

A phenotypic screening platform utilising human spermatozoa identifies new compounds with contraceptive activity

SHORT REPORT

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Abstract

There is an urgent need to develop new methods for male contraception, however a major barrier to drug discovery has been the lack of validated targets and the absence of an effective high-throughput phenotypic screening system. To address this deficit, we developed a fully-automated robotic screening platform that provided quantitative evaluation of compound activity against two key attributes of human sperm function: motility and acrosome reaction. In order to accelerate contraceptive development, we screened the comprehensive collection of 12,000 molecules that make up the ReFRAME repurposing library, comprising nearly all the small molecules that have been approved or have undergone clinical development, or have significant preclinical profiling. We identified several compounds that potently inhibit motility representing either novel drug candidates or routes to target identification. This platform will now allow for major drug discovery programmes that address the critical gap in the contraceptive portfolio as well as uncover novel human sperm biology.

Introduction.

An effective and comprehensive family planning strategy is fundamental to delivery on the United Nations Sustainable Development Goals (Starbird *et al.*, 2016; Goodkind *et al.*, 2018), however the current contraceptive portfolio is suboptimal. For example, it is estimated that more than 214 million women in developing countries have an unmet need for contraception which results in 89 million unintended pregnancies and 48 million abortions every year, with substantial impacts on maternal and child health and poverty levels (Guttmacher Institute, 2017). To achieve the UN targets it is critical that involvement of men be considered of equal importance as women (Hardee *et al.*, 2016). However, it is noticeable that no effective, reversible and widely-available form of contraception has been developed for men since the condom and as such the burden falls largely on the woman. The development of drugs that can be used in the male would address a critical gap in the contraceptive portfolio.

Progress in developing new male contraceptives has been slow for a plethora of reasons. Despite decades of research, safe and effective hormonal approaches have yet to be realised and viable alternatives remain elusive (Page and Amory, 2018). Developing a drug for any purpose is difficult but one that blocks sperm function is a particular challenge as spermatozoa don't divide, transcribe or translate, have specialized organelles, are highly motile and continually

produced in large numbers. Moreover, as the production of fertilization competent cells is necessarily different between species, it is essential to use a human system early in the drug discovery cascade to increase the chances of translational success.

Though some potential sperm-specific contraceptive drug targets have been identified (Lishko and Mannowetz, 2018), our relatively poor understanding of the spermatozoon makes target-agnostic strategies more attractive (Barratt *et al*, 2017). Phenotypic drug discovery is undergoing a renaissance in a number of therapeutic areas because it can uncover novel biology in an unbiased way. Retrospective analysis of drug approvals shows the approach to be more successful in first-in-class medicine discovery than previously appreciated (Swinney and Anthony, 2011; Moffat *et al.*, 2017). For male contraceptive drug discovery, a limiting barrier has been the absence of a scalable drug screening system using spermatozoa.

To address this unmet need we developed the first high-throughput phenotypic sperm screening platform where an image-based kinetic analysis module, tracking movement, is coupled to a flow cytometry module that detects the acrosome reaction (AR), using a fully-automated robotic system. The bespoke computational pipeline quantitatively evaluates compound activity against both attributes thus achieving unprecedented throughput. The platform was used to identifying inhibitors from a unique 12,000 molecule ReFRAME (Repurposing, Focused Rescue, and Accelerated Medchem) library, which represents the most comprehensive collection of drugs available, as it contains nearly all the small molecules that have achieved regulatory approval and others that have undergone varying degrees of clinical development or have had significant preclinical profiling (Janes *et al.*, 2018). This advance opens up the possibility of accelerated male contraceptive development by allowing drug repurposing, target identification as well as screening of chemical diversity libraries for novel medicinal chemistry start points.

Results

Phenotypic Assay Development

Our automated platform (Figure 1A) allows for the identification of relatively fast-acting compounds with a rapid plate-processing time (~75 minutes/ 384-well plate), where the motility assay runs for ~30 minutes followed directly by the AR assay (~ 45 minutes). Automated cell tracking (Figure 1B & Supplementary Figure 1A) using time-lapse images allowed kinetic parameter quantification on >200 cells/well (Figure 1C). The kinetics show good agreement with clinical

systems (Supplementary Figure 1B) and a comparable distribution was observed between experimental days, plates and positions (Supplementary Figure 1.1 A) with any small differences between days stemming from donor pool variance. Importantly, spermatozoa were found to be tolerant to DMSO (~4%), far above typical screening concentration of 0.1% (Supplementary Figure 1C). To determine the optimal screening batch size spermatozoa were dispensed every 30 minutes for 150 minutes observing only a small decrease in motility (~10%) after 4 plates were screened (Supplementary Figure 1D). A flow cytometry-based assay measuring AR (Figure 1D) was run directly afterwards (Figure 1E, F & Supplementary Figure 1E).

Screening of the ReFRAME library, confirmation of hits by dose response.

The ReFRAME library (11,968 compounds) was supplied in “assay-ready” imaging plates and was solubilised prior to addition of sperm. A total of 63 compounds decreased motility (Figure 2 A, B with examples in Figure 2C, E and supplementary movies 1 & 2). High assay quality was achieved throughout with a Z'-factor between 0.4-0.8 (Figure 2D). 14 compounds were selected as AR⁺ hits (Figure 3 A with examples in B, C). Motility hits were confirmed with resupplied material in dose-response experiments. There was a dose-dependent decrease in motility for 29 compounds, with EC₅₀ values as low as 0.05 μM and effect sizes ranging from 15-100% (Figure 2 F, G; Supplementary Figure 2, Supplementary Table 1). Amongst the confirmed hits were the aldehyde dehydrogenase inhibitor, Disulfiram (70% maximum reduction at 10 μM) and a putative platelet aggregation inhibitor, KF-4939 (100% max. reduction; EC₅₀=0.49μM), and a range of other compounds having very modest effect and/or showing low potency (See Supplementary Figure 2, Supplementary Table 1). In the AR screen, 9 of the resupplied compounds had a dose-dependent effect with EC₅₀ values as low as 0.4 μM (See Supplementary Figure 3, Supplementary Table 2). However, following the orthogonal assay triaging none of the AR hits were found to be true positives (see examples in Supplementary Figure 3.1 D).

Discussion.

A major barrier in the search for new male contraceptives has been the lack of an effective high throughput phenotypic screening system. This study presents the first platform based on imaging and flow cytometry that measures two fundamental aspects of sperm behaviour – motility and AR, allowing for the rapid screening of compound collections. A significant challenge was to create a system for the tracking of these highly motile cells that was fully automated and

scalable to allow throughput at sufficient speed. Moreover, concomitant assessment of a second functional attribute, AR, was developed in order to maximise use of the biological material. Assessment of motility required novel implementation of tracking algorithms. Importantly, the kinetic outputs were equivalent to those from the “industry-standard” CASA, which is very low throughput and unsuitable for screening more than a few compounds at one time. Significant workflow optimisation resulted in good assay robustness and a high hit confirmation rate following the primary screen. For AR detection, a flow cytometry approach was taken based on established methods (Mortimer *et al.*, 1987). However, the presence of assay-interfering compounds in screening libraries (potential fluorescent compounds including DNA intercalators) necessitated further triaging steps (see Materials and Methods). This combination of triaging approaches meant that, in this screen, no AR compounds were suitable for progression. Overall, although this was a complex biological assay, it achieved acceptable throughput with the potential to increase batch size for larger screening campaigns.

The advantage of using the ReFRAME collection was the potential to repurpose drugs that are already approved for other indications or are at earlier stages of clinical progression. The associated compound annotations and safety data offers the potential to help accelerate the development of an effective male contraceptive. In the motility assay the hit rate of ~0.2% is typical for a cell-based assay, however surprisingly a relatively high number of hits were not deemed suitable for compound progression. Examples included potentially toxic mercury-containing compounds (phenylmercuric borate and mercufenol chloride); antiseptics and antibiotics including tyrothricin, or compounds with impacts on fundamental biology e.g. the microtubule stabiliser and chemotherapeutic, docetaxel. However, several hits are potentially of interest. One is Disulfiram (70% maximum reduction at 10 μ M), a drug that is well tolerated, has long been used for treating alcohol dependency and has previously been shown to inhibit sperm motility (Lal *et al.*, 2016). Disulfiram is unlikely to be developable as a suitable contraceptive drug due to its side-effects when alcohol is consumed but has potential to facilitate mode-of-action studies. A recent report identified 3-Phosphoglycerate dehydrogenase (PHGDH) as a target for disulfiram (Spillier *et al.*, 2019) although the relevance to sperm function is unclear. The identification of KF-4939, a putative platelet aggregation factor (PAF) inhibitor, as a potent hit (100% max. reduction EC_{50} = 0.5 μ M) may also be of interest since PAF has been used to improve sperm function in male infertility treatments (Roudebush *et al.* 2004). It is noteworthy that like disulfiram, KF-4939 also contains a disulphide linkage indicating that its biological effect maybe mediated by modifying sulfhydryls on proteins (Yamada *et al.*, 1985). Further thiol-containing compounds, ethanedithiol (98% max reduction; EC_{50} =2.5 μ M) and the anti-fungal benzothiazole, Ticlatone (96% max. reduction EC_{50} = 4.4 μ M) were also potent hits, suggesting free cysteines might play a critical role in motility.

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142 Moreover, the Toll-like receptor 7/8 ligand (Resiquimod; R848) recently shown to preferentially reduce the motility of X
143 chromosome-bearing mouse sperm by suppressing ATP production (Umehara *et al*, 2019) was a hit in our screen,
144 demonstrating that this phenotypic screening approach has the potential to uncover new aspects of sperm biology.

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146 In summary, our high-throughput phenotypic platform allows for the screening of bioactives including natural products,
147 focussed small molecule libraries and chemical diversity sets, which can provide novel start-points for male
148 contraceptive drug development.

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Material and Methods

Ethical approval

Written consent was obtained from each donor in accordance with the Human Fertilization and Embryology Authority (HFEA) Code of Practice (version 8) under local ethical approval (13/ES/0091) from the Tayside Committee of Medical Research Ethics B.

Development of methods for Motility and Acrosome Reaction (AR).

A pre-existing automated cell-based phenotypic screening platform was adapted that utilises a Yokogawa CV7000 Cell Voyager high-throughput microscope able to image 384 multiwell plates under full environmental control at high speed. For high throughput screening with human spermatozoa to be effective and yield meaningful hits, the system needed to replicate, as close as possible, the manual workflow currently used in an andrology lab, so that the effect of large numbers of compounds on viable human sperm could be performed. In order to achieve this goal, we investigated a number of factors that could impact the quality of the data such as: plate type; concentration and number of spermatozoa per well; logistics of spermatozoa preparation and handling; dispense speed for getting spermatozoa into 384 well plates; timing of compound addition; temperature control; image acquisition parameters; algorithm choice for motility assessment and data management and, evaluating the overall speed of data analysis. A flow cytometry-based assay and data analysis workflow was developed to measure acrosomal status. The goal was to be able to screen a 384-well plate in both assays consecutively within 90 minutes of sperm dispensing using a fully automatic robotic system. Data was analysed for inter- and intra-plate variation (both technical and biological replicates) and signal-to-background and variance measured to demonstrate the assay was reproducible and robust. The assay was shown to be scalable and all the workflows optimal. Once established, the system was validated by screening the ReFRAME library (Janes *et al.*, 2018) and hits confirmed by dose-response analysis.

Sperm handling

Control semen samples were obtained from volunteer donors with normal sperm concentration, motility and semen characteristics (WHO, 2010) and no known fertility problems. Samples were obtained by masturbation after 48–72 h of sexual abstinence. After ~30 min of liquefaction at 37°C, cells were prepared using a discontinuous density gradient procedure (DGC). Semen was loaded on top of a 40%-80% suspension of Percoll (Sigma Aldrich, UK) diluted with non-capacitation medium using Minimal Essential Medium Eagle, supplemented with HEPES, Sodium lactate and Sodium Pyruvate to achieve a similar buffer as described previously (Tardif *et al.*, 2014). We routinely pooled samples from 3-5 donors for use in each screening batch run in order to reduce donor-to-donor variability. Samples were obtained and analysed in line with suggested guidance for human semen studies where appropriate (Björndahl *et al.*, 2016).

