynestrenol, norethindrone, ethisterone, norgestrol acetate, gestrinone, chlormadinone acetate, norgestrel(-)-D, ethynodiol diacetate, mifepristone, budesonide, adrenosterone, ethinylestradiol, megestrol acetate, ethinylestradiol 3-methyl ether, 3 α ,21-dihydroxy-5 α -pregnan-20-one, 5 α -pregnan-3 α -ol-20-one, 19-norandrosterone, androsterone, 5 α -pregnan-20-one, 5 α -androstan-3-one, 5 α -pregnane-3,20-dione, androstenedione, tanaproget, BB_NC-00182 and dissolved at a stock concentration of 20 mM in DMSO. To implement the pharmacophore model, the

steroids were purchased from ChemDIV (California, United States) under the following ID numbers: N017-0003, N039-0025, N050-0010, 3647-1627, N044-0005, 0449-0076, 2361-0084, N039-0043, N050-0013, N039-0021, 0449-0077, N017-0007, 8011-0433, N050-0008, 3529-0011, 3529-0046, 5263-0009, N050-0018, and 3 β ,21-dihydroxy-5 α -pregnan-20-one, 21-hydroxypregnenolone and MolPort-001-728-767 were purchased from MolPort (Riga, Latvia), and promegestronone was purchased from BOC Sciences (NY, United States) and dissolved at a 20 mM stock concentration in DMSO.

Experimental design

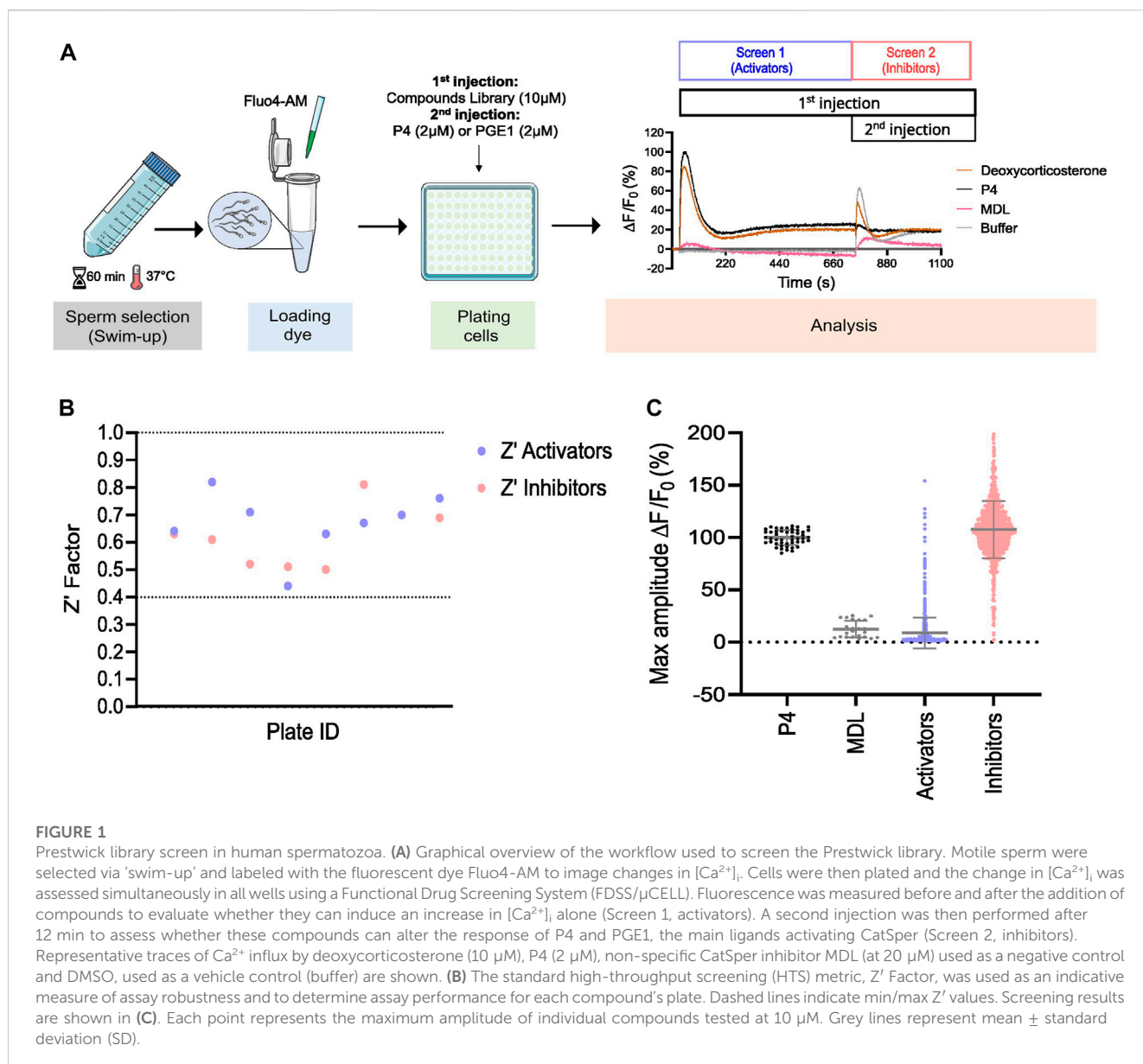
The experimental design is summarized in [Figure 1](#). Briefly, motile spermatozoa were selected by swim-up and labeled with the fluorescent calcium indicator Fluo-4 a.m. to image changes in $[Ca^{2+}]_i$. After centrifugation to remove excess dye, Fluo-4 AM loaded cells were distributed in a 384-wells plate to obtain ~125,000 cells/well. After measuring basal fluorescence, a first injection of compounds was performed in order to evaluate their effect on $[Ca^{2+}]_i$ alone. This first screen is therefore considered as a screen to search for 'activators'. Fluorescence was recorded for 12 min during which sperm cells were continuously exposed to compounds. P4 or PGE1 were then added in the same wells to evaluate whether the tested compounds can reduce the ligand-induced activation of CatSper. This second screen was therefore considered to be a search for CatSper 'inhibitors'.

Sample collection and preparation

Human semen samples were collected from healthy donors by masturbation after a recommended sexual abstinence period of minimum 2 days. Semen samples were allowed to liquefy at 37°C for 15–30 min. Motile spermatozoa were selected by a 'swim-up' procedure as previously described ([Strünker et al., 2011](#)). Briefly, 1 mL of semen was deposited in a 45°-inclined 50 mL Falcon tube with 4 mL of Human Tubular Fluid (HTF) medium containing: 93.8 mM NaCl, 4.7 mM KCl, 0.2 mM $MgSO_4$, 0.37 mM KH_2PO_4 , 2.04 mM $CaCl_2$, 20.98 mM HEPES, 2.78 mM glucose, 21.4 mM Na-lactate, 4 mM $NaHCO_3$, 0.33 mM Na-pyruvate, and the pH was adjusted to 7.35. Motile sperm were allowed to swim at 37°C for 1 hour. The supernatant was collected in 15 mL Falcon tubes and washed twice by centrifugation at 700 x g for 20 min. Sperm were supplemented with 3 mg/mL HSA. Written informed consent was obtained from all donors prior to donation and approved by the Cantonal Ethics Committee of the State of Geneva (CCER #14-147).

