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Derivatives of a benzoquinone acyl hydrazone with activity against Toxoplasma gondii

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ABSTRACT

Toxoplasma gondii is an obligate intracellular parasite with global incidence. The acute infection, toxoplasmosis, is treatable but current regimens have poor host tolerance and no cure has been found for latent infections. This work builds upon a previous high throughput screen which identified benzoquinone acyl hydrazone (KG8) as the most promising compound; KG8 displayed potent in vitro activity against T. gondii but only marginal in vivo efficacy in a T. gondii animal model. To define the potential of this new lead compound, we now describe a baseline structure-activity relationship for this chemotype. Several derivatives displayed IC50's comparable to that of the control treatment pyrimethamine with little to no cytotoxicity. The best of these, KGW44 and KGW59, had higher metabolic stability than KG8. In an in vivo T. gondii murine model, KGW59 significantly increased survivorship. This work provides new insights for optimization of this novel chemotype.

1. Introduction

Toxoplasma gondii is a protozoan parasite capable of infecting virtually all classes of warm-blooded vertebrates. Initial infection often occurs from contaminated food and water or exposure to oocysts shed by members of the Felidae family. Upon host infection, the disease toxoplasmosis can occur. Acute toxoplasmosis is frequently described as mild flu-like symptoms, but healthy individuals will often be asymptomatic, while transition to a life-long latent infection is common (Tenter et al., 2000).

The immunocompromised populations who carry the parasitic infection require lifelong prophylactic treatment to prevent parasite induced encephalitis. Primary infections that occur in mothers during pregnancy can cause congenital toxoplasmosis in the fetus due to the ability of the parasite to cross the placenta. Such fetal infections can result in disastrous birth defects such as hydrocephaly and other malformations, causing T. gondii to be leading cause of birth defects worldwide (Tenter et al., 2000).

Current treatments exist to control acute toxoplasmosis, but the issues remain with poor host tolerance and frequent severe complications (Fung and Kirschenbaum, 1996). Front-line treatment most commonly includes pyrimethamine in combination with sulfadiazine but is associated with bone marrow toxicity and allergic reactions (Fung and Kirschenbaum, 1996). Some symptoms have been mitigated with the co-administration of folinic acid. Because of this, there is an imperative need for new therapeutics to be developed to combat acute infection. No FDA approved treatment has yet to be established for congenital toxoplasmosis, but it has been treated with clindamycin with varying degrees of success (McFarland et al., 2016).

While acute stage toxoplasmosis has been mitigated with current treatments, the latent infection has remained resistant to clearance and is presently life-long (Neville et al., 2015). Research efforts have produced few promising lead candidates against the chronic stage (Neville et al., 2015; McFarland et al., 2016).

A high-throughput phenotypic screen (HTS) of a chemical library produced a small collection of drug-like compounds with demonstrated...
efficacy against both *Plasmodium falciparum* and *T. gondii* in vitro (Guiguemde et al., 2010). The three compounds with the highest potency against *T. gondii* were further characterized as potential leads for further optimization and development (Sanford et al., 2018). The most promising of these was KG8 (Fig. 1). Given that kinases are thought to be promising *T. gondii* drug targets (Kamau et al., 2012), it is interesting to note that the closely related compound poloxin (Fig. 1), a derivative of thymoquinone, is an inhibitor of host polo-like kinase 1 (Liao et al., 2010). KG8 and closely related analogues have been previously shown to inhibit cyclooxygenase-2 and 5-lipoxygenase (Misra et al., 2013) in the host, and potentiate nerve growth factor (Eguchi et al., 2000). KG8 is also an acyl hydrazone derivative of the antibiotic isoniazid; however, the latter was inactive against *T. gondii* in vitro (Sanford et al., 2018). These data may suggest a host target of KG8, or potentially a parasite homolog target.

We now describe our initial work to assess the potential of this chemotype by the generation of a small library of chemical analogues to increase potency and selectivity against *T. gondii*. We identified two promising derivatives (KGW44 and KGW59) and assessed their physicochemical and ADME profiles and their in vivo efficacy in a *T. gondii* animal model.

