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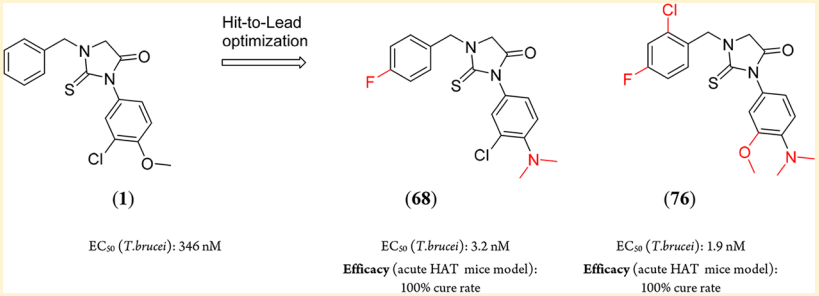
1-Benzyl-3-aryl-2-thiohydantoin Derivatives as New Anti-*Trypanosoma brucei* Agents: SAR and in Vivo Efficacy

Andriy Buchynskyy,[†] J. Robert Gillespie,[‡] Zackary M. Herbst,[‡] Ranae M. Ranade,[‡] Frederick S. Buckner,*[,‡] and Michael H. Gelb*[,†]

[†]Department of Chemistry, University of Washington, Seattle, Washington 98195, United States

[‡]Department of Medicine, University of Washington, Seattle, Washington 98109, United States

Supporting Information



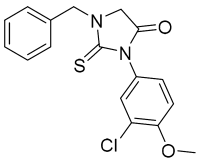
ABSTRACT: A high throughput screening and subsequent hit validation identified compound **1** as an inhibitor of *Trypanosoma brucei* parasite growth. Extensive structure−activity relationship optimization based on antiparasitic activity led to the highly potent compounds, 1-(4-fluorobenzyl)-3-(4-dimethylamino-3-chlorophenyl)-2-thiohydantoin (**68**) and 1-(2-chloro-4-fluorobenzyl)-3-(4-dimethylamino-3-methoxyphenyl)-2-thiohydantoin (**76**), with a *T. brucei* EC_{50} of 3 and 2 nM, respectively. This represents >100-fold improvement in potency compared to compound **1**. In vivo efficacy experiments of **68** and **76** in an acute mouse model of Human African Trypanosomiasis showed a 100% cure rate after 4 days of oral treatment at 50 mg/kg twice per day.

KEYWORDS: *Human African Trypanosomiasis, "sleeping sickness", Trypanosoma brucei inhibitor, thiohydantoins, hit-to-lead optimization*

HAT (Human African Trypanosomiasis) or sleeping sickness is one of the most neglected diseases of human, caused by parasites of *Trypanosoma brucei* species, and spread through the bite of infected tsetse flies. Most of the morbidity is due to the late neurological stage when parasites cross the blood−brain barrier. Without treatment, the disease is invariably fatal. The WHO approved treatment for late-stage HAT is nifurtimox−eflornithine combination therapy (NECT), which requires intravenous administration, skilled staff, and inpatient monitoring.[1] A new drug, effective for late stage disease, which is nontoxic and orally administered, is urgently needed. Moreover, this drug should be affordable by national health systems in the disease-endemic countries located in sub-Saharan Africa.

In the course of a high throughput phenotypic screen for compounds that inhibit *T. brucei* growth in vitro,[2,3] we discovered the substituted 2-thiohydantoin (**1**) (Figure 1).

Compounds with the 2-thiohydantoin moiety have been investigated for a variety of applications, including hypolipidemics,[4] anticarcinogenics,[5] antimutagenics,[6] antithyroidals,[7] antivirals,[8−10] antimicrobials,[11,12] antiulcer, and anti-inflammatories,[13] as well as herbicides.[14] Enzalutamide is an FDA approved anticancer drug with a 2-thiohydantoin pharmacophore and is an androgenic receptor antagonist.[15] Despite their use in multiple disease indications, 2-thiohydantoins should not be regarded as promiscuous inhibitors, but rather as a scaffold with a distinct intermolecular interaction profile that can be usefully exploited for drug development.[16]



(1)
EC_{50} (*T.brucei*): 346 nM
Cytotoxicity (CRL-8155): >50000 nM
Microsome $T_{1/2}$ (mouse): 12 min
IC_{50} (CYP3A4): 4015 nM

Figure 1. Screening hit, a 2-thiohydantoin (compound **1**).

Different synthetic methods to prepare 2-thiohydantoin and its derivatives have been described. The most commonly used

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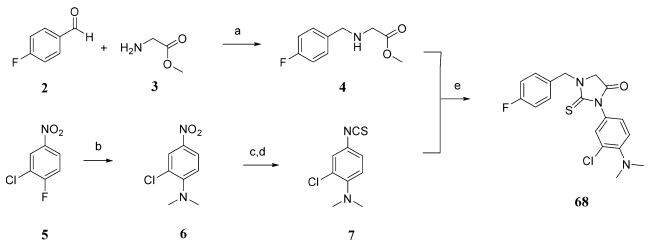
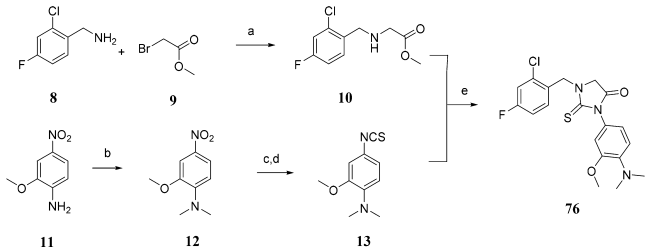
methods are the treatment of α-amino acids with acetic anhydride followed by ammonium thiocyanate[17] or the coupling reaction between α-amino acid derivatives and isothiocyanate.[18,19] We have used the isothiocyanate route to prepare our thiohydantoin library, as it is the most suitable for our purposes. *N*-Benzyl substituted glycine esters and isothioocyanates were prepared as reagents for the synthesis of 1,3-disubstituted 2-thiohydantions (Schemes 1 and 2).