High throughput measurements of changes in $[Ca^{2+}]_i$

Cells were loaded with the fluorescent Ca^{2+} indicator Fluo-4 AM at a final concentration of 2.5 μ M for 30 min at 37°C. Excess dye was washed via centrifugation at 700 x g for 20 min and sperm were resuspended in HTF medium to obtain a final concentration of

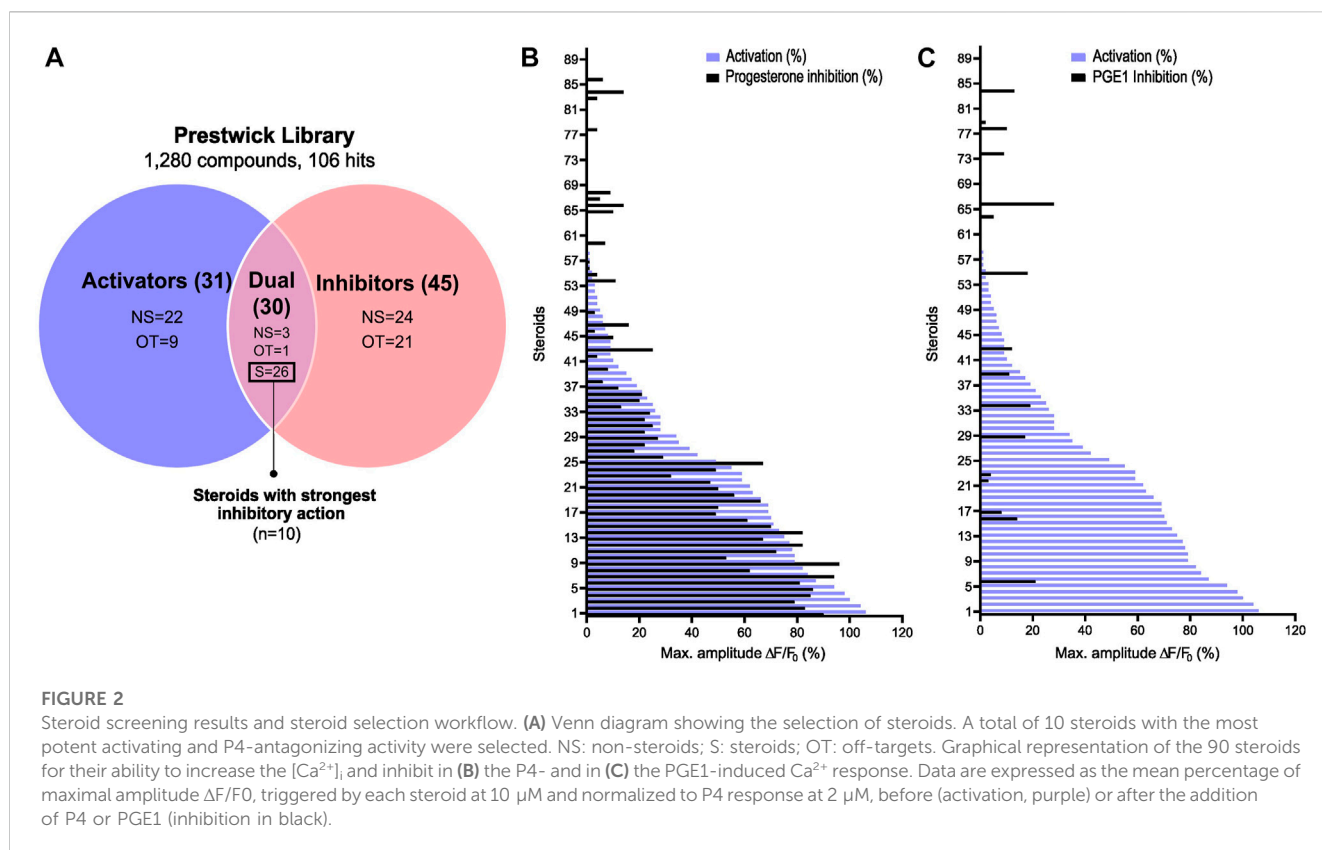


6.25 × 10⁶ cells/mL. 20 µL of cells (corresponding to ~125,000 cells) loaded with Fluo-4 a.m. were dispensed in a 384-wells plate (Corning, NY, United States) to allow fluorescence measurement. The dye was excited at 480 nm, and emission was recorded at 530 nm. The change in [Ca²⁺]_i was assessed simultaneously in all wells using a Functional Drug Screening System (FDSS/µCELL) (Hamamatsu Phototonics K.K., Yokohama, Japan) at 37°C. Basal fluorescence was first measured for 30 s. A volume of 5 µL of compounds was then added to obtain a final concentration of 10 µM and P4 or PGE1 were used as positive control at a final concentration of 2 µM corresponding to a value close to saturation (EC₈₀). P4 and PGE1 were tested at a near-saturating concentration in the HTS in order to evaluate a potential additive or synergistic effect induced by the compounds. Non-specific CatSper inhibitor MDL 12330A was used as a negative control at final concentration of 20 µM while DMSO was used as a vehicle control. For alkalization-induced Ca²⁺ measurement, NH₄Cl was used as a positive control at a final concentration of 10 mM. Fluorescence was

recorded for a total of 16.5 min: 30 s before injection, 12 min after the first injection of the tested compounds, and 4 min after the second injection of either P4 or PGE1. The Prestwick library was tested in technical duplicate. Ca²⁺ response curves were normalized using the following formula $\frac{(F-F_0)}{F_0} \times 100$, where F_0 is the average of the basal fluorescence prior to the addition of the compound of interest and F corresponds to the fluorescence value at each time point. The $\Delta F/F_0$ of the vehicle control (DMSO) was subtracted from all the other values to adjust for dilution and pipetting artifacts. The values of all compounds were normalized to their respective controls.

Assessment of acrosome reaction

The evaluation of acrosome reaction in live human sperm cells was performed using a modified version of a previously described method (Zoppino et al., 2012). Briefly, swim-up recovered motile



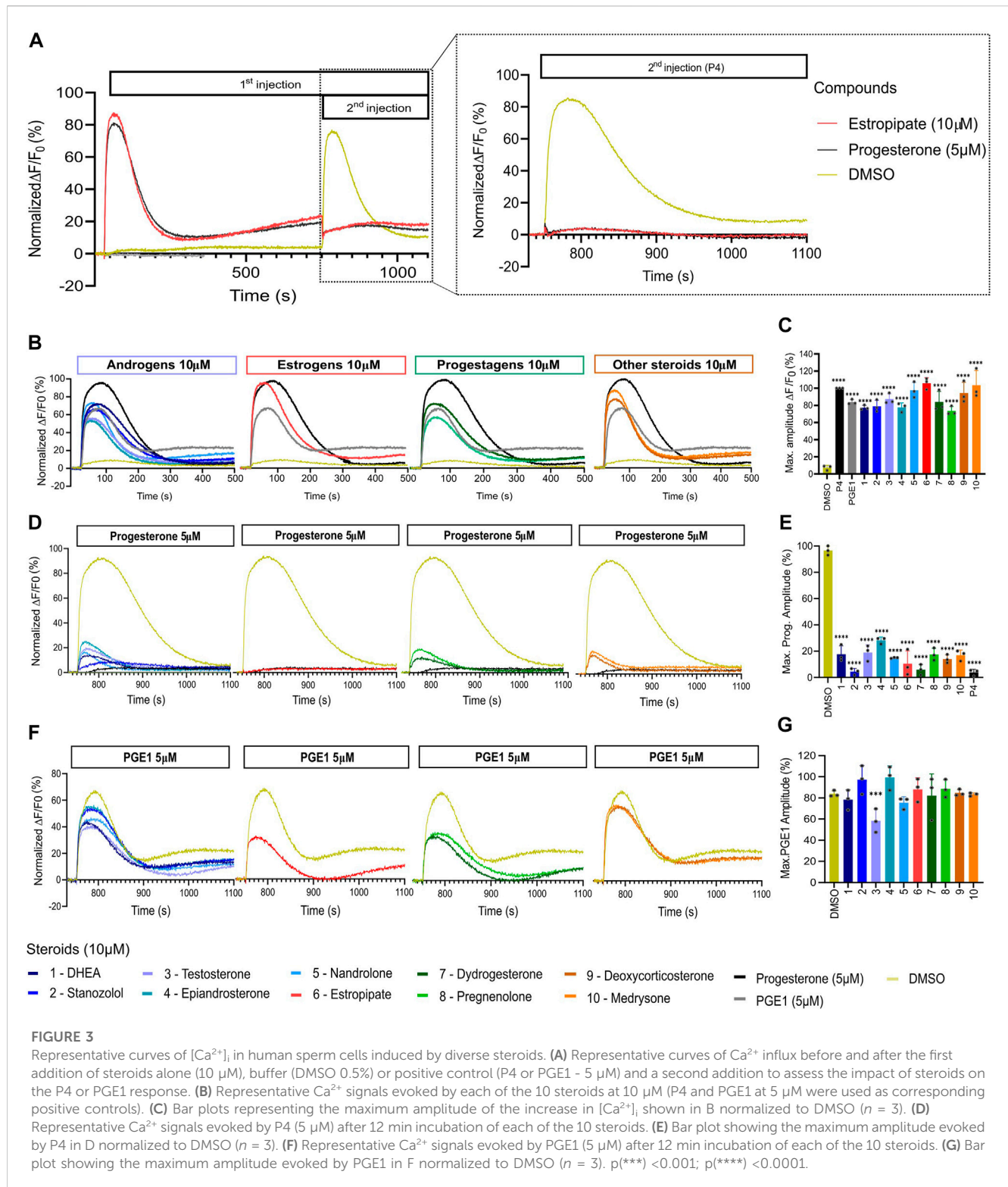
spermatozoa were allowed to capacitate for 4 h at 37°C in capacitating media (HTF+) containing: 93.8 mM NaCl, 4.7 mM KCl, 0.2 mM $MgSO_4$, 0.369 mM KH_2PO_4 , 2.04 mM $CaCl_2$, 20.98 mM HEPES, 2.78 mM glucose, 21.4 mM Na-lactate, 25 mM $NaHCO_3$, 6.6 μM Na-pyruvate, and the pH was adjusted to 7.35. Capacitated sperm were then incubated for 30 min with 5 $\mu g/mL$ *Pisum sativum* agglutinin conjugated with fluorescein isothiocyanate (PSA-FITC) and 10 $\mu g/mL$ Hoechst-33342. Samples were centrifuged at 700 x g for 10 min at room temperature to remove excess dye and resuspended in HTF+. Sperm were then incubated for 45 min in the absence (DMSO) or presence of the steroids at 10 μM , or positive control P4 at 10 μM . The ionophore A23187 was also used as a positive control and incubated with sperm at 2 μM for 30 min only. PI (0.5 $\mu g/mL$) was then added to all samples which were analyzed using BDFACSaria. FITC-PSA fluorescence was detected by excitation at 475 nm and emission at 560/35 nm, PI was detected by excitation at 530 nm and emission at 675/75 nm and Hoechst was detected by excitation at 346 nm and emission at 460 nm. Hoechst-positive and PI-negative cells were classified as live cells. Doublet exclusion was performed by two-dimensional dot plot analysis of forward-scatter width (FSC-W) versus forward-scatter height (FSC-H), and side-scatter width (SSC-W) versus side-scatter height (SSC-H) on the pre-selected singlets. Data were collected from 40,000 PSA-FITC negative events per sample to define the sperm population. Based on the selected population, PI-negative and PSA-FITC-positive cells were selected as live acrosome reacted cells, whereas PI-negative and PSA-FITC-negative cells were selected as live acrosome intact cells.