Motility assay

Sperm cells were incubated for 3 hours at 37°C after DGC, transferred to the robotic platform (HighRes Biosolutions

Inc.) and maintained at 37°C with gentle stirring (100 rpm) in a water bath on top of a magnetic stirrer. Approximately 10,000 spermatozoa were dispensed per well using a MultiDrop Combi (ThermoFisher) into pre-warmed assay-ready plates. Spermatozoa were incubated with the compounds for 10 mins (thus favouring fast acting compounds) at 37°C in the CV7000 microscope prior to the commencement of imaging.

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191 Time-lapse Imaging

192 A CV7000 Cell Voyager high-content imaging system (Yokogawa) was used as it allowed full environmental control, 193 sufficient contrast using simple brightfield optics and a fast acquisition rate (up to 45 frames per second). Using a 20x 194 lens with bright field illumination (0.11 ms exposure, 3% lamp power), time-lapse image series were acquired (24 frame 195 in 0.5 sec) at two positions per well (with a 400 µm gap between positions to eliminate double counting of sperm). 196 Image acquisition across a 384-well plate with such settings takes ~17 minutes.

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198 Sperm Tracking

199 A Python implementation of the particle tracking algorithm originally developed by others (Crocker and Grier, 1996) 200 was employed (Trackpy v0.4.1 Allan *et al.*, 2018). The algorithm determines the position of every sperm head in each 201 frame and links the position over time creating tracks. We optimized the algorithm parameters for our imaging data to 202 exclude sperm cross-tracks and avoid detecting non-sperm particles. The image data (~17,000 files amounting to 21GB 203 per plate) can be processed within 30 minutes using a standard desktop PC (Intel® Core™ i5-6600 CPU, 3.3 GHz, 8 Gb 204 RAM) and can also be parallelized on a compute cluster with minimal effort.

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206 Acrosome assay

207 A flow cytometry-based assay was developed employing an iQue Screener (Sartorius) with Alexa488-conjugated peanut 208 agglutinin (PNA) (Mortimer et al., 1987) and general sperm viability (using propidium iodide, Pi). This assay was run 209 directly after motility assessment. Controls (DMSO and the Ca²⁺ ionophore A23187) were added using an acoustic 210 dispenser (Echo 555, Labcyte Inc.) and incubated for 10 minutes at 37°C in the SteriStore (HighRes Biosolutions Inc.). 211 PNA488 (ThermoFisher Scientific, Cat. No. L21409, stored as 1 mg/mL stock) was then added to achieve a 1:1000 final 212 dilution and propidium iodide (ThermoFisher Scientific, Live/Dead Sperm Viability kit, Cat. No. L7011, stored as 2.4 mM 213 solution) was added to achieve a 1:2000 final dilution using a liquid handler (Tempest, Formulatrix). After addition of 214 dyes, plates were incubated for 10 minutes at 37°C in the SteriStore before sampling using an iQue Screener (2 sec sip 215 time per well with pump setting of 45 rpm). This resulted in a sample processing time of about 25 minutes per 384-well 216 plate. Cells were categorised as either: unstained cells; “dead” cells (Pi+ PNA-); Acrosome-reacted but “dead” cells (Pi+ 217 PNA+); or Acrosome-reacted “live” cells (Pi- PNA+). This method is capable of detecting a shift of populations (Pi+ and 218 PNA+) upon induction of AR using A23187. The AR assay was performed as an agonist screen (scoring for induction of 219 AR in live cells compared to DMSO controls) with the expectation that compounds could be found that induce AR beyond 220 levels of induction with A23187.

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222 Controls and QC criteria

223 For the motility assay DMSO was used as a vehicle control (negative control) and Pristimerin (Merck, Cat. No. 530070,
224 stored as 10 mM stock in DMSO; final concentration of 20 μ M) as a positive control. For the acrosome assay we added
225 a calcium ionophore (A23187, Sigma-Aldrich, Cat. No. C7522, stored as 10 mM stock in DMSO, used at a final
226 concentration of 10 μ M) as a positive control and DMSO as a negative control. We calculate a Z'-factor (Z'; Zhang, 1999)
227 for each plate using positive and negative controls for the motility assay and observed Z'-factors ranging between 0.4-
228 0.8. In addition we performed a visual check of heatmaps for every plate to detect edge effects.

230 Data analysis and normalization

231 **Motility:** custom R scripts were written to calculate standard sperm kinetic parameters (Mortimer *et al.*, 2015). Those
232 parameters allow classification of sperm into standard WHO classes: progressively motile (PM) (where VAP > 25 μ m/sec
233 AND STR > 80%); non-progressively motile (NPM) (where VAP > 5 μ m/sec OR VSL > 11 μ m/sec), and immotile (IM). In
234 addition to calculating kinetic parameters, we established a workflow to generate movies of time-lapse videos with
235 overlapping sperm tracks using R and FFMPEG (FFmpeg Developers. Available from <http://ffmpeg.org>). We expressed
236 results as % of control VCL. This was defined as VCL_median (cpd)/VCL_median(DMSO) * 100. Hit selection criteria was
237 15% reduction of VCL. VCL was chosen as the main kinetic measurement as it had an acceptable Z' value and is
238 independent of path averaging. Wells with autofocus errors or compounds which did not dissolve properly (resulting in
239 sperm cells being stuck in one location but moving normally in the rest of the well) were excluded from analysis.

240 **Acrosome:** iQue Screener Data was exported as FCS format and processed using the following *Bioconductor* packages
241 for analysing flow cytometry data: flowCore, flowDensity, flowWorkspace, ggcyto (see <https://bioconductor.org>). These
242 packages allow handling flow cytometry data as objects, which can then be compensated, gated and visualized
243 efficiently. In addition we use HDF5 format (www.hdfgroup.org) for storing processed and averaged data. Percent
244 events for each population (Pi+ PNA+, Pi- PNA+, Pi+ PNA- and Pi- PNA-), normalized to total well events, were calculated.
245 Hits were defined as compounds which induce AR beyond 15% (maximum level of induction achieved with positive
246 control). Wells with irregularities (low in Pi+ or Pi+PNA+ population, or below 200 events) have been excluded from
247 analysis.

248 Compound Screening

249 We screened the ~12,000 compound ReFRAME (Repurposing, Focused Rescue, and Accelerated Medchem) library
250 (Janes *et al.*, 2018; and www.reframedb.org) supplied by CALIBR at the Scripps Institute. This unique library consists of
251 bioactives and approved drugs that have been assembled from the literature, drug databases and by patent mining.
252 Compounds were spotted (12.5 nL, final assay concentration ~6 μ M) into 384-well black-sided optical imaging plates
253 (CellCarrier, PerkinElmer) at CALIBR and were shipped on dry ice to the screening centre in Dundee and stored at -20°C
254 until required. Immediately prior to screening plates were thawed, controls were added using the acoustic dispenser
255 (Echo 555, Labcyte), and 10 μ L warm media added followed by shaking on a plate shaker for 10 seconds. Plates were
256 then incubated for 30 min at 37°C to solubilise the compounds and prewarm the plates prior to the addition of live
257 sperm.

All experiments were performed with pools of donors (3-5 per batch of 4 screening plates) and results normalised to DMSO-treated wells (16 wells per plate).

The ReFrame library has been screened for cytotox (using CellTiterGlo) in HEK293 and HepG2 cells (see www.reframedb.org) with some of the hit compounds displaying activity in one or both assays (Supplementary Figure 2), information which helps inform compound progression decisions.

Dose response experiments

Assay-ready 384-well CellCarrier plates with 8-point curves (10 μ M highest concentration) were resupplied by CALIBR in duplicate and processed as described above. Curves were fitted using the R package *dr4pl* (<https://cran.r-project.org/web/packages/dr4pl/index.html>) using a 4-parameter logistic fit option.

Triaging of AR hits.

Given the prevalence of a range of interfering compounds amongst the library hits, two approaches were used for triaging. These additional triaging steps, after the primary screen, allowed for the elimination of intrinsically fluorescent compounds and compounds fluorescing in the presence of biological material. Screening plates were excited at 488 nm and read at 520 nm and 670 nm in a M1000Pro multimode plate reader (Tecan) after flow cytometry. In addition, when performing dose-response curve follow up on resupply material a replicate set of compound-dosed cells were processed with no-dyes added. This identified the small number of compounds whose fluorescence is indistinguishable from a true positive. In addition, visual confirmation of the appropriate pattern of acrosome staining was performed by microscopy.

Figure Legends

Figure 1

Phenotypic Assay workflows

(A) Graphical summary of modular screening workflow where motility measurement is followed by acrosome reaction (AR) measurement allowing a screening throughput of >1400 compounds per donor pool (B) Steps in imaging and analysis: human sperm are recorded with brightfield illumination (first panel) then sperm heads detected using a particle tracking algorithm (second panel) which are then tracked across the timelapse series of images (third panel) and subsequently classified (fourth panel). Each panel contains a zoomed-in subsection of the field. Colour coding for tracking distance and kinetic classification is shown in the panel insets: *Tracking* panel - rainbow gradient (showing progression over time); *Classification* panel - red for immotile (IM), yellow for non-progressively motile (NPM) and blue for progressively motile (PM). Scale bars: 100 μ m (main images), 5 μ m (insets). (C) Sperm counts per well after microscopy and detection shown in a combined violin/box plot. Colors: purple violin outline (probability density of values), yellow dots (outliers of boxplot). (D) Graphical summary of the expected populations determined by flow cytometry based on distribution of cells measured with FL3-A (Pi) vertical axis and FL1-A (PNA-488) horizontal axis: dead cells (upper left, Pi+ PNA-); dead and acrosome-reacted (upper right, Pi+ PNA+); unstained/live/non-reacted (lower left)

and live acrosome-reacted (lower right, Pi-PNA+). (E) Example flow cytometry data comparing sperm treated with the Ca^{2+} ionophore (A23187) which induces AR (left panel) with sperm from DMSO-treated well (right panel). Colors indicate event density. (F) Combined violin/box plot data showing flow cytometry event counts per well. Colors and label as in (C).

Supplementary Figure 1

Further characterisation of phenotypic assays

(A) Example data for a single sperm track with x and y coordinates being used to calculate standard sperm kinetics. Color represents the cumulative distance travelled in microns. Acronyms: VCL (curvilinear velocity), ALH (amplitude of lateral head displacement; maximum value), STR (straightness ratio). (B) Comparison of data from the standard computer-assisted semen analysis (CASA) with the high-throughput system using a Bland-Altman plot of VAP, VCL, and VSL. Colors: turquoise line (mean of differences), dashed purple lines (limit of agreement, mean of differences $\pm 1.96 * \text{SD}$). (C) Effect of DMSO on sperm motility (VCL) relative to untreated wells. (D) Effect of pre-dispense incubation time on sperm motility (% reduction in VCL). Colors: green line (arbitrary 10% cut-off). (E) Plots of FL3-A vs FL1-A for the flow cytometry assay controls using acrosome specific PNA-488 dye (FL-1) and cell viability marker propidium iodide (Pi, FL-3) upon addition of A23187 Ca-ionophore. Panels from left to right: with ionophore and acrosome stain; with ionophore and propidium iodide only, with ionophore but unstained. Colors: red rectangles show population gates; colored hexagons show event density (bin size = 40).

Supplementary Figure 1.1

Screening consistency over time analysis

(A) Distribution of average path velocities (VAP) for sperm motility classes (IM = immotile; NPM = non-progressively motile; PM = progressively motile), using data from two positions within each well (Position 1, Position 2), represented as combined box/violin plot. Each box/violin plot is a summary of a screening day ($n = 4$, 384-well plates). Colors: each screening day is represented as a different color; grey = boxplot outliers. (B) Distribution of numbers of tracked sperm per well across screening days, represented as combined box/violin plot. Each box/violin plot is a summary of one screening plate. Colors as in (A). (C) Distribution of events identified in the flow cytometry assay, represented as combined box/violin plots. Each box/violin plot is a summary of a screening plate. Colors as in (A).