### Table 1
IC$_{50}$ values of KG8 and derivatives against *T. gondii* strains and HFF.

| Derivative ID | RH *T. gondii* IC$_{50}$ (μM) | PRU *T. gondii* IC$_{50}$ (μM) | HFF IC$_{50}$ (μM) |
|--------------|-------------------------------|-------------------------------|------------------|
| KG8$^1$     | 2.3                           | 2.5                           | 28.0             |
| KGW15       | 2.0                           | –                             | > 100            |
| KGW42       | 2.0                           | –                             | > 100            |
| KGW43       | 3.8                           | –                             | > 100            |
| KGW64$^a$   | 0.91                          | 78                            | > 100            |
| KGW44$^a$   | 0.20                          | 0.99                          | > 100            |
| KGW54       | 1.2                           | 2.2                           | > 100            |
| KGW65$^a$   | 0.66                          | > 100                         | > 100            |
| KGW45       | 40                            | –                             | > 100            |
| KGW66$^a$   | > 100                         | –                             | > 100            |
| KGW68$^a$   | 3.8                           | –                             | > 100            |
| KGW72       | 13                            | –                             | > 100            |
| KGW73       | > 100                         | –                             | > 100            |

Derivatives of KG8 (Sanford et al., 2018$^1$) were screened against both RH-dTom and Pru-dTom *T. gondii*.

$^a$ Compounds that were effective in inhibiting *T. gondii* viability were selected for further cell viability screening. All compounds were also screened in a cell viability screen against human foreskin fibroblasts (HFF). No compound was highly toxic against HFF.

$^b$ Compounds KGW64-68 were found to be auto fluorescent and were excluded from further analysis.

### Table 2
Expanded mammalian cell viability screen results and point mutagenicity.

| Derivative ID | NR-9456 IC$_{50}$ (μM) | U2OS IC$_{50}$ (μM) | HEK293 IC$_{50}$ (μM) | HCO4 IC$_{50}$ (μM) | Ames Assay |
|--------------|-------------------------|---------------------|-----------------------|---------------------|------------|
| KG8$^1$     | > 100                   | > 100               | 0.97                  | 28                  | Negative   |
| KGW44       | > 100                   | > 100               | > 100                 | > 100               | Negative   |
| KGW59       | > 100                   | > 100               | > 100                 | > 100               | Negative   |

Derivatives of KG8 (Sanford et al., 2018$^1$) that were identified as effective in the preliminary screen (Table 1) were further screened for possible cell viability inhibition in NR-9456, U2OS, and HEK293. Potential point mutagenicity was also screened for with the use of a bacterial reversion assay (Ames Assay).
2. Methods

2.1. Experimental compounds

The synthesis of the KG8 derivatives is described in the supplemental material section.

2.2. Cell line maintenance

Human foreskin fibroblasts (HFF), human osteosarcoma cells (U-2OS), human embryonic kidney cells (HEK-293), human liver cells (HC-04), and murine macrophages (NR-9456) were all obtained from ATCC. HFF, U-20S, and all T. gondii strains (RH-dTom, ME49 and PRU) used were maintained in DMEM media (Lonza) supplemented with 10% heat inactivated Hyclone bovine serum (GE Healthcare Life Sciences), 2 mM L-glutamine (GE Healthcare Life Sciences), 100 μg/mL penicillin and streptomycin (Corning), 10% Medium 199 (Corning) and gentamicin sulfate (Corning) at 36.5 °C with 5% CO2. HEK-293 and HC-04 were maintained in the same medium without the addition of Medium 199. Macrophage NR-9456 was grown with the addition of 1 mM sodium pyruvate as recommended.

2.3. Parasite strains

RH (Type I) and PRU (Type II) strain T. gondii with inserted fluorescent transgene dimerized Tomato (dTom) were used for in vitro growth inhibition experiments. ME49 (Type II) strain T. gondii was used for the in vivo survivorship challenge in a murine model.

2.4. T. gondii growth inhibition in vitro screens (IC50)

HFF cells were plated in 96 well plates at approximately 20,000 cells per well and allowed to grow until confluent. Once confluency was reached, 2000 RH-dTom (or 8000 PRU-dTom) tachyzoites were added to each well and the plates were incubated at 37 °C for 24 h. Cells were then washed and infected with 2000 RH-dTom tachyzoites. (b.) Isolated RH-dTom tachyzoites were pretreated with compounds at 10 μM for 4 h at room temperature, then 2000 parasites were used to infect HFF. Fluorescent readings were taken 5 days post-infection for both experiments. (p value = **0.00007; *0.002).