Sperm penetration in viscous media

The ability of sperm to penetrate in viscous media similar in viscosity to that found in the female reproductive tract was assessed using the modified Kremer test, as previously described (Rahban et al., 2021). Briefly, swim-up recovered and capacitated sperm cells at $3 \times 10^6/mL$ were incubated in the absence (DMSO) or presence of steroids (10 μM), or P4 (5 μM) as a positive control for 1 h at 37°C. A glass capillary ($0.2 \times 4.0 \times 50$ mm, CM scientific, UK) was filled with 1% (w/v) methylcellulose (MC, 4000 centipoises cP) prepared with HTF media supplemented with 3 mg/mL HSA, respective compounds (10 μM), P4 (5 μM), or DMSO. The glass capillary tube was then sealed on one end with wax (Vitrex, UK) and added to the sperm cells on the open end. Sperm penetration was assessed after 1 h of incubation at 37°C by counting sperm at 1 cm using a microscope with a $\times 10$ objective.

Statistical analysis

Data are shown as mean \pm standard deviation (SD) with “n” referring to the number of independent experiments performed using sperm samples from ≥ 3 different donors. Statistical analysis and fitting of dose-response relations were performed using GraphPad Prism 8.1.1 (Prism, La Jolla, United States). To generate sigmoidal curves, the concentrations were converted to their respective log values, and the data were normalized using

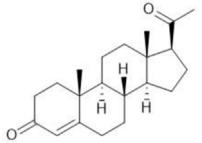
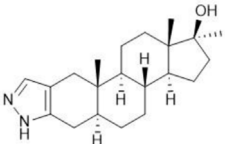
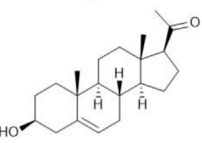
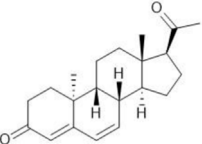
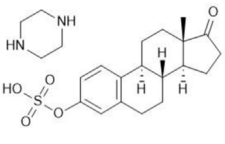
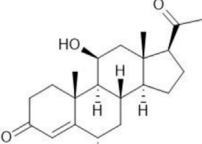


the following formula: $\frac{(100-x)}{(F_{max})^x}$, where F_{max} is the maximum value of the positive control. A four-parameter Hill equation with a variable Hill coefficient was fitted to generate the EC_{02} , EC_{50} , and IC_{50} . Statistical significance between control and treated conditions was evaluated using a one-way analysis of variance (ANOVA), followed by Dunnett’s test. A p -value < 0.05 was considered significant.

Pharmacophore and statistical modelling

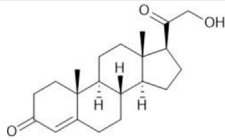
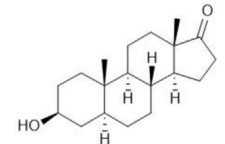
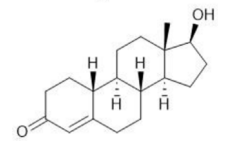
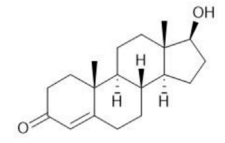
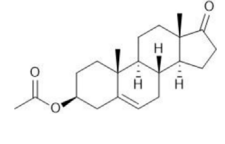
In order to build a ligand-based model that could be used to screen *in silico* for additional steroid-based compounds with an agonist effect, we started by constructing an initial pharmacophore model and a semi-parametric zero-inflated beta regression model using the 90 steroids identified through the screening of the

TABLE 1 Summary table of EC₅₀ and IC₅₀ values of the ten selected steroids. All EC₅₀ and IC₅₀ were generated following dose-response experiments in the absence (DMSO) or presence of steroids ranging from 90 μM to 0.3 nM. EC₅₀/IC₅₀ units are in μM. ND: No data available.

Common name	Chemical structure	Steroid class	EC ₅₀ (±SD)	IC ₅₀ (±SD)	Maximum serum level	Physiological presence	Common use	References
Progesterone		Progestagen	0.003 (±0.001)	0.006 (±0.002)	3 nM (male) 60 nM (female)	Yes	Birth control	Löfgren and Bäckström (1990)
Stanozolol		Androgen	0.285 (±0.328)	0.194 (±0.045)	ND	No	Anabolic-Androgenic Steroids (AAS)	ND
Pregnenolone		Progestagen	0.073 (±0.016)	0.199 (±0.008)	12 nM	Yes	Endometriosis	Hill et al. (1999)
Dydrogesterone		Progestagen	0.130 (±0.103)	0.245 (±0.042)	52 nM	No	Luteal insufficiency infertility	Abdel-Hamid et al. (2006)
Estropipate		Estrogen	0.161 (±0.017)	0.288 (±0.010)	0.29 nM	No	Ovarian failure	Barnes and Levrant (2007)
Medrysone		Corticosteroid	0.240 (±0.103)	0.569 (±0.275)	ND	No	Anti-inflammatory	ND

(Continued on following page)

TABLE 1 (Continued) Summary table of EC₅₀ and IC₅₀ values of the ten selected steroids. All EC₅₀ and IC₅₀ were generated following dose-response experiments in the absence (DMSO) or presence of steroids ranging from 90 μM to 0.3 nM EC₅₀/IC₅₀ units are in μM. ND: No data available.