Figure 2

ReFRAME Library Screening: Motility Assay Results

(A) Primary screening results of the motility assay. Each dot represents a well (either compound or control well) showing % of DMSO control (VCL). Positive controls (Pristimerin), negative controls (DMSO) and individual compound datapoints are shown. The dashed line showing the 15% of control (reduction in VCL) – the cut-off for primary hit selection. Total number of compounds = 11,903, excluding wells with auto-focus errors and with “sticky” compounds which have been excluded from analysis. See Figure_2_Source_Data.H5 along with Figure_2_Code.R. (B) Assay robustness metrics: Z'-factor values for all screening plates is shown. Dashed lines indicate min/max Z' values. (C) Tracking data visualizations of 3 example wells showing sperm tracks of both imaging positions (Position 1 [Pos. 1] and Position 2 [Pos. 2] respectively) within the wells. A DMSO control well (left panels “Control”) shows a large number of progressively motile (PM) sperm (yellow) with few non-progressively motile (NPM) sperm (green and very few immotile

(IM) sperm (purple) – this is in contrast to the shorter tracks and higher levels of NPM and IM in the middle panel (“20% reduction”) for a compound that shows 20% inhibition of motility (i.e. 80% of control) and the right hand panel (“80% reduction”) for a compound showing 80% inhibition (i.e. 20% of control), showing almost all cells are in the IM and NPM classes. (D) Histogram of sperm tracks quantification of the data show in (C). (E) Summary of motility assay hit rate (0.5%) and reconfirmation rate (0.24%). (F-G) Dose response confirmation of two hits. 8-point 3-fold dilution curves are shown with 10 μ M as the highest concentration. two data points per concentration (n=2, data point is mean $\pm$ SD). Each curve is a 4-parameter logistic fit. Each plot shows estimated values Hill Slope and EC₅₀. The chemical structure of the hit compound is shown as well as some annotation and physicochemical properties. Physicochemical properties were calculated using RDKit, Python and KNIME: SlogP = partition coefficient (Wildman and Crippen, 1999); TPSA is the Topological Polar Surface Area (Ertl *et al.*, 2000); MW is the exact Molecular weight; QED= Quantitative Estimate of Drug-likeness (Bickerton *et al* 2012).

Supplementary Figure 2

Dose response and additional data for primary motility hits

Compound names and nominal target/functional annotation shown on left along with chemical structure and physicochemical properties. The 8-point dose response curves for each hit with estimated Hill slope, EC₅₀ and Efficacy [% max reduction] are shown on the right. Two data points per concentration (n=2); data points are Mean $\pm$ SD. A 4-parameter logistic fit has been performed using R package: *dr4pl*.

Calculated EC₅₀ values and Efficacy (% max effect) in the motility assay are shown in the right hand section. CC₅₀ values for HEK293 and HepG2 CellTiter-Glo cytotoxicity assays performed by CALIBR (www.reframedb.org) are shown in the right hand section. Note: 0 = inactive in cytotoxicity assay. The chemical structure of the hit compound is shown as well as some annotation and physicochemical properties (for nomenclature description see Figure 2). See Supplementary_Figure_2_Source_Data.csv along with Supplementary_Figure_2_Code.R.

Figure 3

ReFRAME Library Screening: AR Assay Results

(A) Results of primary screening of the library using the acrosome assay (live cells, acrosome reacting, Pi- PNA+ population). Each dot represents a well (either compound or control well). Shown is % Events (number of events in Pi- PNA+ gate relative to total events in the sampled well). Datapoints for compounds, negative controls (DMSO) and positive controls (A23187) are shown. Black dashed line = 15% threshold for primary hits selection. See Figure_3_Source_Data.csv along with Figure_3_Code.R. (B-C) Data from two example wells: a DMSO well (left panel) and a well with 65% Pi- PNA+ population (right panel). (D) Summary of acrosome assay results before and after triage.

Supplementary Figure 3

Dose response and additional data for primary AR hits

Compound names and nominal target/functional annotation shown on left along with chemical structure and physicochemical properties. The 8-point dose response curves for each hit with estimated Hill slope, EC₅₀ and Efficacy [% max induction] values are shown on the right. Two data points per concentration (n=2); data points are Mean $\pm$ SD. A 4-parameter logistic fit has been performed using R package: *dr4pl*. CC₅₀ values for HEK293 and HepG2 CellTiter-Glo cytotoxicity assays performed by CALIBR (www.reframedb.org) are shown in the right hand section. Note: 0 = inactive

in cytotoxicity assay. Physicochemical properties were calculated using RDKit, Python and KNIME: SlogP = partition coefficient (Wildman and Crippen, 1999); TPSA is the Topological Polar Surface Area (Ertl *et al.*, 2000); MW is the exact Molecular weight; QED= Quantitative Estimate of Drug-likeness (Bickerton *et al.* 2012). See Supplementary_Figure_3_Source_Data.csv along with Supplementary_Figure_3_Code.R.

Supplementary Figure 3.1

Further analysis of AR screening data and triaging strategy.

Results are shown of primary screening data for two of the other populations in the flow cytometry assay: **(A)** Acrosome reaction positive and propidium iodide positive (PI+ PNA+) events and **(B)** acrosome reaction negative and propidium positive (PI+ PNA-) events. Shown is % Events (number of events relative to total events per well). Datapoints for compounds, negative controls (DMSO) and positive controls (A23187) are shown. See Figure_3_Source_Data.csv along with Figure_3_Code.R. **(C)** Control experiment showing microscopy images of A23187-induced acrosome-reacted sperm. Shown are individual channels (greyscale) for acrosome signal stained with PNA-Alexa488 (green band in merged image) and DNA stained with Propidium iodide indicating lack of cell viability (purple in merged image). Scale bar = 10 μ M. **(D)** Orthogonal assay to eliminate fluorescent compounds: Two primary hits were analyzed using fluorescence microscopy without addition of staining reagents: top panels show the non-specific fluorescence of Merbromin and in bottom panels fluorescence of Manoalide which stains only significantly in the midpiece/tail. Grey scale images are shown in the left two panels and a merged image on the right. Key: green channel = Ex 488 nm with Em bandpass filter BP 525/50 nm; red channel = Ex 561 nm; Em Bandpass filter BP600/37 nm). Scale bar = 10 μ M.

Supplementary Movie 1

Movie was generated using a brightfield image sequence for a typical control well (left pane), a well where a compound reduced motility by 20% (middle pane) and one where it reduced it by 80% (right pane).

Supplementary Movie 2

Movie was generated using a brightfield image sequence for a typical control well (left pane), a well where a compound reduced motility by 20% (middle pane) and one where it reduced it by 80% (right pane). Tracking is overlayed in each panel. Colour coding is detailed in Figure 2.

Supplementary Table 1: Compounds that had a significant effect on sperm motility. Summary of dose response experiments of primary motility hits with estimated EC50 and Efficacy [% reduction] values. Information and names were provided by Calibr. See Supplementary_Table_1_Source_Data.csv.

Supplementary Table 2: Compounds that had a significant effect on Acrosome Reaction. Summary of dose response experiments of primary acrosome hits with estimated EC50 and Efficacy [% increase] values. Information and names were provided by Calibr. Note that none of these compounds passed orthogonal counter screening and are considered as assay interfering compounds/false positives. See Supplementary_Table_2_Source_Data.csv.

Source Data Files:

Figure_2_Source_Data.H5

<https://datadryad.org/stash/share/06d75FZ6GiPmme3HnKnkyTFbgKJ2mV0UVRaN-gVkoVE>.

Primary screening data motility assay

Figure_3_Source_Data.csv

Primary screening data acrosome assay

Supplementary_Figure_2_Source_Data.csv

Dose response confirmation data motility assay

Supplementary_Figure_3_Source_Data.csv

Dose response confirmation data acrosome assay

Supplementary_Table_1_Source_Data.csv

Data of Supplementary Table 1

Supplementary_Table_2_Source_Data.csv

Data of Supplementary Table 2

Supplementary Files:

Figure_2_Code.R

R Code for Figure 2 primary motility assay

Figure_3_Code.R

R Code for Figure 2 primary acrosome assay

Supplementary_Figure_2_Code.R

R Code for Supplementary Figure 2 dose response confirmation motility assay

Supplementary_Figure_3_Code.R

R Code for Supplementary Figure 3 dose response confirmation acrosome assay

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Authors' roles

518 FG performed the sperm preparation, HTS screening and analysis of the data. CLRB and PDA designed the study, assisted
 519 with interpretation of the data and obtained funding. All authors contributed to the construction, writing, and analysis
 520 of data. All authors approved the final manuscript.

