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Novel *Toxoplasma gondii* inhibitor chemotypes

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ABSTRACT

We profiled three novel *T. gondii* inhibitors identified from an antimalarial phenotypic high throughput screen (HTS) campaign: styryl 4-oxo-1,3-benzoxazin-4-one KG3, tetrahydrobenzo[*b*]pyran KG7, and benzoquinone hydrazone KG8. These compounds inhibit *T. gondii in vitro* with IC_{50} values ranging from 0.3 to 2 μM, comparable to that of 0.25 to 1.5 μM for the control drug pyrimethamine. KG3 had no measurable cytotoxicity against five mammalian cell lines, whereas KG7 and KG8 inhibited the growth of 2 of 5 cell lines with KG8 being the least selective for *T. gondii*. None of the compounds were mutagenic in an Ames assay. Experimental $gLogD_{7.4}$ and calculated PSA values for the three compounds were well within the ranges predicted to be favorable for good ADME, even though each compound had relatively low aqueous solubility. All three compounds were metabolically unstable, especially KG3 and KG7. Multiple IP doses of 5 mg/kg KG7 and KG8 increased survival in a *T. gondii* mouse model. Despite their liabilities, we suggest that these compounds are useful starting points for chemical prospecting, scaffold-hopping, and optimization.

1. Introduction

The protozoan parasite *Toxoplasma gondii* (*T. gondii*) is the most common parasitic infection in the world with a 30–50% prevalence in the human population [1]. The initial infection frequently occurs upon ingestion of contaminated food and water or exposure to environmentally persistent oocysts shed by infected members of the Felidae family [2–3]. Upon the initial exposure to *T. gondii*, acute toxoplasmosis can occur subclinically (approximately 25%), or can be manifested as self-resolving flu-like symptoms [2,4]. The parasite will often persist in a slow-growing cyst stage of infection under continual host immune surveillance. When the host immune system is weakened, the latent infection (chronic toxoplasmosis) can re-activate and cause severe tissue damage such as toxoplasmic encephalitis [5]. Special populations such as HIV and AIDS patients require life-long prophylactic treatment to control parasitic latent infections, but such treatment does not lead to clearance of the parasite.

Pyrimethamine remains the most effective treatment and is commonly used in combination with sulfonamides [6]. Pyrimethamine administration can lead to dose-dependent bone marrow toxicity in the treated host, which may be partially controlled by co-administration of folinic acid supplements [7]. In addition, patients can be allergic to the adjunctive medication sulfadiazine [8]. While acute toxoplasmosis is treatable with these drugs, the latent infection remains recalcitrant to therapy of any length [9]. For these reasons, there exists a need to identify better drugs for treatment of toxoplasmosis and ultimately clearance of infection [9–10].

In 2010 Guigemende et al. performed a cell-based high-throughput screen (HTS) to identify novel antimalarial chemotypes. This work identified several compounds that show promise against both *Plasmodium falciparum (P. falciparum)* and *T. gondii*. As these two apicomplexan parasites display close evolutionary relationships, they may share similar drug sensitivities [11]. However, this screen suggested that *T. gondii* may be more chemoresistant: while 561 *P. falciparum* hits emerged from a chemical library of over 300,000 compounds, only 23 had measurable activity *against T. gondii*[12].

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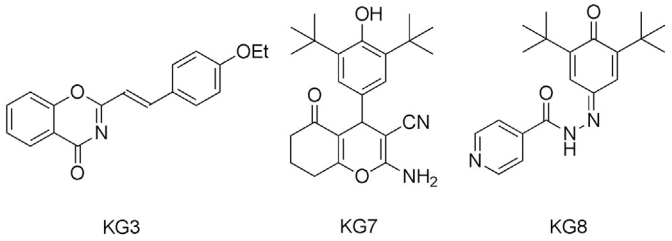

KG3 KG7 KG8

Fig. 1. Chemical structures. Styryl 4-oxo-1,3-benzoxazin-4-one (KG3), Tetrahydrobenzo [*b*]pyran (KG7), and Benzoquinone hydrazone (KG8).

From this filtered group of compounds with activity against both parasites [12], three were selected for further characterization with *T. gondii*: styryl 4-oxo-1,3-benzoxazin-4-one SJ000033445 (KG3), tetrahydrobenzo[*b*]pyran SJ000144280 (KG7), and benzoquinone hydrazone SJ000296485 (KG8) (Fig. 1). Each of these chemical structures appeared to be singleton scaffolds, making their characterization useful for further work in generating derivatives to optimize activity. In addition to verifying *in vitro* activity against multiple strains of *T. gondii*, the mammalian cell cytotoxicity profile was expanded, *in vitro* ADME properties were profiled, and mutagenicity was assessed. Lastly, the compounds were examined *in vivo* for their capacity to increase survivorship following an acute lethal challenge with *T. gondii* tachyzoites. Our evaluation of these compounds demonstrates statistically significant but incomplete *in vivo* survivorship following acute parasite infection, likely hampered by metabolic instability.

2. Materials and methods

2.1. Compounds

Compounds were obtained from ChemDiv (San Diego, California).