Common name	Chemical structure	Steroid class	EC ₅₀ (±SD)	IC ₅₀ (±SD)	Maximum serum level	Physiological presence	Common use	References
Deoxycorticosterone		Mineralocorticoid	0.302 (±0.062)	0.523 (±0.081)	0.19 nM	Yes	Adrenocortical insufficiency	Oddie et al. (1972)
Epiandrosterone		Androgen	0.209 (±0.033)	0.628 (±0.102)	9 nM	Yes	AAS	Lewis et al. (1997)
Nandrolone		Androgen	0.306 (±0.033)	0.711 (±0.109)	19 nM	No	AAS; Anemia	Bagchus et al. (2005)
Testosterone		Androgen	0.407 (±0.062)	1,107 (±0.157)	11.6 nM	Yes	Sexual dysfunction (men); hot flashes (women)	Groepenhoff et al. (2021)
Dehydroisoandrosterone 3-acetate (DHEA)		Androgen	0.593 (±0.043)	1,745 (±0.488)	53 nM	Yes	Depression, Supplement	Habib et al. (2021)

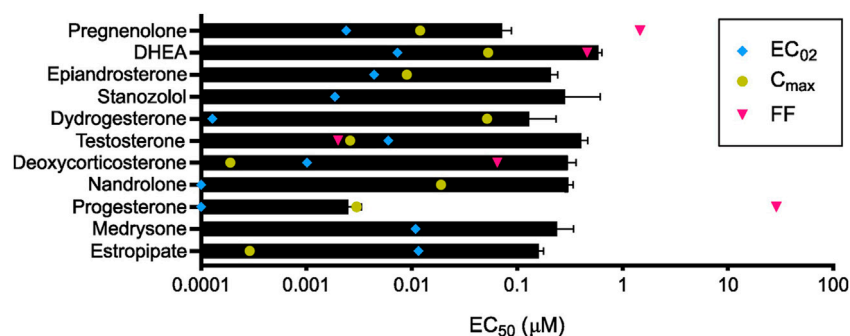


FIGURE 4

Steroids and their pharmacological concentrations. Calculated *in vitro* half maximal effective concentration (EC₅₀, black) compared with reported maximum serum concentration (C_{max}, yellow), average concentration in follicular fluid (FF, pink), and calculated minimum effective concentration (EC₀₂, blue) ($n = 3$). C_{max} was not found to be reported in the literature for medrysone and stanozolol as well as the FF concentration for epiandrosterone, stanozolol, dydrogesterone, nandrolone, medrysone, and estropiate.

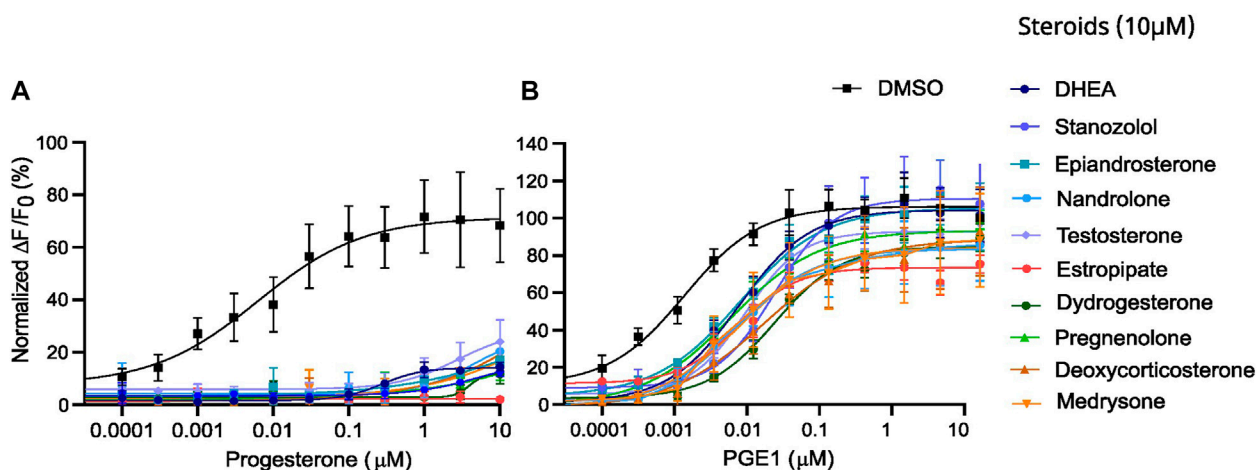
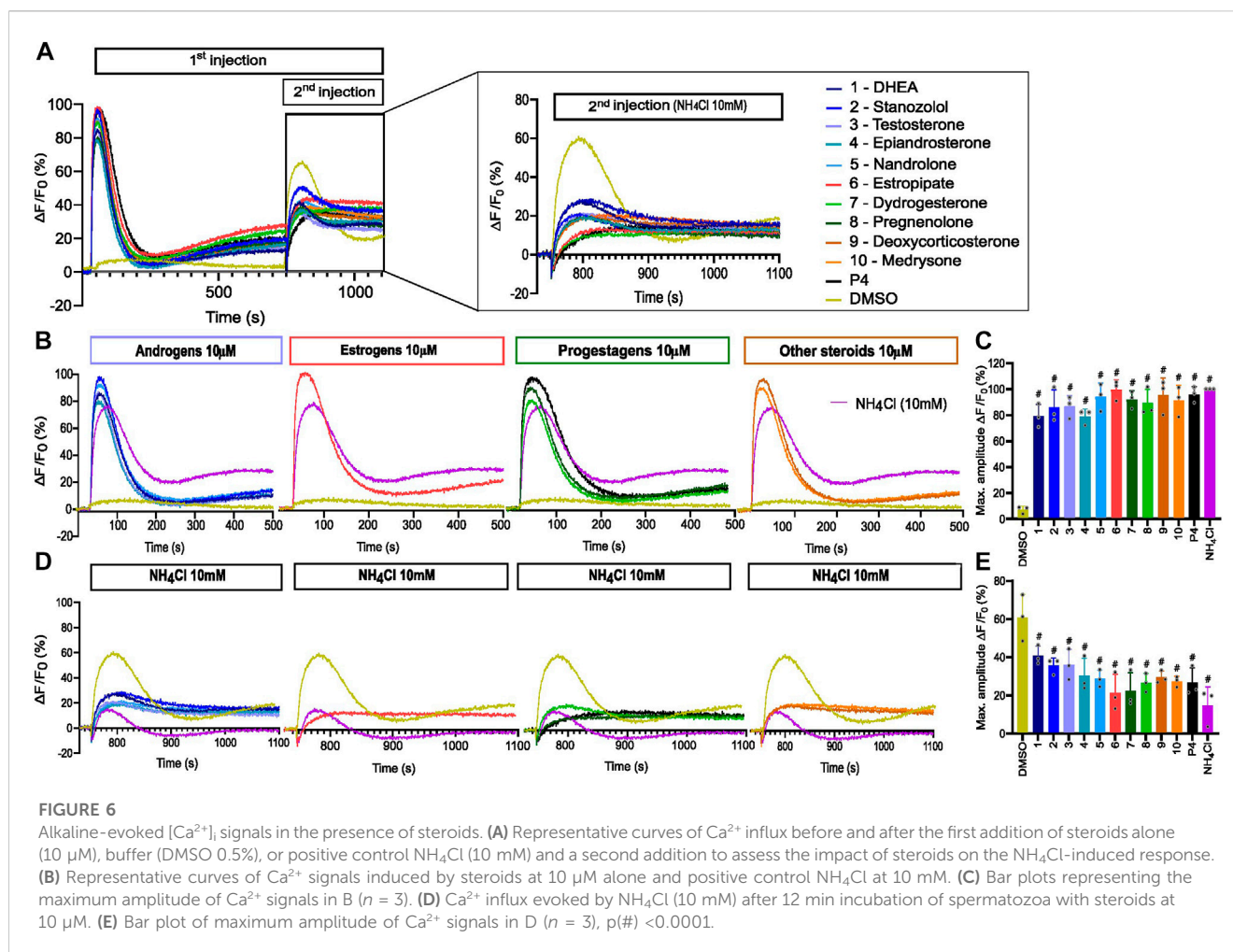


FIGURE 5

Desensitization experiments with increasing doses of P4 and PGE1 in the presence of a fixed dose of steroids. Dose-response curves of (A) the P4-induced and (B) the PGE1-induced Ca²⁺ response in the absence (DMSO) and presence of steroids at 10 μM. Data are presented as the mean of 3 independent experiments with error bars representing the standard deviation (SD).