521

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532

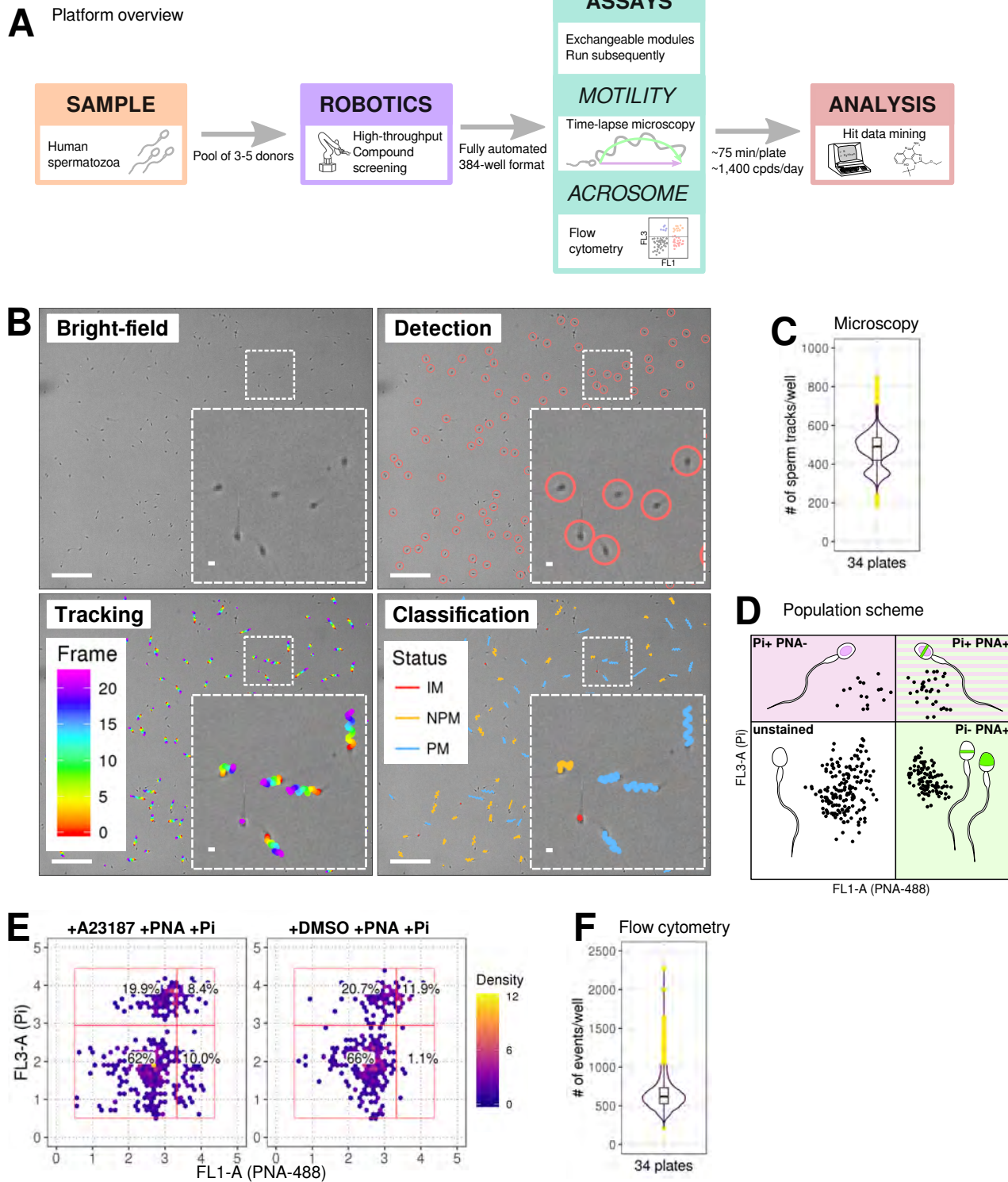
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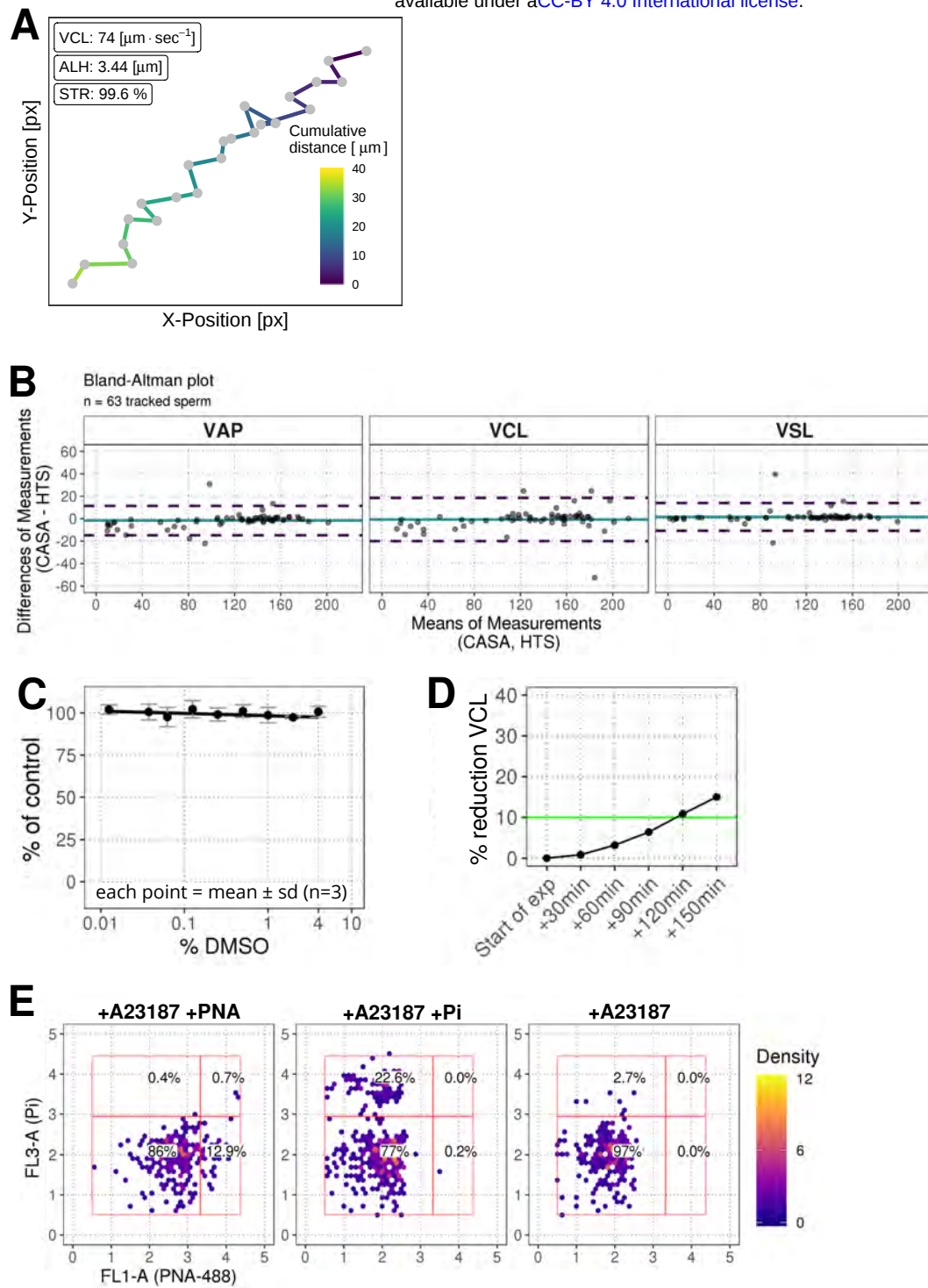
536 Council and Scottish Universities Life Science Alliance. PDA/NPSC receives funding from Janssen Pharmaceutica NV.
537
538 **Conflict of interest**
539 CLRB is Editor for RBMO, has received lecturing fees from Merck, Pharmasure and Ferring and is on the Scientific
540 Advisory Panel for Ohana BioSciences. PDA, FG had no COI.

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Figure 1



Supplementary Figure 1



Supplement Figure 1.1

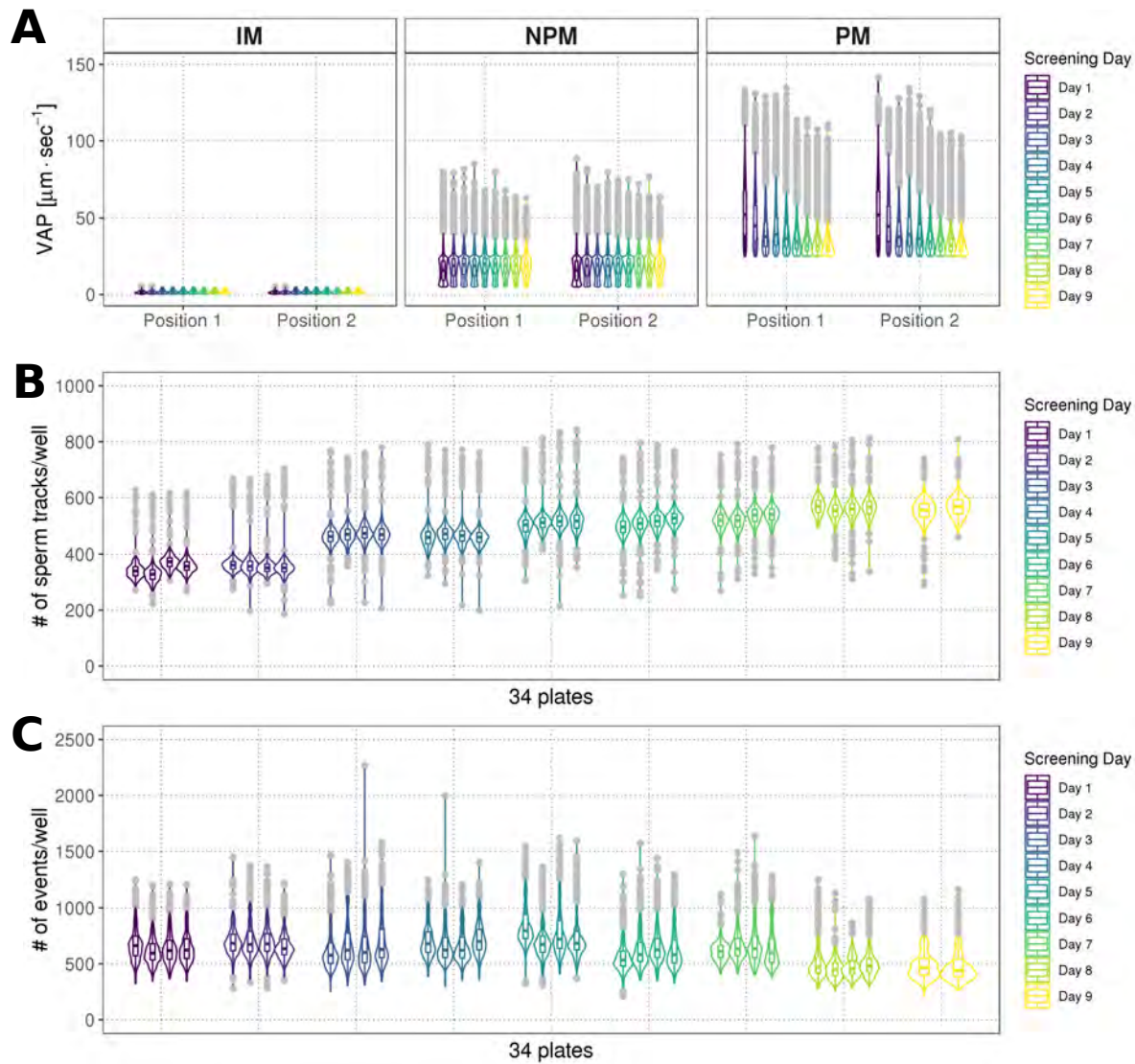
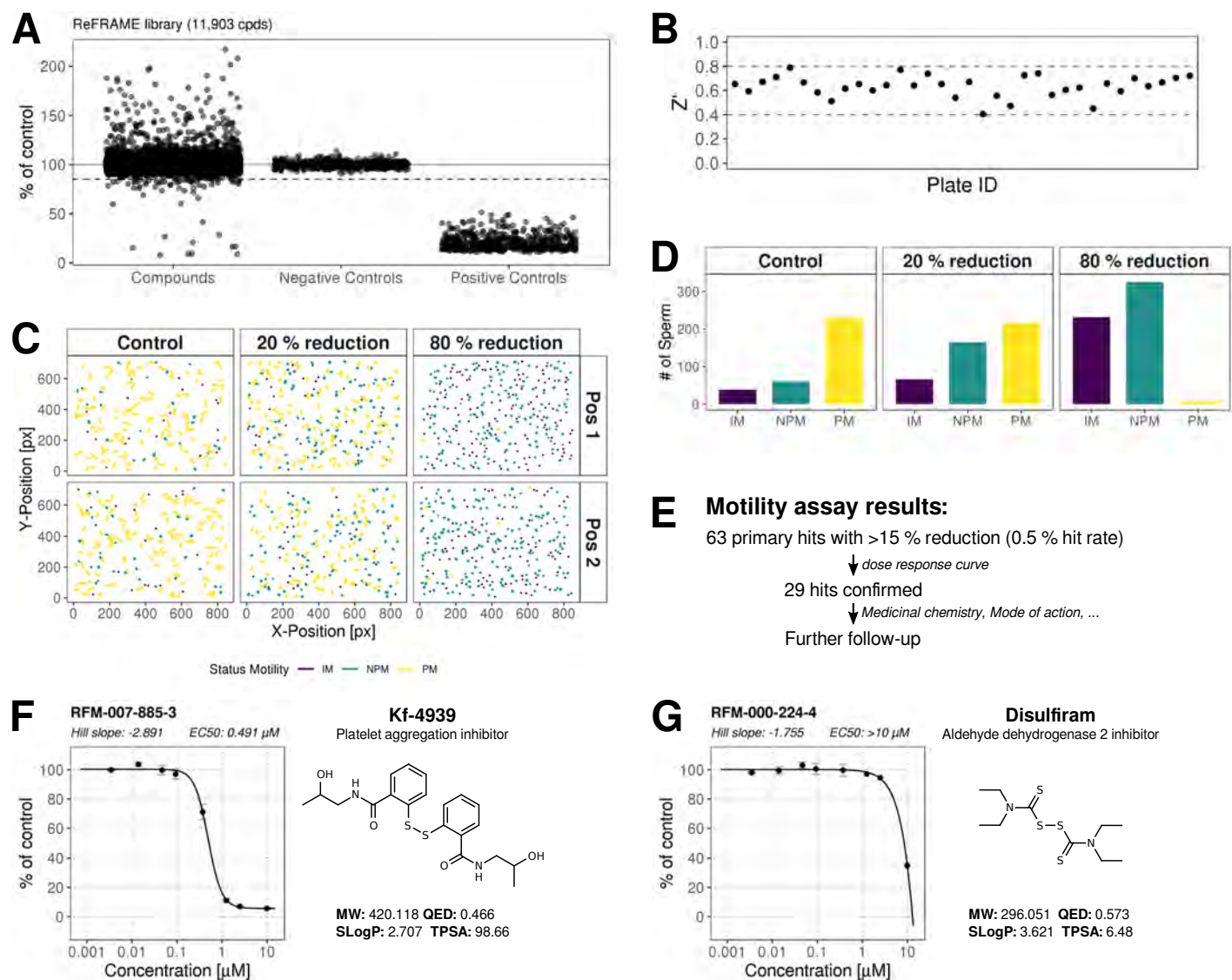


