ChemistrySELECT

Supporting Information

Identification and Screening of Selective WEE2 Inhibitors to Develop Non-Hormonal Contraceptives that Specifically Target Meiosis

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Experimental Section

All compounds were assessed by a combination of nuclear magnetic resonance and mass spectrometry. Purity analyses were completed by the parent company and/or in-house assessment. Suppliers and purities are as follows: Sigma-Aldrich (St. Louis, MO): 6-(2,6-dichlorophenyl)-8-methyl-2-((4-morpholinophenyl)amino)pyrido[2,3-*d*]pyrimidin-7(8*H*)-one (**12**, 98% HPLC), 6-(2,6-dichlorophenyl)-2-((4-fluoro-3-methylphenyl) amino)-8-methylpyrido[2,3-*d*]pyrimidin-7(8*H*)-one (**14**, >98% HPLC); Tocris (Minneapolis, MN): 6-(2,6-dichlorophenyl)-2-((4-(2-(diethylamino) ethoxy)phenyl)amino)-8-methylpyrido[2,3-*d*]pyrimidin-7(8*H*)-one (**2,** >99% HPLC), 4'-(5-((3-((cyclopropylamino)methyl)phenyl)amino)-1*H*-pyrazol-3-yl)-[1,1'-biphenyl]-2,4-diol (**3**, >97% HPLC), 9-hydroxy-4-phenyl pyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-dione (**8**, >98%); Activate Scientific (Prien, Germany): 6-(2,6-dichlorophenyl)-8-methyl-2-((3-(methylthio) phenyl)amino)pyrido[2,3-*d*]pyrimidin-7(8*H*)-one (**15**, >95% HPLC), 3-((6-(2,6-dichlorophenyl)-8-methyl-7-oxo-7,8-dihydropyrido[2,3-*d*]pyrimidin-2-yl)amino)benzoic acid (**16**, >95% HPLC); Cayman (Ann Arbor, MI): 6-(2,6-dichlorophenyl)-2-((3-(hydroxymethyl)phenyl)amino)-8-methylpyrido[2,3-*d*]pyrimidin-7(8*H*)-one (**17**, >95% HPLC); Toronto Research Chemicals (Ontario, Canada): 6-(2,4-difluorophenoxy)-2-((1,5-dihydroxypentan-3-yl)amino)-8-methylpyrido[2,3-*d*]pyrimidin-7(8*H*)-one (**13**, 98% HPLC); Chembridge Corporation (San Diego, CA): methyl 2-(2-(benzylamino)-5-methyl-7-oxo-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-6-yl)acetate (**7)**, methyl 2-(2-((4-(benzyloxy)benzyl)amino)-5-methyl-7-oxo-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-6-yl)acetate (**22)**, methyl 2-(2-((3,4-dimethoxybenzyl)amino)-5-methyl-7-oxo-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-6-yl)acetate (**21)**, methyl 2-(5-methyl-2-((3-nitrobenzyl)amino)-7-oxo-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-6-yl)acetate (**24)**, methyl 2-(2-((2-chloro-6-fluorobenzyl)amino)-5-methyl-7-oxo-4,7-dihydro-[1,2,4] triazolo[1,5-*a*]pyrimidin-6-yl)acetate (**23)**, methyl 2-(5-methyl-7-oxo-2-((thiophen-2-ylmethyl)amino)-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-6-yl)acetate (**25)** , all >90-95%.