Prestwick library (see [Supplementary Figure S1](#) for the details on the construction of the models). Given the known importance of different physicochemical properties of compounds for their binding to charged porins in bacteria, including their polarity ([Acosta-Gutierrez et al., 2018](#)), we developed a physicochemical property-based filtering criterion relying on a statistical regression model. This model was then used in combination with our pharmacophore model to increase the probability of the *in silico* screening. The regression model assumes a zero-inflated beta distribution to model the agonist effect, which is the dependent variable, while it considers the original physicochemical properties and their second and third-order interactions between them as independent variables. The QikProp tool of the Schrödinger suite (QikProp, Schrödinger, LLC, New York, NY, 2021) was used to calculate the physicochemical properties of each compound. Of the 10 physicochemical properties selected using the Adaptive Lasso procedure ([Zou, 2006](#)) and included in the model, seven were

identified as significant when considering a confidence level of $1 - \alpha = 0.95$, most of which were the result of interaction between variables. Out of these variables, only one was an original variable, while the rest of the significant variables were interactions between two or three initially measured properties. To select the most optimal pharmacophore and statistical model combination, we used a blind dataset of 18 compounds whose agonist effect was predicted by the pharmacophore and statistical model prior to their experimental testing. Among the different pharmacophore models considered, the one with the two hydrophobic sites and one acceptor site, combined with a beta zero-inflated model with adaptive-Lasso selected covariates was considered the most optimal ([Supplementary Figure S1](#)). In particular, the combination of the two models was able to identify five true positives and one false positive out of the six compounds. The results of the blind test demonstrated that the combination of the



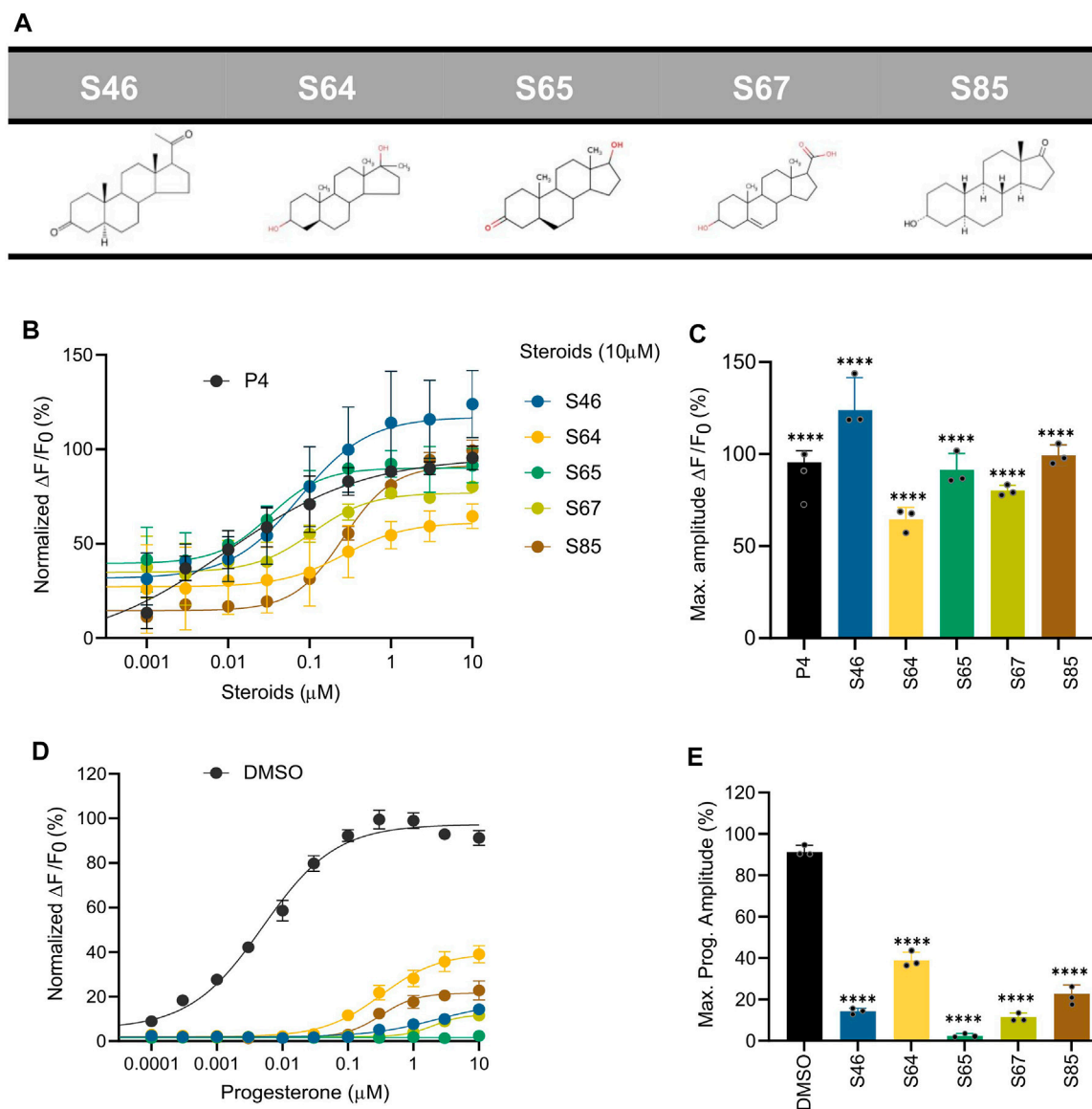
two models was crucial to achieving a good prediction ratio, as considering either model alone led to several false positives. Using this combination of models, we screened *in silico* the GeDB ($n = 187$), ChemDiv ($n = 498$), and ZINC ($n = 98$) libraries after querying only for compounds with a steroidal core. For all the compounds screened virtually, 30 additional steroids were tested: the top 30 with the highest pharmacophore fitting score and predicted agonist effect, and five that were expected to have no effect but were included for further validation of the model were purchased and tested experimentally.

Results

The Prestwick library screen and confirmation of hits by dose-response

To evaluate the potential effect of synthetic and physiological compounds including steroids on Ca^{2+} influx, we screened the Prestwick library which consists of 1,280 compounds from various therapeutic classes. Our HTS assay was designed to screen for compounds that can induce a Ca^{2+} influx (screen 1, activators) and/or can alter the P4- and

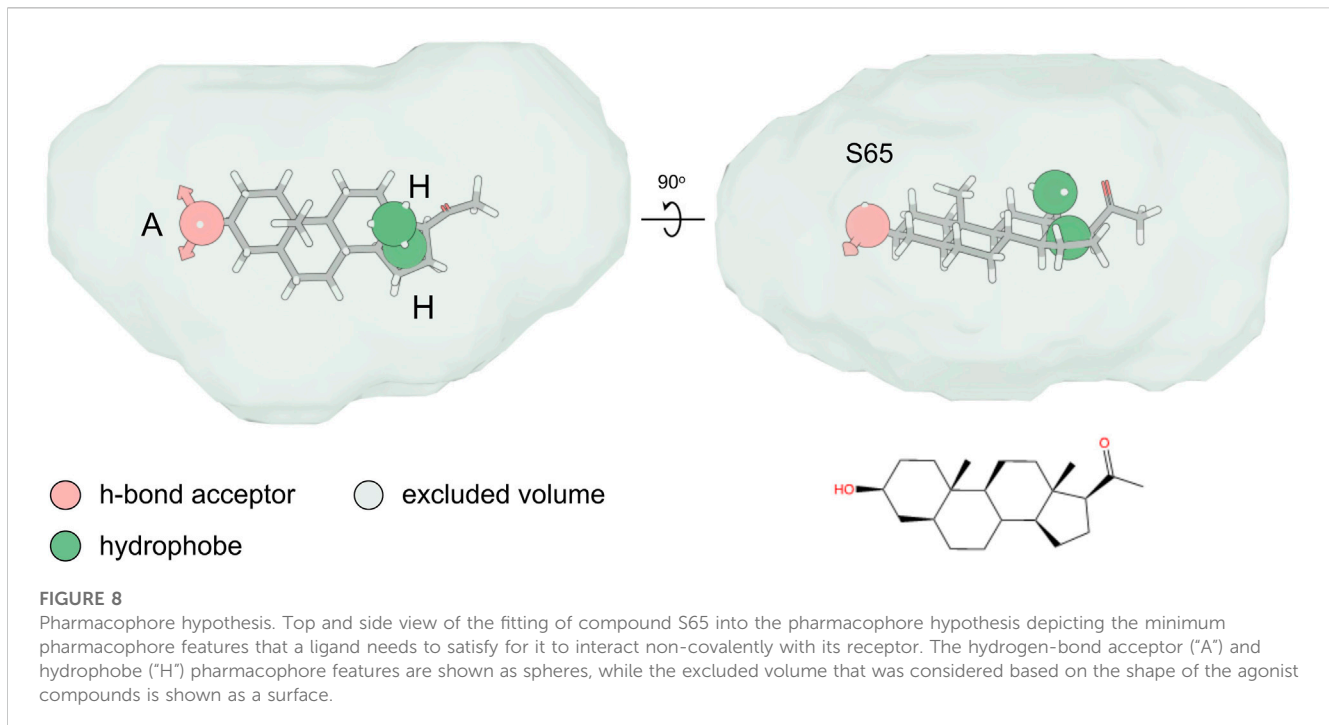
PGE1-induced Ca^{2+} influx (screen 2, inhibitors) (Figure 1A). Compound plates were validated with a Z' -factor > 0.4 (Figure 1B). Hit selection was based on an arbitrary cutoff of 30% activation and/or inhibition compared to the controls (Figure 1C). We identified 106 (8.3%) hits, of which 31 were putative activators, 45 were putative inhibitors, and 30 had a dual effect (Figure 2A). Interestingly, the compounds with dual activating and inhibiting effects were mostly steroids. All 26 of them reduced the P4- but not PGE1-induced Ca^{2+} influx (Figure 2B, C). The hit steroidal compounds were validated by dose-response curves (DRC) in technical duplicates and biological triplicates and their EC_{50} s as well as their IC_{50} s for the P4-induced response were measured (Supplementary Table S1). Interestingly, of the 90 steroids in the Prestwick library, more than half (53%) were able to both activate and inhibit P4-induced Ca^{2+} influx in human sperm (Figure 2B). In contrast, inhibition of PGE1-induced Ca^{2+} influx was rarely observed with these steroids (Figure 2C). For the next part of this study, the 10 steroids with the strongest dual activation and inhibition potential were selected for further investigation. These were the synthetic steroids stanozolol, estropipate, dydrogesterone, medrysone, and the natural steroids dehydroepiandrosterone acetate (DHEA), testosterone, epiandrosterone, nandrolone, pregnenolone, deoxycorticosterone.



