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A. G. Sanford
University of Nebraska at Omaha

T. T. Schulze
University of Nebraska at Omaha

L. P. Potluri
University of Nebraska at Omaha

G. F. Watson
University of Nebraska at Omaha

E. B. Darner
University of Nebraska at Omaha

See next page for additional authors

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Derivatives of a benzoquinone acyl hydrazone with activity against *Toxoplasma gondii*

A.G. Sanford[a,1,2], T.T. Schulze[a,1,2], L.P. Potluri[a,3], G.F. Watson[a], E.B. Darner[a], S.J. Zach[a], R.M. Hemsley[a], A.I. Wallick[a], R.C. Warner[a], S.A. Charman[c], X. Wang[b], J.L. Vennerstrom[b], P.H. Davis[a,*]

[a] Department of Biology, University of Nebraska at Omaha, Omaha, NE, USA
[b] College of Pharmacy, University of Nebraska Medical Center, Omaha, NE, USA
[c] Centre for Drug Candidate Optimisation, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Australia

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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 IC_{50}'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

* Corresponding author. University of Nebraska at Omaha, Allwine Hall 427, 6001 Dodge Street, Omaha, NE 68182-0040, USA.
E-mail address: pdavis@unomaha.edu (P.H. Davis).
[1] These authors contributed equally to this work.
[2] Current address: Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska, USA.
[3] Current address: Division of Infectious Diseases, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts, USA.

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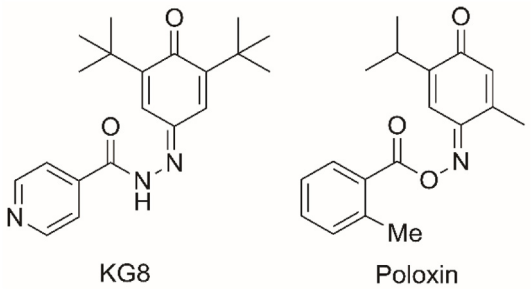

Fig. 1. KG8 and poloxin structures.

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[b]	0.91	78	> 100
KGW44[a]	0.20	0.99	> 100
KGW54	1.2	2.2	> 100
KGW65[b]	0.66	> 100	> 100
KGW45	40	–	> 100
KGW66[b]	> 100	–	> 100
KGW68[b]	3.8	–	> 100
KGW59[a]	0.13	1.4	> 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).

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 cyclooxgenase-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.

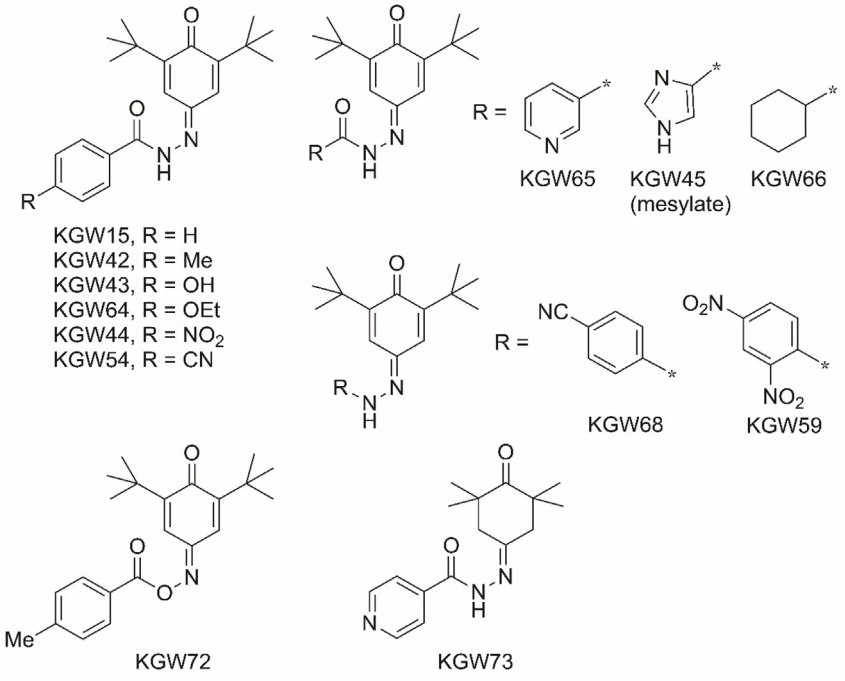

Fig. 2. KGW structures.

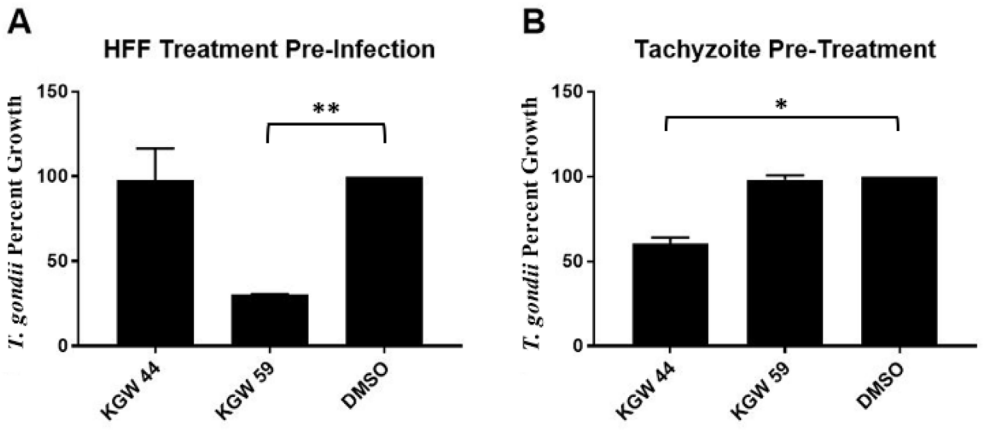

Fig. 3. Host cell pre-treatment and extracellular tachyzoite exposure results. (a.) HFF cells were pretreated with compounds at 10 μM and allowed to incubate at 37 °C for 24 h. Cells were then washed and infected with 2000 RH-dTom tachyzoites. (b.) Isolated RH-dTom tachyzoites were pretreated with compounds 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).