Immunoassay for WEE kinase inhibitor activity. A CycLex WEE1 Kinase Assay/Inhibitor Screening Kit (Cat# CY-1172; MBL International, Woburn, MA) was used to evaluate candidate WEE2 inhibitors for inhibition of CDK1 phosphorylation activity and to determine differences in specificity against WEE2 compared to WEE1. Manufacture guidelines were followed for a standard assay as directed and performed in duplicate. Briefly, either 10 µL of 40 mUnits of human WEE1 protein (Cat# CY-E1172; MBL International) or 5 µg of recombinant human WEE2 (Schönbrunn Lab, Moffitt Cancer Center, Tampa, FL) were added to wells pre-coated with CDC2 (CDK1) containing the tyrosine 15 residue, and co-cultured with ATP and either 1 µM of selected inhibitor (n=24) or assay buffer (control) at 30 °C for 60 min. Wells containing assay buffer + ATP and no protein were used as negative controls and all wells contained 100 µL total reaction volume. Wells were washed and a primary anti-phospho-tyrosine monoclonal antibody (Cat# PY-39; MBL International) was added to detect only the phosphorylated form of tyrosine 15. Following a 60 min incubation at ambient temp, wells were washed again and a horseradish peroxidase (HRP) conjugated anit-mouse IgG secondary antibody was used to bind the primary antibody. After a final incubation for 60 min at ambient temp, wells were washed and tetramethylbenzidine was added as a chromogenic substrate that reacts with the peroxidase in HRP to produce a blue color. Wells were allowed to develop for 10 min before adding sulfuric acid to stop the reaction. Absorbance was measured at dual wavelengths of 450/540 nm and relative WEE kinase activity was determined **(Figures 5, 7, 8)**.

Bovine oocyte *in vitro* fertilization (IVF) with WEE2 inhibitors. IVF was performed in the presence of selected inhibitors to evaluate biological activity against WEE2 to block fertilization. Immature bovine oocytes (n=1,133) were obtained commercially from DeSoto Biosciences (Seymour, TN) and shipped overnight to the laboratory at 38.5 °C in 2 mL of medium designed to allow the oocytes to resume meiosis and mature to metaphase II during transport, as previously described.[1] Included in the medium was either 1 µM or 10 µM of inhibitors **7**, **2**, **12**, **15**, or **16** solubilized in DMSO. No inhibitors or 1% DMSO treatments were also included as controls. After 20 h of culture, mature oocytes were transferred into a standard *in vitro* fertilization protocol with continued exposure to the same concentration of inhibitor as shipment and maturation and inseminated with 2 x 10^6 sperm/mL isolated from cryopreserved semen samples.[2] Presumptive zygotes were removed from culture 20 h post insemination and repeatedly passed through a fine bore pipette to remove surrounding cumulus support cells and cultured in KSOMaa (Cat# IVL04; Caisson Labs, Smithfield, UT) for 96 h to allow sufficient time to observe mitotic divisional cleavage. The proportional data of embryo formation, judged by mitotic cleavage, was analyzed by Shapiro-Wilk and found to be normally distributed ($p=0.872$) with equal variance ($p=0.474$) then compared to control groups (DMSO only and no treatment Control) by One way ANOVA and significant differences were detected ($p<0.001$). Multiple comparisons to the controls were performed by a Bonferroni t-test with $p < 0.05$ to determine which treatment groups had a significant effect on WEE2 inhibition.

Flow cytometry based somatic cell proliferation assay with WEE2 inhibitors. Somatic cells were cultured with selected WEE2 inhibitors to determine biological specificity to block WEE2 activity but not WEE1. HEK 293 cells were cultured at 37 °C with 5% CO_2 in humidified air in DMEM medium (Cat# 30-2003; ATCC, Manassas, VA) with 10% fetal bovine serum (Cat# 30-2020; ATCC) and 1 µM of selected WEE inhibitor. Additional cell populations were cultured with 1% DMSO to serve as a control. Samples of cell cultures were taken every 24 h up to 6 days and fixed in 1.85% formaldehyde solution then held in phosphate buffered saline + 10% fetal bovine serum until analysis. Prior to the start of the experiment, all cultures were transiently exposed to Cell Trace Violet dye (Cat# C34557; ThermoFisher, Waltham, MA), a membrane permeable fluorophore that diminishes in concentration within the cell after each progressive division. Fixed samples were analyzed by flow cytometry and emission of the dye was measured at 405/450 nm in at least 20,000 events. Assays were performed in duplicate. By measuring the reduction in fluorescent emission over time, effects on mitotic cell division were tracked where inhibited cells maintained more of the dye compared to unaffected populations or untreated cells.

[1] N. W. Santiquet, J. R. Herrick, A. Giraldo, J. P. Barfield, W. B. Schoolcraft, R. L. Krisher, *J. Assist. Reprod. Genet.* **2017**, 1-8.
[2] J. J. Parrish, *Theriogenology* **2014**, *81*, 67-73.