Structurally diverse steroids modulate human CatSper

The 10 selected steroids were classified into 4 classes: androgens, estrogens, progestagens, and ‘others’, and they all induced an increase in $[Ca^{2+}]_i$ similar to P4 with estropiate inducing the highest increase in $[Ca^{2+}]_i$ (Figures 3A, D). All 10 steroids were tested in dose-response experiments and revealed a dose-dependent increase in $[Ca^{2+}]_i$ (Supplementary Figure S2). More specifically, estropiate, pregnenolone, and

dydrogesterone induced a dose-dependent increase in $[Ca^{2+}]_i$ with effective concentrations as low as 10 nM. Stanozolol and nandrolone also induced a 30% increase in $[Ca^{2+}]_i$ at low concentrations in the nanomolar range (Supplementary Figure S2). EC_{50} values ranged from 0.073 μM for pregnenolone to 0.5 μM for DHEA (Table 1). To determine the lowest activating concentration, EC_{02} was calculated for all steroids, and compared with the reported maximum concentration found in blood serum (C_{max}) and the reported average concentration of steroids found in the follicular fluid (FF) (Figure 4). This



comparison highlighted that the effective concentration at which progesterone, pregnenolone, dydrogesterone, epiandrosterone, nandrolone, and DHEA activate CatSper *in vitro* is within the biological range of their concentration measured *in vivo*. In the case of nandrolone, progesterone, and dydrogesterone, the EC_{02} values were more than 100 times lower than the C_{max} reported value in the literature (Table 1; Figure 4). All steroids tested significantly reduced the P4 response with IC_{50} values ranging from 0.19 μ M to 1.75 μ M (Supplementary Figure S2; Table 1). This indicates that P4 is the most potent steroid tested. Interestingly, a strong correlation was observed between the steroids inducing the highest increase in $[Ca^{2+}]_i$ and the ones that reduce the P4 response the most potently (Supplementary Figure S3). This decrease was not class-specific, suggesting that different functional groups can be accommodated in the binding site. Steroids did not alter the PGE1-induced increase in $[Ca^{2+}]_i$ suggesting that steroids act on the same binding site as that of P4 but not that of PGE1 (Figures 3C, F).

Steroids compete for the same binding site as progesterone

To determine whether the strong inhibitory effect of the 10 steroids on P4-induced Ca^{2+} influx was due to competitive binding, we performed cross-desensitization experiments by challenging pre-incubated sperm cells with steroids at 10 μ M with increasing doses of P4 (Figure 5A). The P4-induced increase in $[Ca^{2+}]_i$ was reduced significantly by the presence of the 10 steroids tested. In contrast, the same experiment performed with increasing doses of PGE1 revealed a slight shift in the EC_{50} without a decrease in maximum response,

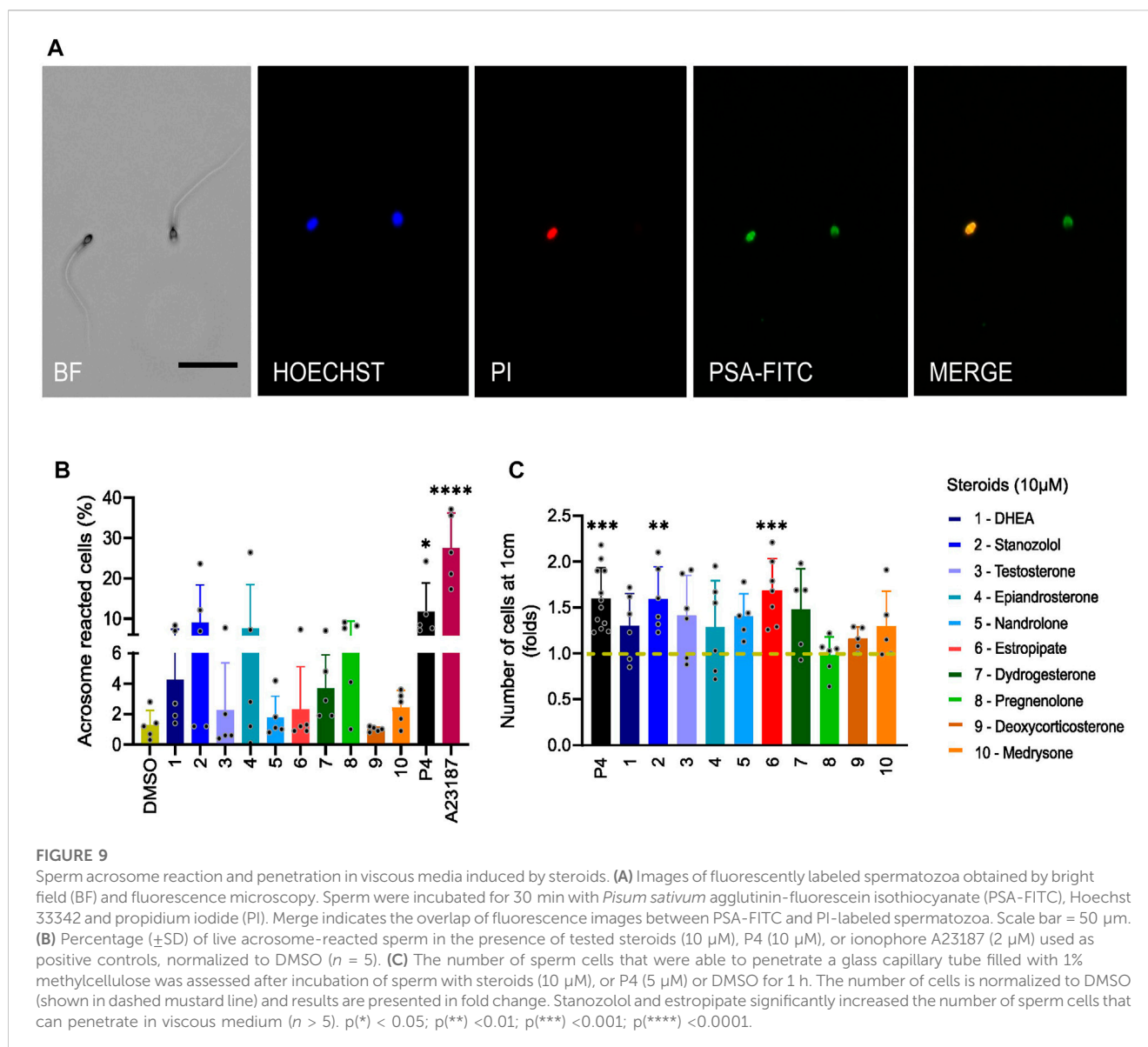
indicating that steroids reduce the potency of PGE1 but not its efficacy. (Figure 5B).

Steroids suppress pH-induced activation of CatSper

Besides ligand activation, CatSper can be weakly activated by changes in membrane voltage or alkalization of intracellular pH (pH_i) (Brown et al., 2019; Wang et al., 2021). Therefore, the 10 selected steroids were evaluated for their ability to affect CatSper-mediated Ca^{2+} influx induced by intracellular alkalization via the weak base NH_4Cl . All tested steroids almost completely suppressed the Ca^{2+} signals evoked by NH_4Cl at 10 mM (Figure 6). We also measured changes in pH_i in the presence of the 10 steroids. None of the steroids induced an increase in intracellular pH (Supplementary Figure S4), indicating that CatSper activation by steroids is not triggered by the increase in pH_i .