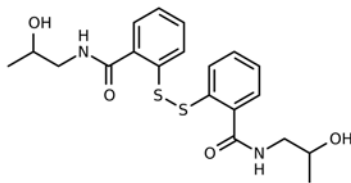
Figure 2



Supplementary Figure 2

Kf-4939

Platelet aggregation inhibitor



MW (RDKit)
420.118

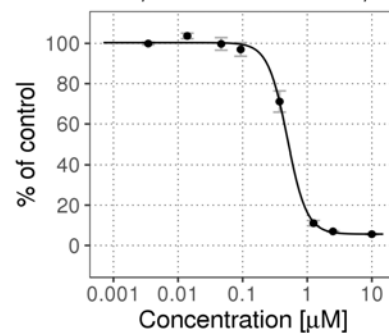
QED (RDKit)
0.466

SlogP (RDKit)
2.707

TPSA (RDKit)
98.66

RFM-007-885-3

Hill slope: -2.891 EC50: 0.491 μ M



EC50 [μ M]
0.49

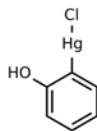
Efficacy
[% reduction]
100

Hek CC50 [μ M]
2.526

HepG2 CC50 [μ M]
0

Mercufenol Chloride

Antiseptic



MW (RDKit)
329.974

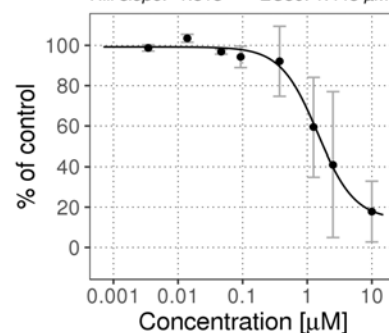
QED (RDKit)
0.769

SlogP (RDKit)
1.254

TPSA (RDKit)
20.23

RFM-006-841-7

Hill slope: -1.518 EC50: 1.448 μ M



EC50 [μ M]
1.45

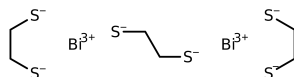
Efficacy
[% reduction]
98

Hek CC50 [μ M]
0.595

HepG2 CC50 [μ M]
2.721

Bismuth Ethanedithiol

Unidentified pharmacological action



MW (RDKit)
392.932

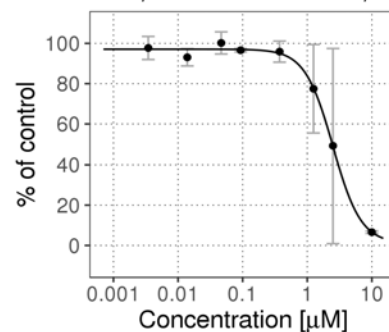
QED (RDKit)
0.58

SlogP (RDKit)
2.382

TPSA (RDKit)
0

RFM-011-751-1

Hill slope: -1.998 EC50: 2.499 μ M



EC50 [μ M]
2.5

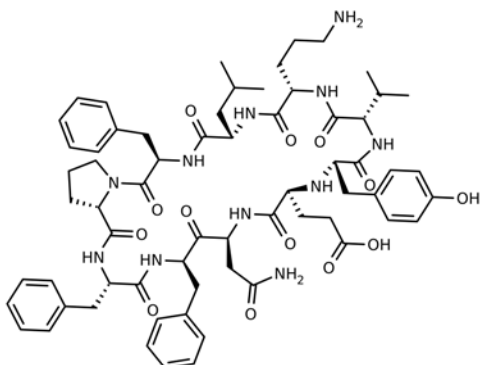
Efficacy
[% reduction]
98

Hek CC50 [μ M]
2.716

HepG2 CC50 [μ M]
1.141

Tyrothricin

Antibiotic



MW (RDKit)
1227.633

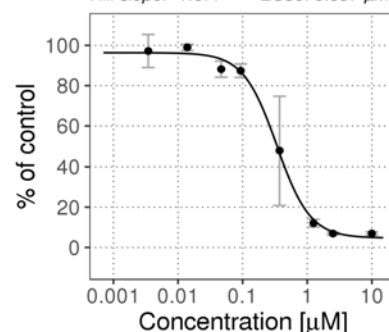
QED (RDKit)
0.063

SlogP (RDKit)
1.138

TPSA (RDKit)
379.75

RFM-011-941-5

Hill slope: -1.677 EC50: 0.337 μ M



EC50 [μ M]
0.34

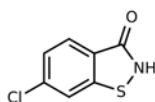
Efficacy
[% reduction]
97

Hek CC50 [μ M]
1.791

HepG2 CC50 [μ M]
0

Ticlatone

Antibiotic



MW (RDKit)

184.97

QED (RDKit)

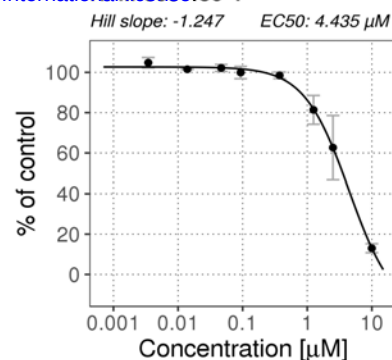
0.671

SlogP (RDKit)

2.243

TPSA (RDKit)

32.86



EC50 [μM]

4.43

Efficacy

[% reduction]

96

Hek CC50 [μM]

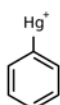
2.259

HepG2 CC50 [μM]

0

Phenylmercuric Borate

Antiseptic



MW (RDKit)

279.009

QED (RDKit)

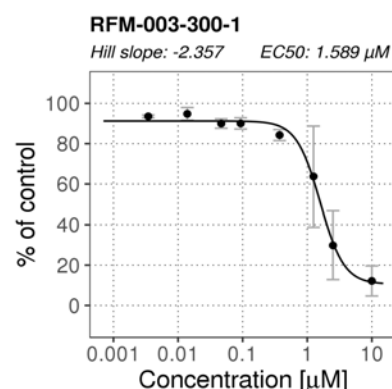
0.617

SlogP (RDKit)

0.859

TPSA (RDKit)

0



EC50 [μM]

1.59

Efficacy

[% reduction]

91

Hek CC50 [μM]

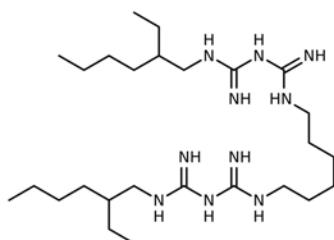
0.687

HepG2 CC50 [μM]

0

Alexidine

Phospholipase inhibitor



MW (RDKit)

508.469

QED (RDKit)

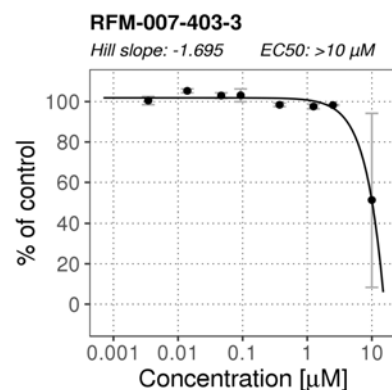
0.071

SlogP (RDKit)

4.256

TPSA (RDKit)

167.58



EC50 [μM]

> 10

Efficacy

[% reduction]

86

Hek CC50 [μM]

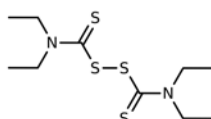
1.453

HepG2 CC50 [μM]

1.506

Disulfiram

Aldehyde dehydrogenase 2 inhibitor



MW (RDKit)

296.051

QED (RDKit)

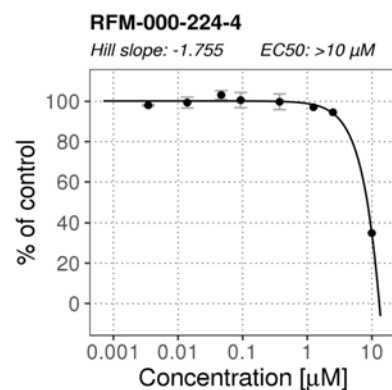
0.573

SlogP (RDKit)

3.621

TPSA (RDKit)

6.48



EC50 [μM]

> 10

Efficacy

[% reduction]

70

Hek CC50 [μM]

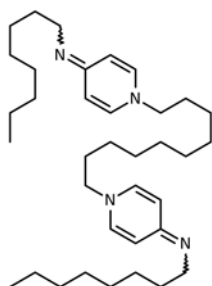
7.159

HepG2 CC50 [μM]

0

Octenidine

Antibacterial



MW (RDKit)

550.497

QED (RDKit)

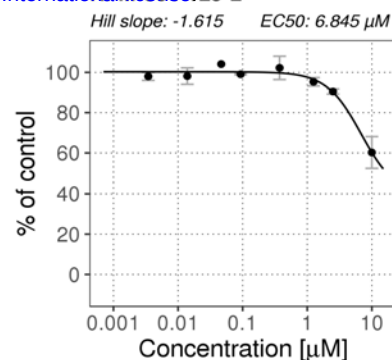
0.11

SlogP (RDKit)

9.633

TPSA (RDKit)

34.58



EC50 [μM]

6.85

Efficacy

[% reduction]

54

Hek CC50 [μM]

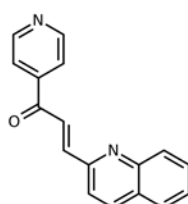
0.798

HepG2 CC50 [μM]

1.763

Pfk-015

PFKFB3 inhibitor



MW (RDKit)

260.095

QED (RDKit)

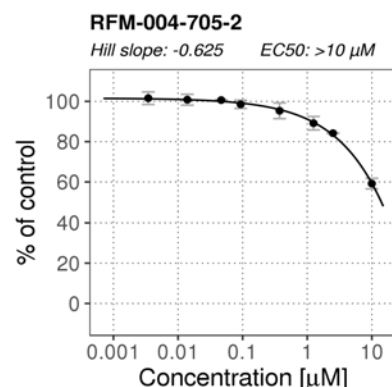
0.534

SlogP (RDKit)

3.526

TPSA (RDKit)

42.85



EC50 [μM]

> 10

Efficacy

[% reduction]

47

Hek CC50 [μM]

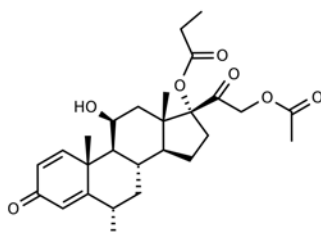
1.382

HepG2 CC50 [μM]

0.95

Methylprednisolone Aceponate

Antiinflammatory



MW (RDKit)

472.246

QED (RDKit)

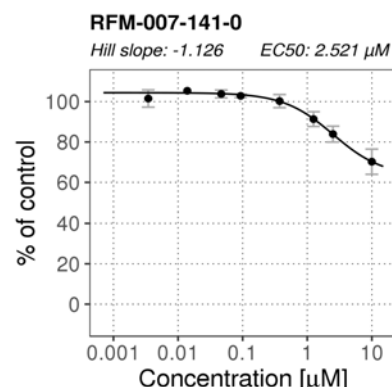
0.612

SlogP (RDKit)

3.335

TPSA (RDKit)

106.97



EC50 [μM]

2.52

Efficacy

[% reduction]

45

Hek CC50 [μM]

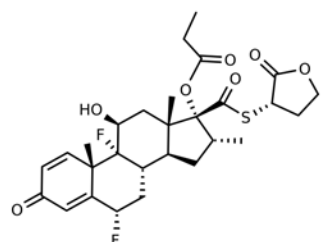
0

HepG2 CC50 [μM]

0

Zoticasone Propionate

Glucocorticoid receptor agonist



MW (RDKit)

552.199

QED (RDKit)

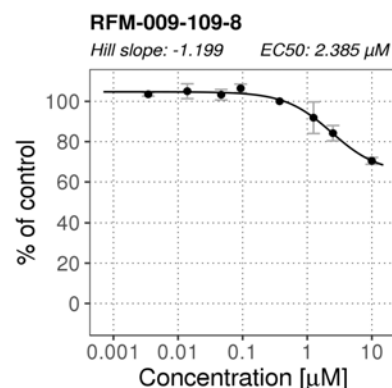
0.525

SlogP (RDKit)

3.819

TPSA (RDKit)

106.97



EC50 [μM]

2.38

Efficacy

[% reduction]

39

Hek CC50 [μM]

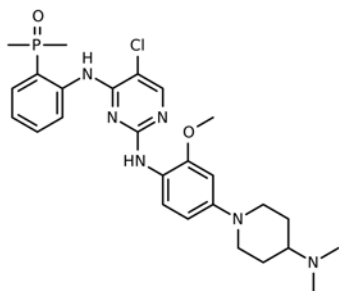
0

HepG2 CC50 [μM]