Table 3
Physiochemical parameters, solubility, and plasma protein binding of top KGW compounds.

Identifier	cPPB[a] (% Bound)	PSA[b] (A^2)	gLogD (pH 7.4)	$Sol_{6.5}$ [c](ug/mL)
KG8[1]	> 99.5	71.4	3.9	3.1–6.3
KGW44	84.3	101.7	4.9	< 1.6
KGW59	ND	127.7	> 5.3	< 1.6

(ND: No chromatographic peak under cPPB conditions) (Sanford et al., 2018[1]).
[a] Plasma protein binding was estimated using a chromatographic method.
[b] PSA values were calculated through the ChemAxon chemistry cartridge with JChem from Excel.
[c] Kinetic solubility was determined by nephelometry after 30 min at room temperature.

Table 4
Metabolic stability of KGW44 and KGW59.

Compound ID	Microsome species	$T_{1/2}$ (min)	$CL_{int,\ in\ vitro}$ (μL/min/mg protein)	Predicted E_H
KG8[1]	Human	9	129	0.88
	Mouse	< 2	> 886	N/A
KGW44	Human	83	21	0.45
	Mouse	9	99	N/A
KGW59	Human	111	16	0.38
	Mouse	37	46	0.50

Metabolic stability was assessed in both human and mouse liver microsomes. The half-life ($T_{1/2}$), intrinsic clearance *in vitro* (CL_{int}), and the predicted hepatic extraction ratios (E_H) were determined (Sanford et al., 2018[1]).

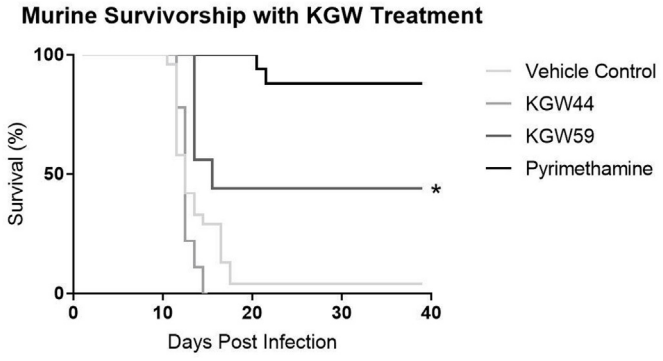

Fig. 4. *In vivo* survival following compound treatment in a murine model. Swiss-Webster mice were infected with 5000 ME49 *T. gondii* tachyzoites and then subsequently treated with respective compound for 10 days at 5 mg/kg. Survival was quantified over 40 d using a Kaplan-Meier curve. (p value = 0.004*).

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-20S), 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), HyClone 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% CO_2. 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 (IC_{50})

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 (48 h for PRU) to allow for infection. Media was then replaced, and compounds dissolved in DMSO (Fischer Scientific) were added at increasing concentration from 0 to 100 μM. The concentration of DMSO did not exceed 1% in all assays to prevent cytotoxicity. All concentrations were performed in duplicate and pyrimethamine was used as a positive control. A fluorescent reading was then taken with a BioTek Snergy HT plate reader at 530/25 excitation and 590/25 emission for 5 days. IC_{50}s were determined after day 5 where half of the relative fluorescent units (RFU) were seen.

2.5. Mammalian cell line cytotoxicity screens (IC_{50})

Mammalian cell viability was determined using human foreskin

fibroblasts (HFF), osteosarcoma (U-20S), 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% CO_2 for 4 h. All concentrations were performed in duplicate. A fluorescent reading was then taken with a BioTek Snergy 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* IC_{50} 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 10e6 tachzyoites/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 assesed 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 coefficient values were then analyzed at pH 7.4 through chromatographic retention properties against standard compounds with known values through gradient HPLC. Chromatography-based protein binding values (cPPB) were then determined by correlation of known chromatographic retention properties by using a human albumin column against a standard series of compounds with known cPPB values (Valko et al., 2003). To determine metabolic stability, compounds were incubated with liver microsomes at 1 μM from both humans and mice at 37 °C at a protein concentration of 0.4 mg/mL. Reactions were initiated by the addition of an NADPH-regenerating system. Reactions were then quenched 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 institutionally 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 IC_{50s} 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 IC_{50} 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 (IC_{50} = 0.99 μM) was the most potent derivative, followed by KGW59 (IC_{50} = 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* IC_{50}) 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 ($p = 0.00007$; 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 ($p = 0.002$; Fig. 3). From these data, it can be reasoned that KGW59 may be taken up by the host cell to effect *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 cPPB 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 *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 *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 *in vivo 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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