Structure-activity relationship of agonist steroid compounds

In the absence of a resolved structure for human CatSper, we used pharmacophore modelling and machine learning to search *in silico* for additional steroid compounds with a strong activating effect as well as define the steroid structure-activity relationship (SAR) responsible for the CatSper activation. We first constructed a pharmacophore model for the steroids with agonist activity that we identified in our initial screening (Figures 2B, C), and then combined them with a statistical regression model capable of relating their physicochemical properties to their activity profile.



After validating the ability of our combined model to identify compounds that could increase the Ca^{2+} influx against a blind dataset, we used it to screen 783 compounds *in silico*. Of the 20 top compounds that we selected and tested experimentally, 11 showed activity in the high nanomolar range (Supplementary Table S1). Of particular interest are the synthetic steroids S46 and S65 (Figure 7A). These two compounds potentially increased $[\text{Ca}^{2+}]_i$ in human sperm (EC_{50} of 32 nM and 73 nM, respectively) at concentrations close to P4 (EC_{50} of 2.5 nM) (Figures 7B, C). These two compounds also significantly decreased the response to P4 (IC_{50} of 46 nM and 138 nM, respectively) (Figures 7D, E). Among the different physicochemical properties that we considered for the construction of the statistical regression model to predict the agonist effect of new steroids, we found a significant positive correlation between the predicted IC_{50} value for human Ether-à-go-go-Related Gene (hERG) K^+ channel block (QLogHERG) and the number of amide groups and the square of the dipole moment divided by the molecular volume (dip^2/V). This finding is not

unexpected as dip^2/V is a crucial term in the free energy of solvation of a dipole with volume V . Additionally, dip^2/V and the octanol/water partition coefficient ($\text{QLogP}_{o/w}$), as well as dip^2/V and the y -component of the dipole moment, were found to be significantly positively correlated with the dependent variable, in this case, the agonist effect. The coefficients of the properties used in the estimated model can be found in Supplementary Table S2. From our model, it is clear that the desolvation of the steroids and their dipole moment in the direction of the electric field inside the channel with respect to their volume are key properties that need to be fine-tuned when designing new steroid analogues that target CatSper. To investigate the SAR of steroid activation of the CatSper channel, we evaluated a wide range of steroids with different functional groups in each of the steroid rings for their ability to induce Ca^{2+} influx in human sperm. Our results showed that the CatSper channel was activated by multiple steroids with various structural modifications. Structural analysis of the steroids provides useful insights into substitutions that can improve the potency of

P4 analogues (Figure 8). In particular, various substitutions on the A-ring could be tolerated, including a pyrazole ring (stanozolol, Supplementary Table S1), resulting in moderate CatSper activators. Interestingly, the removal of the double bond present on the A-ring of P4 also increased the agonist activity of some compounds such as pregnenolone, S67, S85, and S45 (Figures 7B, C; Supplementary Table S1). This suggests that some flexibility in this ring may facilitate stronger interactions with the channel residues. In agreement with previous reports (Carlson et al., 2022b), methylation at the C19 position on the B-ring does not appear to be important for the activity, whereas our results indicate that methylation at the C7 position on the same ring can be tolerated. Any significant substitution on the C-ring rendered the steroids inactive or unable to bind at all. Only steroids with a hydroxyl group at the C13 or C14 position seemed tolerable in our dataset. Finally, bulky aliphatic substitutions at the C17 position of the D-ring led to a dramatic decrease in agonist activity, but small substitutions with hydrogen bond donors had a modest activating effect (deoxycorticosterone, stanozolol, norgestimate, Supplementary Table S1), suggesting that this part of the steroid may play a key role in stabilizing binding and thus activating CatSper. Notably, structural modifications on the D-ring have been reported to convert agonists to antagonists (Carlson et al., 2022b) and, therefore, this region can be exploited in the design of potent steroidal antagonists of CatSper (Supplementary Figure S1).

Steroids do not affect the acrosome reaction but Stanozolol and estropipate increase sperm penetration in viscous media

We finally investigated the effect of the 10 selected steroids on the acrosome reaction (AR) in human sperm (Figure 9A). All steroids showed similar results to negative control DMSO except stanozolol, epiandrosterone, and pregnenolone. These steroids induced acrosome reaction in more than 10% of the cells, but this increase was not significant because of the large variation in response among donors. P4 only slightly increased the AR in consistency with previously published data (Baldi et al., 1991; Prajapati, et al., 2022). We also assessed sperm penetration in a viscous medium using a modified Kremer's test (Figure 9B). At a distance of 1 cm, the majority of the steroids tested increased the number of sperms that can penetrate in viscous media, although only stanozolol and estropipate induced a significant increase in cell number similar to P4. In addition, we evaluated the sperm motility in the presence and absence of the selected steroids at 10 μM using Computer Assisted Sperm Analysis (CASA) (Supplementary Figure S5). Incubation of spermatozoa with the selected steroids for 1 hour did not alter total, progressive, and hyperactive motility (Supplementary Figure S5). Cell viability was also assessed by flow cytometry of PI incubated cells (Supplementary Figure S6). Our results show that steroids at 10 μM were not cytotoxic.

Discussion

CatSper is the main entry point for Ca^{2+} ions in human spermatozoa, but it is also a promiscuous channel that can be modulated by a wide variety of natural and synthetic molecules

(Spehr et al., 2003; Tavares et al., 2013; Schiffer et al., 2014; Miller et al., 2016; Rehfeld et al., 2016; Birch et al., 2021; Jeschke et al., 2021; Rahban et al., 2021). To investigate the impact of diverse chemical structures on $[\text{Ca}^{2+}]_i$, we developed a HTS Ca^{2+} influx assay in human sperm. We screened 1,280 approved and off-patent drugs from the Prestwick chemical library for their potential to either induce a Ca^{2+} influx or to alter the P4- and PGE1-induced response. We show that a wide range of pharmaceutical compounds belonging to the steroid class, both natural and synthetic, are capable of acting on CatSper. Using a pharmacophore and statistical modelling, we screened *in silico* for steroids that can activate the channel and better understand the molecular characteristics of the steroids that promote CatSper activation.

A recent study on 15 steroids present in the follicular fluid showed that these steroids cause a rapid increase in Ca^{2+} influx in human sperm via CatSper (Jeschke et al., 2021). The classical Ca^{2+} response was abolished in CATSPER2^{-/-} spermatozoa for all 15 steroids, demonstrating that the response depends on Ca^{2+} influx via CatSper. Here, we show that the ability of the steroids to activate CatSper is highly variable, with some steroids activating CatSper at low nanomolar concentrations while others are completely ineffective in inducing a Ca^{2+} response. In particular, 10 steroids had a strong dual activating and inhibiting effect on CatSper and were therefore selected for in-depth analysis of their action. These steroids were either synthetic (stanozolol, estropipate, dydrogesterone, medrysone) and/or physiological (dehydroepiandrosterone acetate - DHEA -, testosterone, epiandrosterone, nandrolone, pregnenolone, deoxycorticosterone) and were all evaluated for their action on P4-, PGE1-, pH- activation of human CatSper. In addition to cross-desensitization experiments, our results showed that all 10 steroids directly modulate the CatSper channel and compete for the same binding site as P4, but not PGE1. These observations are consistent with several scientific reports indicating that numerous steroids such as testosterone, hydrocortisone, estradiol, pregnenolone, dihydrotestosterone, and estrone act as true agonists that activate CatSper and increase $[\text{Ca}^{2+}]_i$ in human sperm (Blackmore et al., 1990; Blackmore et al., 1996; Luconi et al., 1999; Luconi et al., 2004; Rossato et al., 2005; Brenker R. A. et al., 2018; Brenker S. C. et al., 2018; Rehfeld, 2020; Jeschke et al., 2021). These results also demonstrate that steroids and prostaglandins act on distinct binding sites, as previously described (Lishko et al., 2011; Strünker et al., 2011; Miller et al., 2015; Kaupp and Strünker, 2017; Lishko and Mannowetz, 2018).