0

Brigatinib

ALK receptor tyrosine kinase inhibitor

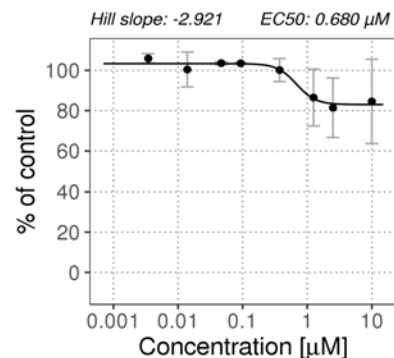


MW (RDKit)
528.217

QED (RDKit)
0.376

SlogP (RDKit)
5.404

TPSA (RDKit)
82.62



EC50 [μM]
0.68

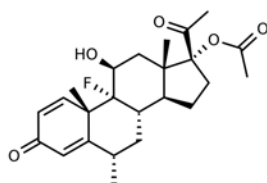
Efficacy
[% reduction]
38

Hek CC50 [μM]
1.867

HepG2 CC50 [μM]
0.136

Fluorometholone Acetate

Antiinflammatory

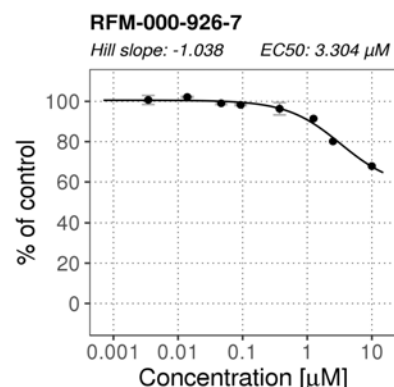


MW (RDKit)
418.216

QED (RDKit)
0.695

SlogP (RDKit)
3.494

TPSA (RDKit)
80.67



EC50 [μM]
3.3

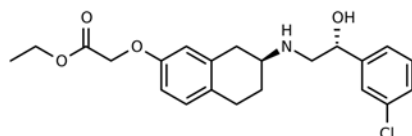
Efficacy
[% reduction]
35

Hek CC50 [μM]
0

HepG2 CC50 [μM]
0

Amibegron Hydrochloride

beta3-Adrenoceptor Agonist

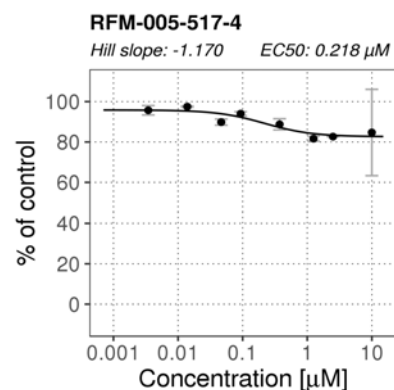


MW (RDKit)
403.155

QED (RDKit)
0.66

SlogP (RDKit)
3.462

TPSA (RDKit)
67.79



EC50 [μM]
0.22

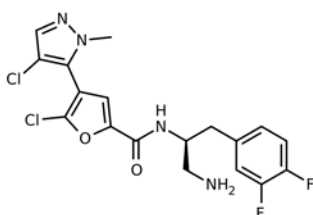
Efficacy
[% reduction]
31

Hek CC50 [μM]
0

HepG2 CC50 [μM]
0

Uprosertib

PKB alpha/Akt1 inhibitor

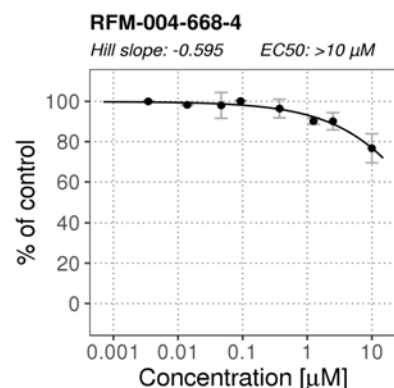


MW (RDKit)
428.062

QED (RDKit)
0.628

SlogP (RDKit)
3.565

TPSA (RDKit)
86.08



EC50 [μM]
> 10

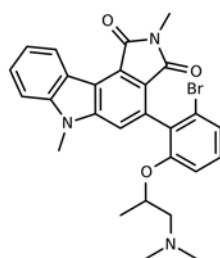
Efficacy
[% reduction]
31

Hek CC50 [μM]
0

HepG2 CC50 [μM]
0

Kf-41399

Chemoprotective agent

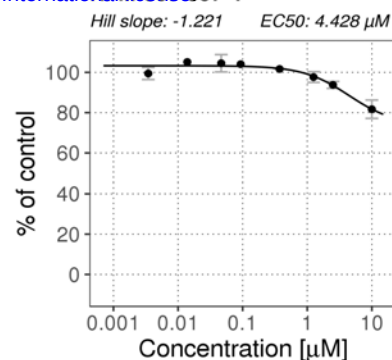


MW (RDKit)
519.116

QED (RDKit)
0.335

SlogP (RDKit)
5.316

TPSA (RDKit)
54.78



EC50 [μM]
4.43

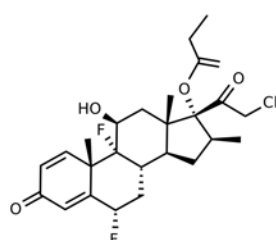
Efficacy [% reduction]
31

Hek CC50 [μM]
0

HepG2 CC50 [μM]
0

Halobetasol Propionate

Antisporiatic

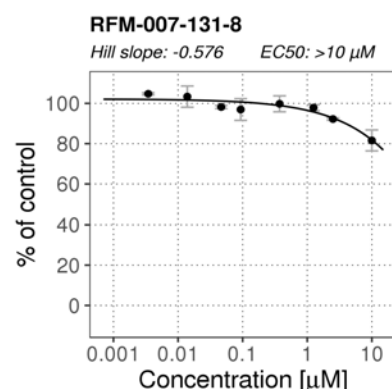


MW (RDKit)
484.183

QED (RDKit)
0.481

SlogP (RDKit)
4.051

TPSA (RDKit)
80.67



EC50 [μM]
> 10

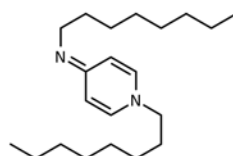
Efficacy [% reduction]
30

Hek CC50 [μM]
0

HepG2 CC50 [μM]
0

Pirtenidine

Antibiotic

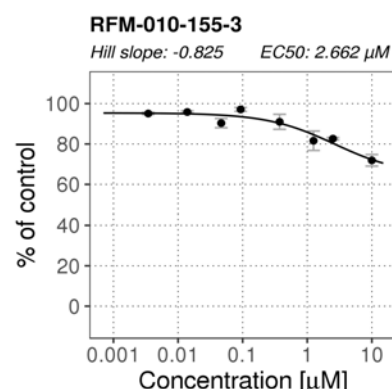


MW (RDKit)
318.303

QED (RDKit)
0.368

SlogP (RDKit)
6.11

TPSA (RDKit)
17.29



EC50 [μM]
2.66

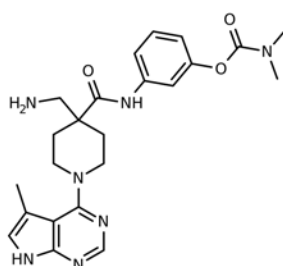
Efficacy [% reduction]
28

Hek CC50 [μM]
1.476

HepG2 CC50 [μM]
0

Lx-7101

LIM Domain Kinase inhibitor

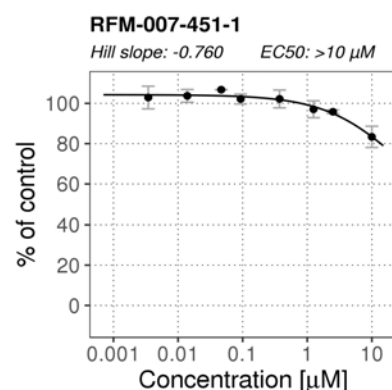


MW (RDKit)
451.233

QED (RDKit)
0.543

SlogP (RDKit)
2.511

TPSA (RDKit)
129.47



EC50 [μM]
> 10

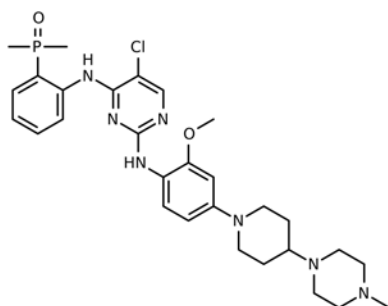
Efficacy [% reduction]
28

Hek CC50 [μM]
0

HepG2 CC50 [μM]
0

Ap26113-analog

ALK receptor tyrosine kinase inhibitor

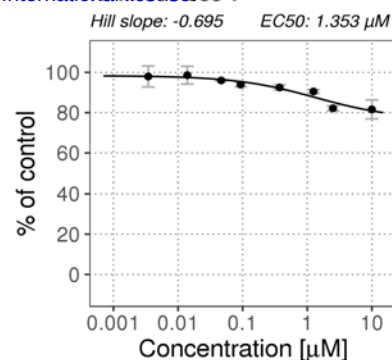


MW (RDKit)
528.217

QED (RDKit)
0.376