2.5. Mammalian cell line cytotoxicity screens (IC50)

Mammalian cell viability was determined using human foreskin fibroblasts. The synthesis of the KG8 derivatives is described in the supplemental material section.
fibroblasts (HFF), osteosarcoma (U-2OS), embryonic kidney (HEK-283), hepatic carcinoma (HC-04), and a murine derived macrophage (NR-9456) cell line that were plated with approximately 20,000 cells per well and let grown until confluent. Once confluent, cells were treated with increasing compound concentrations from 0 to 100 μM for 24 h. This was followed by an Alamar Blue assay with 0.5 mM resazurin at 37 °C with 5% CO2 for 4 h. All concentrations were performed in duplicate. A fluorescent reading was then taken with a BioTek Synergy HT plate reader at 530/25 excitation and 590/25 emission. Host cell viability was determined by comparing the treatments with no compound treatment as 100% viability.

2.6. Bacterial reverse mutation assay

A modified form of the Ames assay (Environmental Bio-Detection Products Inc.) with Salmonella typhimurium (TA100 strain) was used to detect DNA single point mutagenicity. Compounds were tested at concentrations of 3x the averaged T. gondii IC50 values (Table 1) in sets of 48 replicates. A count of revertant colonies was performed and compared to the natural revertant control with the unpaired Student's t-test to assess statistical significance.

2.7. Host cell and extracellular parasite pre-treatment assay

HFF cells were grown in 96 well plates to confluency. 10 μM of respective compound was added to the wells (performed in triplicate). After 24 h of compound exposure, cells were then washed twice with D10 media. Cells were then infected with 20,000 RH tachyzoites/mL and parasite growth was quantified by fluorescence for 5 days post-infection.

To evaluate the effect of compound exposure to free tachyzoites, 10 μM of respective compound was added to RH-dTom tachyzoites isolated from culture and resuspended in D10 media at 106 tachyzoites/mL. Exposure to compound lasted 4 h at 37 °C. After treatment, tachyzoites were centrifuged and washed twice. Confluent HFF in 96 well plates were then infected with 2000 treated tachyzoites per well. Tachyzoite growth was quantified by fluorescence at 5 days post-infection.

2.8. Calculated physicochemical properties

Polar surface area (PSA) and lipophilicity (cLogD) values were assessed by the ChemAxon chemistry cartridge via JChem for Excel Software. Aqueous solubility of the most potent three compounds was determined by dissolving in DMSO and then spiked into either pH 6.5 PBS or 0.01 M HCl (pH 2.0). Samples were then analyzed 30 min after nephelometry to determine a solubility range. Partition coefficients were then determined through LCMS. This was followed by an Alamar Blue assay with 0.5 mM resazurin at varying time points (2, 30, and 60 min) and compound concentrations were then determined through LCMS.

2.9. Efficacy in vivo

Female Swiss Webster mice were obtained from Charles River. At 2 months old, mice were infected intraperitoneally with 5000 ME49 tachyzoites. Simultaneously, treatment began with experimental compounds at 5 mg/kg (n = 10), pyrimethamine at 5 mg/kg (n = 20) or solvent (n = 24) given once per day. Doses was determined for all three compounds by prior screening for toxicity. All experimental compounds and pyrimethamine were dissolved in 5% DMSO, 47.5% Propylene glycol (Sigma-Aldrich), and 47.5% Kollisolv (Sigma-Aldrich). Mice were weighed daily to assess overall health and to monitor weight changes indicative of infection. P values were obtained through the use of IBM STSS Statistics 22 software. All in vivo studies were institutionaly approved and carried out under IACUC #12-062-10.

3. Results and discussion

3.1. Primary screen for RH strain T. gondii and human fibroblast (HFF) cytotoxicity

Thirteen derivatives of KG8 (Fig. 2) were initially screened against the RH strain T. gondii for their potency in growth inhibition, and in human foreskin fibroblasts for host cell cytotoxicity (Table 1). We first discovered that replacing the 4-pyridyl substructure with a phenyl (KGW15) did not lower potency against T. gondii. Further, compounds in which the para position of the phenyl substructure was substituted with electron-donating (KGW42, 43, 64) and electron-withdrawing (KGW44, 54) groups were also quite active with IC50 in the range of 0.20–3.8 μM; of these KGW64 and KGW44 were the most potent. Compound KGW65, the 3-pyridyl isostere of KG8, was marginally more potent than the parent compound. However, replacing the 4-pyridyl substructure with an imidazole (KGW45) or cyclohexyl (KGW66) significantly weakened or abolished activity, respectively. Removal of the acyl hydrazone carbonyl (KGW68) and adding a nitro group (KGW59) increased potency by an order of magnitude. Replacing the acyl hydrazone of KG8 with an acyl oxime ether to afford the poloxin analog KGW72 reduced potency 6-fold. KGW73, a saturated analog of the benzoquinone substructure, was inactive. Finally, none of the compounds decreased HFF viability at up to 100 μM concentration.