Steroids are widely used as medications for a variety of purposes, some on a long-term basis, and have been available for over 50 years (Rice et al., 2017; Kapugi and Cunningham, 2019; Bleecker et al., 2020). Corticosteroids are one of the most commonly prescribed steroids and are often used to reduce inflammation and suppress the immune system in conditions such as asthma, allergic rhinitis, chronic obstructive pulmonary disease, multiple sclerosis, and others (Rice et al., 2017; Ramadan et al., 2019; Bleecker et al., 2020). Synthetic versions of testosterone and estrogen are also used by some athletes (anabolic steroids) and postmenopausal women, respectively (Cole et al., 2019). Our results suggest that steroids such as deoxycorticosterone significantly increased $[\text{Ca}^{2+}]_i$ and reduced more than 86% of the P4 response with an $\text{EC}_{50} = 0.3 \mu\text{M}$ and an $\text{IC}_{50} = 0.5 \mu\text{M}$, respectively. Similarly, androgens and estrogens also acted as both potent activators and inhibitors at low

nM concentrations. Therefore, an important matter to be addressed here is the physiological significance of the pharmaceutical steroid on CatSper activation. To our knowledge, few previous studies have reported on the concentration of steroids in seminal, oviductal, or follicular fluid. Jeschke *et al.* reported the average concentration of 18 steroids measured in the follicular fluid of eight oocytes from four different gonadotropin-stimulated women. Among the 18 steroids, five were steroids that we tested: progesterone, pregnenolone, deoxycorticosterone, DHEA, and testosterone (Jeschke *et al.*, 2021). Here we show that of the 10 steroids analyzed, pregnenolone, dydrogesterone, epiandrosterone, nandrolone, and DHEA are present in sufficient quantities to activate CatSper with EC_{02} below the reported maximum concentration found in blood serum. We show here that these steroids compete for the same binding site, suggesting that they can bind to CatSper *in vivo* and potentially affect P4 binding and downstream sperm function. For fertilization to occur, it is crucial that sperm functions controlled by $[Ca^{2+}]_i$ are triggered appropriately and in a timely manner (Publicover *et al.*, 2007). We therefore argue that binding of these steroids to CatSper before the sperm reaches the oocyte could prevent the optimal binding of P4 and thus the fertilization process. This in turn could reduce the fertility of couples. It has been shown on several occasions that a suboptimal P4-induced Ca^{2+} influx is associated with reduced male fertility and that impaired P4-induced calcium signaling is associated with lower rates of IVF success (Falsetti *et al.*, 1993; Krausz *et al.*, 1995; Forti *et al.*, 1999; Williams *et al.*, 2015; Kelly *et al.*, 2018).

Considering that humans may be exposed to a variety of chemicals that can interfere with CatSper, and these molecules may act additively or synergistically with steroidal compounds to affect CatSper-mediated Ca^{2+} signaling (Schiffer *et al.*, 2014; Rehfeld *et al.*, 2016; Brenker R. A. *et al.*, 2018). To this end, further, *in vitro* and clinical studies are needed to assess the levels of EDCs and other relevant steroids in oviductal fluids and to investigate whether synergistic interactions between these compounds may affect the activity of CatSper. It is worth noting that the antagonizing activity of the steroids on the P4-induced response can also be considered relevant in the field of male contraception (Wang *et al.*, 2021; Rehfeld *et al.*, 2022). Indeed, steroids such as stanozolol and dydrogesterone that strongly reduce the P4 response can serve as a starting point in that direction.

Although all selected steroids had a significant impact on CatSper activation or reduction of the P4-induced response, none affected the AR, while stanozolol and estropipate only slightly increased sperm penetration into viscous media. It is well established that P4 acts on CatSper (Lishko *et al.*, 2011; Strünker *et al.*, 2011). However, the downstream effects of this activation on human sperm function, such as the AR and sperm penetration in viscous media have been highly controversial (Baldi *et al.*, 2009; Tamburrino *et al.*, 2014). Recently, Young and others described nine CatSper-deficient men presenting with couples infertility and failure of Medically Assisted Reproduction (MAR) (Young *et al.*, 2023). They showed that loss of CatSper function abolished spontaneous hyperactivation induced by capacitation and P4, but did not affect AR and sperm penetration in viscous media (Young *et al.*, 2023). This suggests that most of the steroids tested here act on CatSper in a similar way to P4.

To provide structural insights into the activation of CatSper by steroids, we used the dataset we generated on over 90 steroids and

applied pharmacophore modelling and machine learning to define the structure-activity relationship (SAR) associated with the agonist effect of the tested steroids. Due to the wide range of steroids with different functional groups in each of the steroid rings, we were able to refine the SAR for steroid activation of the CatSper channel and provide useful information on substitutions that may enhance the ability of certain steroids to activate CatSper. Our results show that the A-ring of steroids can tolerate various substitutions, including a pyrazole ring, resulting in moderate CatSper activation. Removal of the double bond on the A-ring was shown to increase the activity of some compounds, suggesting that the flexibility of this ring facilitates stronger interactions with the channel residues. Methylation at the C7 position on the B-ring is tolerated, but any significant substitution on the C-ring renders the steroids inactive or unable to bind. Steroids with a hydroxyl group at the C13 or C14 position appeared to be tolerable, and the bulky aliphatic substitutions at the C17 position of the D-ring led to a dramatic decrease in agonist activity. Our results are consistent with those of previous studies that have examined the chemical structures of steroids to determine their affinity for the unknown binding site of CatSper (Jeschke *et al.*, 2021; Carlson *et al.*, 2022a; Carlson *et al.*, 2022b; Xiang *et al.*, 2022). The use of the SAR information allowed us to identify and test several commercially available synthetic steroids in addition to the steroids we initially screened. Some of these steroids were able to increase $[Ca^{2+}]_i$ and inhibit the P4-induced response at nM concentrations, close to P4. The reduced response suggests that this combined pharmacophore and physicochemical properties modelling approach is effective and allows the identification of steroids capable of activating CatSper already at very low concentrations. The study from Carlson *et al.* also investigated the effect of multiple steroids and found that CatSper is activated by a wide range of steroids with diverse structural modifications (Carlson *et al.*, 2022b). They examined the modification of residues throughout the steroid skeleton and found that over 30 steroids are capable of activating CatSper. Interestingly, all modifications reduced potency relative to P4, generally without affecting the maximal extent of Ca^{2+} influx. Here, we have extended this analysis by examining 90 steroidal drugs as well as 30 commercially available synthetic steroids selected by SAR for steroid activation of the CatSper channel. Our data confirm those of Carlson *et al.* as we found that more than half of the steroids tested can activate CatSper and selectively antagonize P4-induced Ca^{2+} influx. Furthermore, the potency of activation or inhibition of the P4 response by these steroids is always lower than that of P4. Our *in silico*-based screening identified several steroidal compounds with high potencies capable of inducing Ca^{2+} influx and inhibiting P4-induced response at a low concentration within the nM range (compounds S65 and S46 with EC_{50} of 32 nM and 73 nM and IC_{50} of 46 nM and 138 nM, respectively). In contrast, the most potent steroids inhibiting P4-induced response identified by Carlson *et al.* such as medroxyprogesterone acetate, levonorgestrel, and aldosterone have IC_{50} values two orders of magnitude higher at 6.6 μ M, 32.3 μ M, and 33.1 μ M, respectively.

In conclusion, our results suggest that diverse steroids act directly on CatSper to activate the channel and compete with P4 for its binding site *in vitro*. If bound to CatSper prior to physiological P4-binding, these steroids might impair the fertilization process. The mechanism of action by which these steroids act on CatSper is however complex and further studies are required to elucidate their potential adverse effect *in vivo*. In a

research setting, their P4-antagonizing activity might serve as a starting point for the development of male contraceptives.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Study was reviewed and approved by the Cantonal Ethics Committee of the State of Geneva (CCER #14-147). All donors gave their written informed consent to participate in this study. The patients/participants provided their written informed consent to participate in this study.

Author contributions

RR and SN designed the study, LW and RR coordinated the study. LW performed experiments; IG and LV performed *in silico* modelling; LW, IG, LV, SG, FG, SN, and RR designed experiments, analyzed and/or interpreted the data, and revised the manuscript critically for important intellectual content, LW, SN, and RR wrote the manuscript. All authors approved the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Swiss Centre for Applied Human Toxicology (SCAHT) and the Département de

l'Instruction Publique of the State of Geneva. S. Guerrier was supported by the SNSF Professorships Grant 211007 and by the Innosuisse Grant 53622.1 IP-ENG. L. Voirol was supported by SNSF Grant 182684.

Acknowledgments

We thank Gregory Schneiter, Cécile Gameiro, and Jean-Pierre Aubry (F.A.C.S platform, University of Geneva) for technical assistance.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2023.1221578/full#supplementary-material>

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