SlogP (RDKit)
5.404

TPSA (RDKit)
82.62



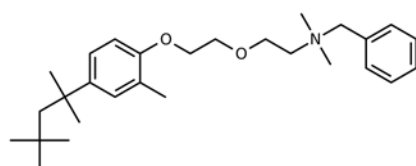
EC50 [μM]
1.35

Efficacy
[% reduction]
24

Hek CC50 [μM]
0

HepG2 CC50 [μM]
0.13

Methylbenzethonium Chloride Antiseptic

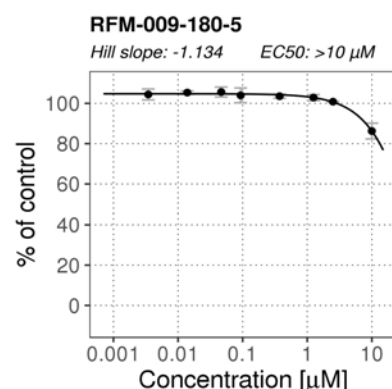


MW (RDKit)
426.337

QED (RDKit)
0.306

SlogP (RDKit)
6.381

TPSA (RDKit)
18.46



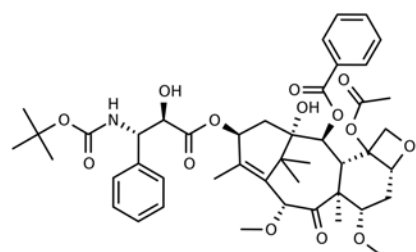
EC50 [μM]
> 10

Efficacy
[% reduction]
24

Hek CC50 [μM]
3.65

HepG2 CC50 [μM]
7.586

Cabazitaxel Tubulin inhibitor

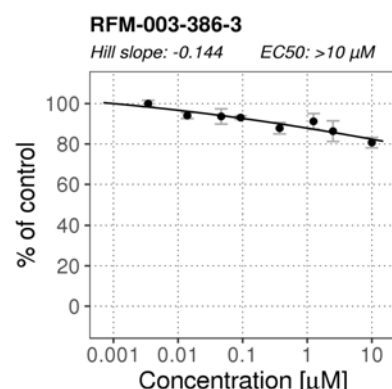


MW (RDKit)
835.378

QED (RDKit)
0.171

SlogP (RDKit)
4.568

TPSA (RDKit)
202.45



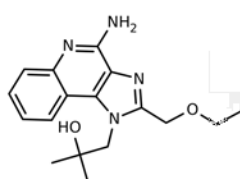
EC50 [μM]
> 10

Efficacy
[% reduction]
23

Hek CC50 [μM]
0

HepG2 CC50 [μM]
0

Resiquimod Immunostimulant

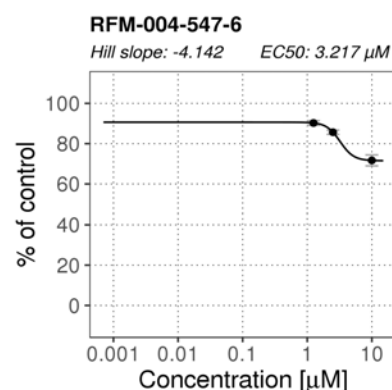


MW (RDKit)
314.174

QED (RDKit)
0.755

SlogP (RDKit)
2.474

TPSA (RDKit)
86.19



EC50 [μM]
3.22

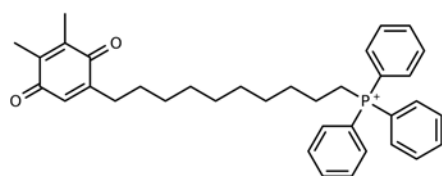
Efficacy
[% reduction]
22

Hek CC50 [μM]
0

HepG2 CC50 [μM]
0

Skq1

Neurologic drug

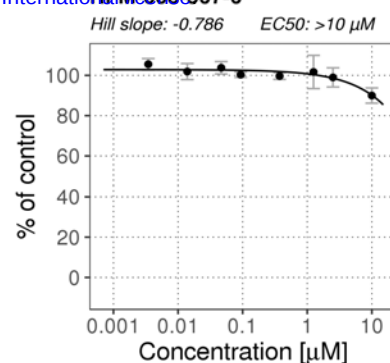


MW (RDKit)
537.292

QED (RDKit)
0.119

SlogP (RDKit)
7.906

TPSA (RDKit)
34.14



EC50 [μM]
> 10

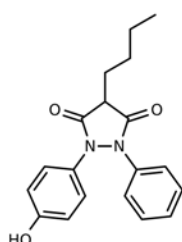
Efficacy
[% reduction]
21

Hek CC50 [μM]
0.778

HepG2 CC50 [μM]
4.41

Oxyphenbutazone

Treatment of Gout

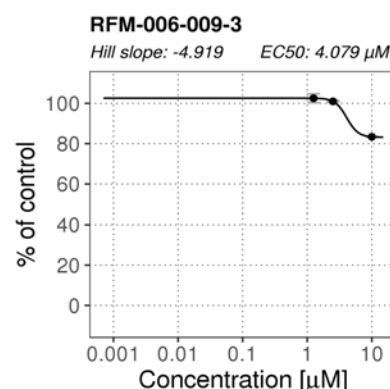


MW (RDKit)
324.147

QED (RDKit)
0.856

SlogP (RDKit)
3.493

TPSA (RDKit)
60.85



EC50 [μM]
4.08

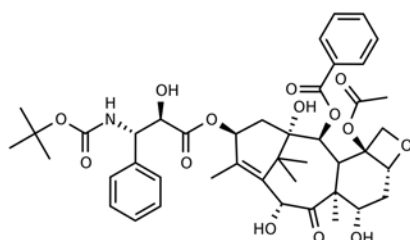
Efficacy
[% reduction]
21

Hek CC50 [μM]
1.572

HepG2 CC50 [μM]
0

Docetaxel

Tubulin inhibitor

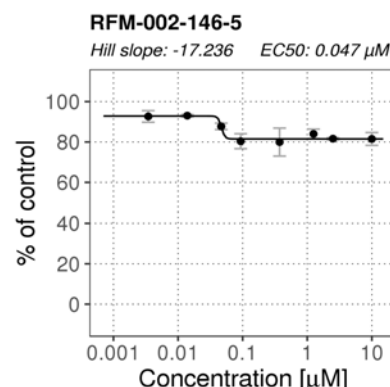


MW (RDKit)
807.347

QED (RDKit)
0.147

SlogP (RDKit)
3.26

TPSA (RDKit)
224.45



EC50 [μM]
0.05

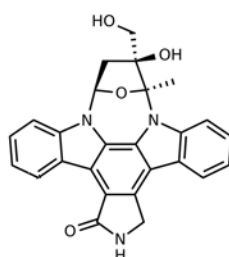
Efficacy
[% reduction]
20

Hek CC50 [μM]
0

HepG2 CC50 [μM]
0

Lestaurtinib

Flt3 inhibitor

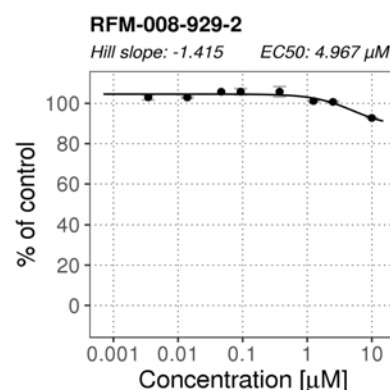


MW (RDKit)
439.153

QED (RDKit)
0.373

SlogP (RDKit)
3.474

TPSA (RDKit)
88.65



EC50 [μM]
4.97

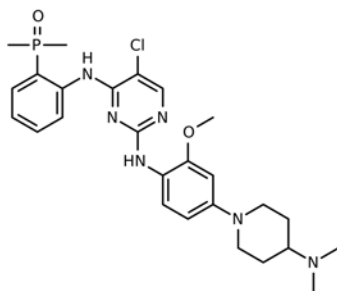
Efficacy
[% reduction]
17

Hek CC50 [μM]
0.551

HepG2 CC50 [μM]
0.029

Brigatinib

ALK receptor tyrosine kinase inhibitor



MW (RDKit)

583.259

QED (RDKit)

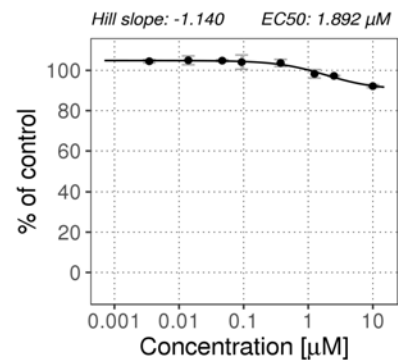
0.352

SlogP (RDKit)

5.09

TPSA (RDKit)

85.86



EC50 [μM]

1.89

Efficacy

[% reduction]

15

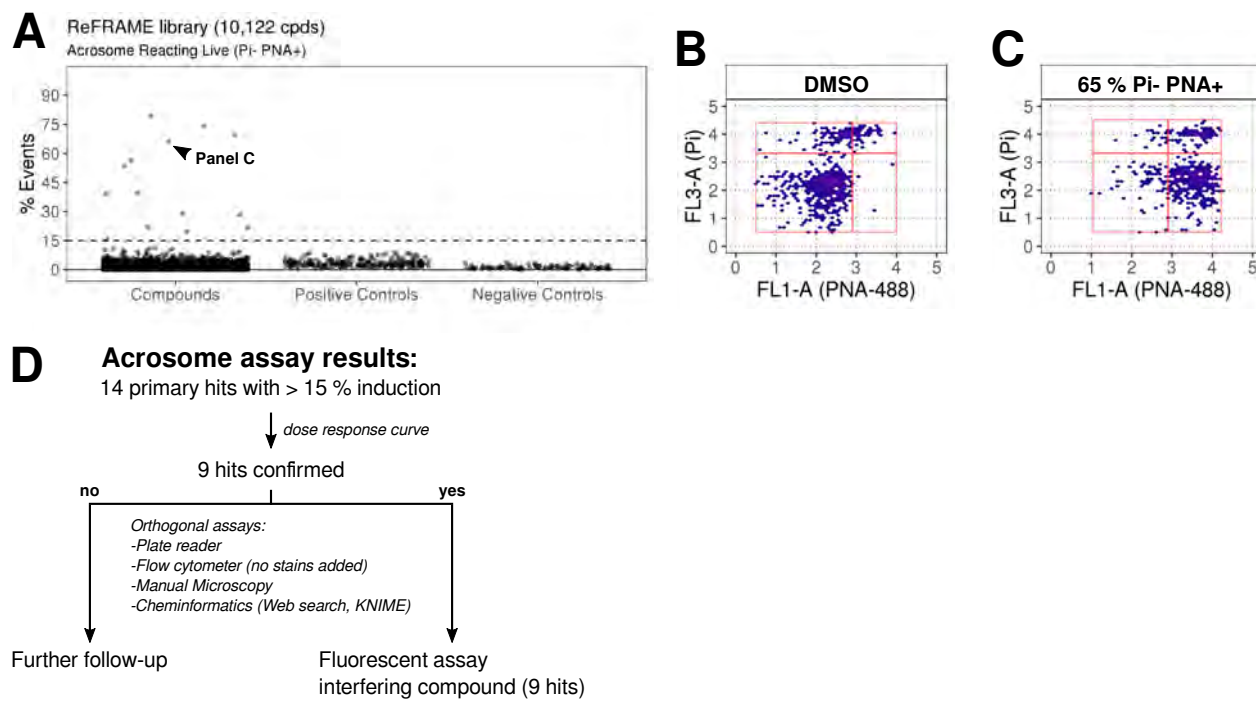
Hek CC50 [μM]

8.066

HepG2 CC50 [μM]