3.2. Screen for activity against T. gondii PRU strain

The five compounds (KGW64, 44, 54, 65, and 59) with IC50 values less than 2 μM against T. gondii strain RH were tested against strain PRU (Type II lineage) which is considered less virulent and more representative of natural infections (Howe and Sibley, 1995). All five compounds were less active against the PRU strain; notably, KGW65 was completely inactive and KGW64 was only weakly active. KGW44 (IC50 = 0.99 μM) was the most potent derivative, followed by KGW59 (IC50 = 1.4 μM). From these data, KGW44 and KGW59 were selected for further profiling.

3.3. Expanded mammalian cell cytotoxicity screen

KGW44 and KGW59 were tested against the U-2OS (osteosarcoma), HEK-293 (kidney), HC-04 (liver) and NR-9456 (macrophage) cell lines to further assess their selectivity against T. gondii (Table 2). Neither compound showed any effects on these cells at concentrations up to 100 μM.

3.4. Bacterial reverse mutation assay

Compounds KGW44 and KGW59 were tested at 0.75 μM (ca. 3x average T. gondii IC50) for their capacity to induce DNA point mutations in a bacterial model (Ames assay) with Salmonella typhimurium strain TA100; neither compound was mutagenic.

3.5. Pre-treatment assays

To probe a possible mode of action, both HFF cells and tachyzoites were pre-treated with 10 μM of KGW44 and KGW59. KGW59 pre-treatment of HFF cells prior to infection with tachyzoites resulted in a...
decrease of parasite growth to 30.2% of control \( (p = 0.00007; \text{ Fig. 3}) \). When tachyzoites were exposed to KGW59 prior to infection no significant change in growth was found. When HFF cells were treated with KGW44 no significant change in growth was measured. However, when tachyzoites were treated with KGW44 prior to infection, percent growth was reduced to 60.3% of control \( (p = 0.002; \text{ Fig. 3}) \). From these data, it can be reasoned that KGW59 may be taken up by the host cell to effect \textit{T. gondii} growth while KGW44 may elicit a direct effect on the extracellular parasite prior to invasion.

3.6. Physicochemical and in vitro ADME

Data in Table 3 indicate that KGW44 and KGW59 were more lipophilic and less soluble than KG8. The three compounds had calculated polar surface area (PSA) values ranging from 72 to 128 \( A^2 \) indicating that polarity would not be a rate-limiting factor for membrane permeability (Palm et al., 1997). Plasma protein binding for KGW44 (84.3%) was considerably lower than that of KG8 which had a cPB value of > 99.5%. Metabolic stability testing in both human and mouse liver microsomes (Table 4) indicated that both KGW44 and KGW59 were considerably more stable than KG8. The intrinsic clearance \( (Cl_{int}) \) and predicted hepatic extraction ratio \( (E_{h}) \) values indicate that all of the compounds were more rapidly metabolized in mouse vs. human liver microsomes. The metabolic instability of these compounds may be a consequence of their twin tert-butyl groups (Barnes-Seeman et al., 2013; Westphal et al., 2015) and electrophilic benzoquinone substructure.

3.7. Efficacy in vivo

KGW44 and KGW59 were tested for their capacity to increase survivorship in Swiss-Webster mice following a lethal challenge with 5000 ME49 strain \textit{T. gondii} tachyzoites (Fig. 4). Compounds were administered IP at 5.0 mg/kg/day for 10 days post-infection. Pyrimethamine was included as a positive control and treated mice displayed 90% survivorship, while 45% of KGW59-treated mice survived, and 0% of KGW44-treated mice survived.

4. Conclusion

To define the potential of benzoquinone acyl hydrazone KG8 as a new lead against \textit{T. gondii}, we performed a baseline structure-activity relationship for this new chemotype. Several derivatives displayed \( IC_{50}'s \) comparable to that of pyrimethamine with little to no cytotoxicity. The best of these, KGW44 and KGW59, had higher metabolic stability than KG8. In an \textit{in vivo} \textit{T. gondii} murine model, KGW59 significantly increased survivorship. This work provides new insights for optimization of this novel chemotype.

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Conflicts of interest

The authors declare that no competing interests exist.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2018.11.001.

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