2.2. Cell maintenance

Human foreskin fibroblasts (HFF) and murine macrophages were obtained from American Type Culture Collection (ATCC). All cell lines and parasite strains were maintained in D10 media which consisted of DMEM media (Lonza) supplemented with 10% heat inactivated Hyclone bovine serum (GE Healthcare Life Sciences), HyClone 2 mM L-glutamine (GE Healthcare Life Sciences), 100 μg/mL penicillin and streptomycin (Corning), 20% Medium 199 (Corning) and gentamicin sulfate (Corning) at 37 °C with 5% CO_2. Type I strain of *T. gondii* constitutively expressing red fluorescent dimerized Tomato ("RH-dTom") and a type II strain, PRU expressing the same fluorophore ("PRU-dTom") were used in assays.

2.3. Cell toxicity assay

Bone marrow derived murine macrophages were allowed to grow until confluent in 96 well plates. Once confluent, an increasing concentration of compound (0 to 100 μM) was added and incubated for 24 h. Alamar blue (10 mM) was then added to each well and incubated for 4 h. A BioTek Synergy HT plate reader was then used to determine fluorescence.

2.4. IC_{50} assay

HFF cells were cultured in 96 well plates at 20,000 cells per well and allowed to grow until confluent. Then 2000 tachyzoites were then added to each well and incubated for 12 h allowing for invasion of host cells. Media was then replaced and compounds were added at increasing concentration from 0 to 100 μM in duplicate. All compounds were dissolved in DMSO; the concentration of DMSO did not exceed 1% in all assays. A fluorescent reading was then taken with a BioTek Snergy HT plate reader at day 5 post-infection.

2.5. Host cell and extracellular parasite pre-treatment assay

HFF cells were cultured in 96 well plates at 20,000 cells per well and allowed to grow until confluent. Once confluent, 10 μM of each compound was added to the wells. After 24 h, media was aspirated and cells were washed three times with D10 media. Cells were then infected with either 2000 RH-dTom or PRU-dTom tachyzoites and fluorescently quantified 5 day post-infection. Assays were performed in triplicate. To evaluate extracellular parasite responses to compound exposure, RH-dTom tachyzoites were isolated from culture and resuspended at 1×10^6 tachyzoites/mL in D10 media. Tachyzoites were treated with 10 μM of compound and incubated at 37 °C for 4 h. After treatment, HFF cells were then infected with treated tachyzoites at 20,000 tachyzoites/mL and tachyzoite growth was fluorescently quantified 5 day post-infection.

2.6. Physicochemical parameters and ADME characteristics

pK_a, PSA, and cLogP/cLogD values were assessed by the ChemAxon chemistry cartridge *via* JChem for Excel Software (version 16.4.11). To determine kinetic aq. solubility, compounds were dissolved into DMSO and then spiked into either pH 6.5 phosphate buffer or 0.01 M HCl (pH 2.0) with the final concentration being DMSO 1%. After 30 min at room temperature, samples were analyzed using nephelometry to determine a solubility range [13]. Partition coefficient values were estimated at pH 7.4 by correlation of their chromatographic retention properties against those of standard compounds with known partition coefficient values by gradient HPLC [14]. Chromatography-based protein binding values (cPPB) were determined by correlation of compound chromatographic retention properties using a human albumin column against those of a standard series of compounds with known protein binding values [15]. To evaluate metabolic stability, compounds (1 μM) were incubated with liver microsomes from humans and mice at 37 °C at a protein concentration of 0.4 mg/mL. Metabolic reactions were initiated by the addition of an NADPH-regenerating system. The reaction was quenched at varying time points (2, 30, and 60 min) and compound concentrations determined by LCMS.

2.7. Bacterial reverse mutation assay

A modified form of the Ames assay (Environmental Bio-Detection Products Inc.) with *Salmonella typhimurium* (TA100 strain) was used in to specifically detect point mutagenicity. Compounds were tested at concentrations of 3 × the averaged *T. gondii* IC_{50} values in sets of 48 replicates. A count of revertant colonies was performed and compared to the natural revertant control with the unpaired Students *t*-test to assess statistical significance (p-value < 0.05).

2.8. In vivo T. gondii studies

Swiss Webster CFW mice (Charles River, Wilmington, MA) were infected by IP injection of 20,000 *T. gondii* RH-dTom tachyzoites. At 24 h post-infection, test compounds dissolved in DMSO and then diluted with water to their respective concentration. All solutions were subsequently treated with hydrochloric acid or sodium hydroxide until dissolved, and the volume of DMSO administered was below the previously established toxic dose [17,18]. Compounds were administered in twice daily IP doses for 7 consecutive days (KG3, n = 3, KG7 and KG8, n = 2). Doses of all three compounds were selected empirically by determining the dose at which drug exposure caused murine toxicity (in all cases, murine hypoactivity) and dividing by 2. Pyrimethamine was included as a positive control (n = 3) and untreated infected mice as the negative control (n = 8). Mice were monitored for toxicity or illness throughout the study. Mouse survival was quantified through a Kaplan-