On Line Supplement for:

Spiroindolone that inhibits PfATPase4 is a potent, cidal Inhibitor of *Toxoplasma gondii* tachyzoites *in vitro* and *in vivo (132 characters ,sp)*

Running Title: Spiroindolone Cidal for T.gondii (on Line Supplement)

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ON LINE SUPPLEMENT

Materials/Methods

Compound synthesis and preparation

Spironindolone was synthesized as described [10] by Reagents 4 Research. Initial 10mM stocks were dissolved in DMSO, and further diluted in culture medium (described below) to desired concentrations. Unless indicated, the final concentration of DMSO in experiments did not exceed 0.1%.

Parasite and Tissue Culture

Confluent monolayers of human foreskin fibroblast (HFF) cells were maintained in Iscoves Modified Dulbecco's Medium supplemented with 10% Fetal Calf Serum, 1% Glutamax, and 1% Penesiillin-Streptomyoisin-Fungisone (IMDM-C). All cultures were maintained at 37^{O}C and 5% CO_2. *T. gondii* tachyzoites of the RH-YFP strain – a genetically modified type I RH strain that expresses the green fluorescent protein[2] (Kindly provided by Boris Streipen, PhD, University of Georgia) and cultured in HFF cells under conditions described above.

In vitro *Challenge Assays*

RH-YFP parasites were lysed from HFF cells by double passage through a 25g needle[1,9]. Confluent HFF cells in 96-well plates (Falcon 96 Optilux Flat-bottom) were infected with parasites at a concentration of 3,500 parasites/well. Parasites were allowed to infect for one hour, after which control and experimental compounds

were added. In standard challenge experiments, parasite proliferation, and compound effectiveness, was assessed 72 hours after initial infection using a Synergy H4 Hybrid Reader (BioTek) and Gen5 1.10 software. Pyrimethamine and Sulfadiazine were used as a positive control, 0.1% DMSO was used as a negative control. In some experiments cultures were in two chamber lab tek chambers, fixation was with methanol, and cultures were stained with Giemsa to demonstrate efficacy using a second method.

In vitro Toxicity Assay

Confluent HFF cells in 96-well plates were treated under the same conditions as in a challenge assay, without being infected by parasites. Cell viability was assessed using the calorimetric assay kit WST-1 (Roche) and the Synergy H4 Hybrid Reader (BioTek). 10% DMSO, known to be toxic to HFF cells, was used as a negative control[1,4].

PPMO

PPMO were designed and utilized as described 4. Herein both translation inhibitory and exon skipping PPMO were utilized with the data in the figure from the studies with translation inhibitory PPMO. They were directed against ATPase 4 and inhibited replication of *T.gondii* . Sequences of PPMO utilized were- Translation blocking AGAGTCAAGATCCGAGGATGCACCA; Exon skipping-ACCAAGCTTCCTCACCCGACTTTTC

In vivo Challenge and Toxicity Assays

RH-YFP parasites were freshly lysed from HFF cells and were used to infect mice intraperitoneally at a concentration of 2000 tachyzoites/mouse. Mice were C57Bl6J mice with a HLA 11 transgene which are readily available in our laboratory. Spironindolone NITD206 (100mg/ml in DMSO) was further diluted into PBS. This suspension was given to mice orally at a 100mg/kg/day dose in a volume of 0.2ml/mouse on day 0 and day 1 after tachyzoite infection. The same volume of DMSO was also diluted in PBS, and used as a negative control. Five days after parasite infection, mice were euthanized, and peritoneal parasites were collected from each mouse by injecting 4 ml of PBS into the peritoneal cavity and collecting the peritoneal fluid. Parasite burden for each mouse was assessed by measuring the fluorescent intensity of the peritoneal fluid using Synergy H4 Hybrid Reader (BioTek) and Gen5 1.10 software. A standard curve with dilutions of parasites was performed4.

Modeling of *T. gondii* ATPase4

A model of *T. gondii* and *P. falciparum* ATPase4 was generated using PHYRE2 and based against the *Squalus acanthias* crystal structure (2ZXE)3,12. A 100% confidence score was given for the structure with 71% coverage of the *T. gondii* sequence. Independent modeling of the *P. falciparum* ATPase4 was also carried out in Phyre2 giving a highly similar structure with 100% confidence and 75%

coverage. Sequence alignments of the *S. acanthias, P. falciparum* and *T. gondii* ATPase4 were carried out using ClustalW13.

Statistical Analyses.

Significance of differences were determined using Student's t test. $P<0.05$ was considered significant. Every experiment was replicated at least twice. response editor AAC01501-