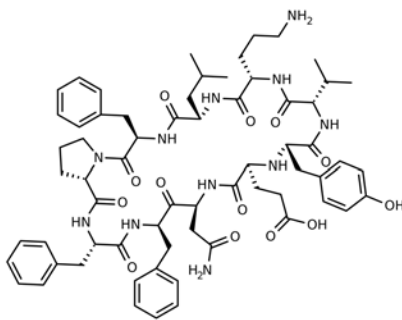
0.435

Figure 3



Supplementary Figure 3

Tyrosinricin Antibiotic

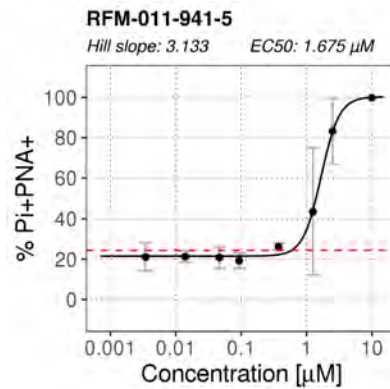


MW (RDKit)
1227.633

QED (RDKit)
0.063

SlogP (RDKit)
1.138

TPSA (RDKit)
379.75



EC50 [μ M]
1.67

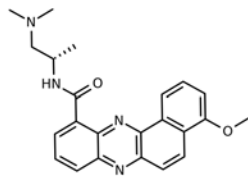
Efficacy [% induction]
84

Hek CC50 [μ M]
1.791

HepG2 CC50 [μ M]
0

MIn 576

DNA topoisomerase II inhibitor

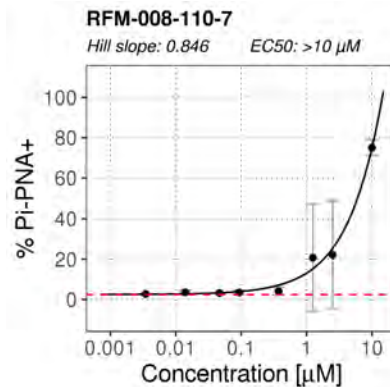


MW (RDKit)
388.19

QED (RDKit)
0.418

SlogP (RDKit)
3.625

TPSA (RDKit)
67.35



EC50 [μ M]
>10

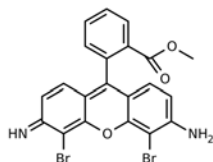
Efficacy [% induction]
76

Hek CC50 [μ M]
0.686

HepG2 CC50 [μ M]
0.574

Th-9402

Immunosuppressant

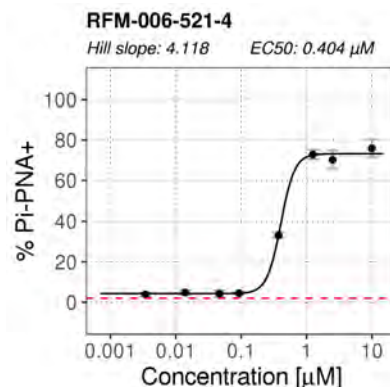


MW (RDKit)
499.937

QED (RDKit)
0.213

SlogP (RDKit)
5.578

TPSA (RDKit)
89.31



EC50 [μ M]
0.4

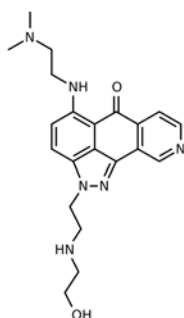
Efficacy [% induction]
76

Hek CC50 [μ M]
0

HepG2 CC50 [μ M]
0

Topixantrone

DNA topoisomerase II inhibitor

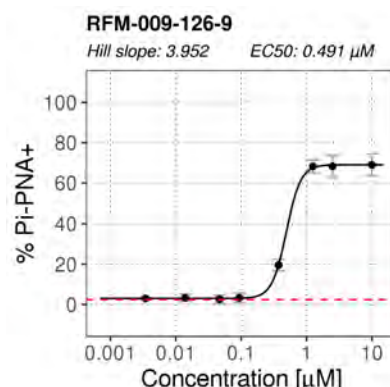


MW (RDKit)
394.212

QED (RDKit)
0.367

SlogP (RDKit)
1.198

TPSA (RDKit)
95.31



EC50 [μ M]
0.49

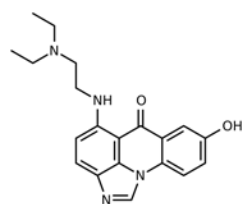
Efficacy [% induction]
72

Hek CC50 [μ M]
1.443

HepG2 CC50 [μ M]
0.658

Imidazoacridinone

DNA topoisomerase II inhibitor

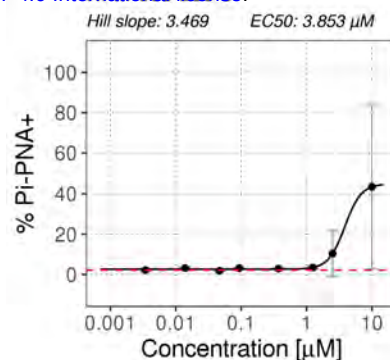


MW (RDKit)
350.174

QED (RDKit)
0.523

SlogP (RDKit)
2.898

TPSA (RDKit)
69.87



EC50 [μM]
3.85

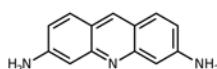
Efficacy [% induction]
71

Hek CC50 [μM]
0.817

HepG2 CC50 [μM]
0.476

Proflavine

DNA synthesis intercalator

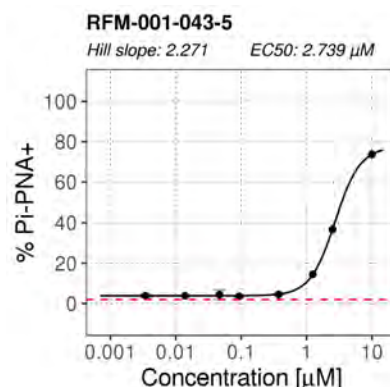


MW (RDKit)
209.095

QED (RDKit)
0.441

SlogP (RDKit)
2.552

TPSA (RDKit)
64.93



EC50 [μM]
2.74

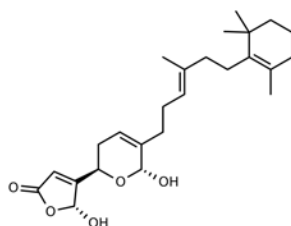
Efficacy [% induction]
71

Hek CC50 [μM]
0

HepG2 CC50 [μM]
0

Manoalide

Ornithin Decarboxylase/PLA2 inhibitor

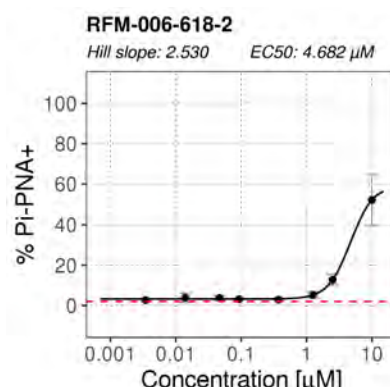


MW (RDKit)
416.256

QED (RDKit)
0.458

SlogP (RDKit)
4.855

TPSA (RDKit)
75.99



EC50 [μM]
4.68

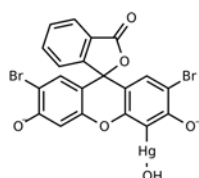
Efficacy [% induction]
59

Hek CC50 [μM]
2.781

HepG2 CC50 [μM]
3.522

Merbromin

Carbonic anhydrase II inhibitor

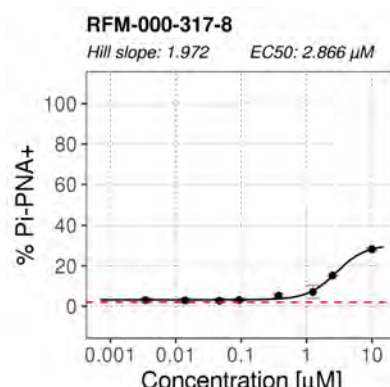


MW (RDKit)
703.841

QED (RDKit)
0.309

SlogP (RDKit)
2.542

TPSA (RDKit)
101.88



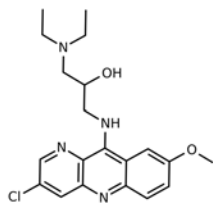
EC50 [μM]
2.86

Efficacy [% induction]
27

Hek CC50 [μM]
0

HepG2 CC50 [μM]
0

Acranil Antiprotzoal

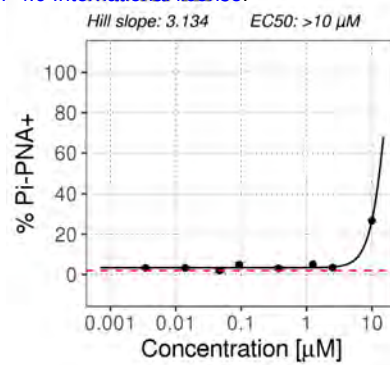


MW (RDKit)
387.171

QED (RDKit)
0.568

SlogP (RDKit)
4.165

TPSA (RDKit)
57.62



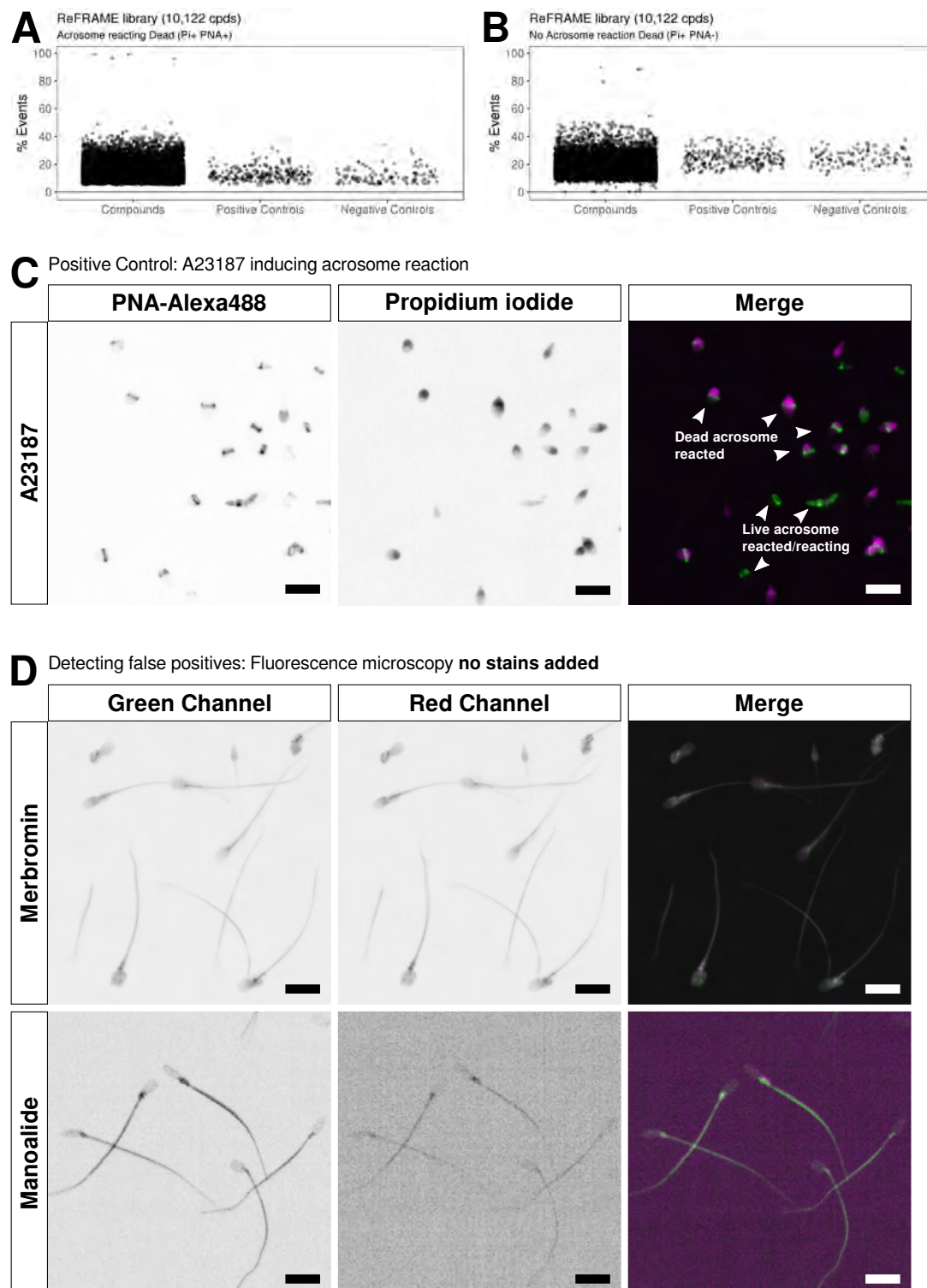
EC50 [μM]
>10

Efficacy [% induction]
25

Hek CC50 [μM]
1.136

HepG2 CC50 [μM]
2.827

Supplementary Figure 3.1



Supplementary Table 1

<i>Name</i>	<i>EC50 [μM]</i>	<i>Efficacy [% reduction]</i>	<i>Information</i>
Kf-4939	0.49	100	Platelet aggregation inhibitor
Mercufenol Chloride	1.45	98	Antiseptic
Bismuth Ethanedithiol	2.5	98	NA
Tyrothricin	0.34	97	Antibiotic
Ticlatone	4.43	96	Antibiotic
Phenylmercuric Borate	1.59	91	Antiseptic
Alexidine	> 10	86	Phospholipase inhibitor
Disulfiram	> 10	70	Aldehyde dehydrogenase 2 inhibitor
Octenidine	6.85	54	Antibacterial
Pfk-015	> 10	47	PFKFB3 inhibitor
Methylprednisolone Aceponate	2.52	45	Antiinflammatory
Zoticasone Propionate	2.38	39	Glucocorticoid receptor agonist
Brigatinib [#]	0.68	38	ALK receptor tyrosine kinase inhibitor
Fluorometholone Acetate	3.3	35	Antiinflammatory
Amibegron Hydrochloride	0.22	31	beta3-Adrenoceptor Agonist
Kf-41399	4.43	31	Chemoprotective agent
Uprosertib	> 10	31	PKB alpha/Akt1 inhibitor
Halobetasol Propionate	> 10	30	Antisporiatic
Pirtenidine	2.66	28	Antibiotic
Lx-7101	> 10	28	LIM Domain Kinase inhibitor
Ap26113-analog	1.35	24	ALK receptor tyrosine kinase inhibitor
Methylbenzethonium Chloride	> 10	24	Antiseptic
Cabazitaxel	> 10	23	Tubulin inhibitor
Resiquimod	3.22	22	Immunostimulant
Oxyphenbutazone	4.08	21	Treatment of Gout
Skq1	> 10	21	Neurologic drug
Docetaxel	0.05	20	Tubulin inhibitor
Lestaurtinib	4.97	17	Flt3 inhibitor
Brigatinib [#]	1.89	15	ALK receptor tyrosine kinase inhibitor

[#] from two different vendors; Information provided by Calibr; NA ... No Annotation

Supplementary Table 2

<i>Name</i>	<i>EC50 [μM]</i>	<i>Efficacy [% induction]</i>	<i>Information</i>
Tyrothricin	1.67	84	Antibiotic
Th-9402	0.4	76	Immunosuppressant
MIn 576	>10	76	DNA topoisomerase II inhibitor
Topixantrone	0.49	72	DNA topoisomerase II inhibitor
Proflavine	2.74	71	DNA synthesis intercalator
Imidazoacridinone	3.85	71	DNA topoisomerase II inhibitor
Manoalide	4.68	59	Ornithin Decarboxylase/PLA2 inhibitor
Merbromin	2.86	27	Carbonic anhydrase II inhibitor
Acranil	21.61	25	Antiprotozoal

Information provided by Calibr