Scheme 1. Representative Synthesis of 2-Thiohydantoin Derivative 68[a]



[a]Reagents and conditions: (a) Et_3N, mol. sieves 3 Å, $NaCNBH_3$, $CHCl_3$, rt, yield 48%; (b) dimethylamine HCl salt, K_2CO_3, DMSO, 80 °C, yield 95%; (c) $SnCl_2$, EtOAc/EtOH, 80 °C, yield 64%; (d) $CSCl_2$, DCM-aq·$NaHCO_3$, 4 °C−rt, yield 84%; (e) EtOH, rt, yield 70%.

Scheme 2. Representative Synthesis of 2-Thiohydantoin Derivative 76[a]



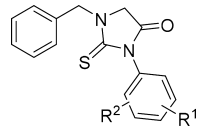
[a]Reagents and conditions: (a) THF, 4 °C−rt, yield 62%; (b) paraformaldehyde, AcOH, $NaCNBH_3$, yield 94%; (c) Pd/C, H_2, MeOH, yield 60%; (d) $CSCl_2$, DCM-aq·$NaHCO_3$, 4 °C−rt, yield 94%; (e) EtOH, rt, yield 84%.

Depending on the commercial availability of starting materials and chemical tractability, N-substituted glycine esters were prepared by reductive amination of aromatic aldehydes with glycine esters (Scheme 1) or by nucleophilic substitution of bromoacetic esters with benzyl amines (Scheme 2). For synthesis of isothiocyanates, not only the commercial availability and chemical tractability but also structural features, such as the presence of a halogen in the aromatic ring, determined the synthetic route. The 3-chloro-4-*N*,*N*-dimethylphenyl isothiocyanate (**7**) was prepared by aromatic nucleophilic substitution of fluorine with dimethylamine, reduction of the nitro group in the presence of halogens (chlorine) with tin chloride, and finally the conversion of the amino group to the isothiocyanate by treatment with thiophosgene in a heterogeneous mixture (Scheme 1). The preparation of 3-methoxy-4-*N*,*N*-dimethylphenyl isothiocyanate (**13**) started with reductive alkylation of 2-methoxy-4-nitroaniline, followed by reduction of the nitro group with hydrogen/palladium catalyst and conversation of the amino group to the isothiocyanate (Scheme 2). The final condensation and subsequent cyclization reaction of the N-substituted glycine ester with isothiocyanates were done in ethanol at room temperature. The corresponding 2-thiohydantoin derivatives were precipitated from the reaction mixture or purified by column chromatography.

Hit compound **1** showed submicromolar activity in *T. brucei* growth inhibition assays, low CYP inhibition activity, and no detected cytotoxicity on the mammalian CRL-8155 or Hep G2 cell lines. Compound **1** displayed moderately rapid degradation in the presence of murine microsomes (Figure 1). Improving *T. brucei* activity and microsomal stability were the first prerequisites for advancing this scaffold to efficacy testing in mouse models of *T. brucei* infection.

We started SAR investigation of this scaffold by modification of the 3-chloro-4-methoxyphenyl moiety. Replacing 3-Cl and/or 4-OMe with hydrogen(s) led to inactive compounds **14**, **15**, and **16** (Table 1). Changing the substitution position of

Table 1. In Vitro Antitrypanosomal Evaluation of *N*-Benzyl-2-thiohydantoins Derivatives (SAR R_1−R_2)



compd	R^1	R^2	*T.brucei* EC_{50} (nM)[a]
1	4-OMe	3-Cl	346
14	4-OMe	H	>10000
15	3-Cl	H	>20000
16	H	H	>10000
17	3-OMe	H	>10000
18	4-Cl	H	>10000
19	2-Cl	H	>20000
20	5-Cl	2-OMe	>10000
21	4-CF_3	3-Cl	>10000
22	4-F	3-Cl	>10000
23	5-Cl	2-F	>10000
24	4-F	H	>10000
25	3-CF_3	5-CF_3	>10000
26	4-$N(Me)_2$	H	1500
27	4-isoPr	H	>10000
28	4-OH	H	>10000
29	4−C(O)Me	H	>10000
30	4-COOH	H	>10000
31	4-OMe	2-OMe	637
32	4-OMe	3-OMe	161
33	4-OMe, 5-OMe	3-OMe	>10000
34	4-$N(Me)_2$	3-Cl	16
35	4-$N(Me)_2$	3-F	125
36	4-$N(Me)_2$	3-OMe	13
37	4-OMe	3-$N(Me)_2$	431

[a]Concentration of compound required to inhibit growth by 50% (EC_{50}) in *T. brucei brucei* strain BF427. Pentamidine isethionate was included as a control for all of the *T. brucei* EC_{50} assays with average ± SEM: 1.15 ± 0.09 nM (n = 32).

chlorine or methoxy group in monoderivatives **17**, **18**, and **19** did not show any positive effects on the compound's activity. The relative position of chlorine and methoxy group was important for the activity as seen by the inactive 5-chloro-2-methoxy analogue (**20**). The results indicated that both substituents had synergetic effects on activity, and their positions in the ring were important. When the methoxy group in **1** was replaced with the trifluoromethyl **21** or the fluoro **22** group, the potency decreased. The combination of

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chlorine and fluorine groups in compound **23** as well as monofluoro substitution in the compound **24** phenyl ring led to inactive compounds. Bis-trifluoromethyl derivative **25** was also inactive.

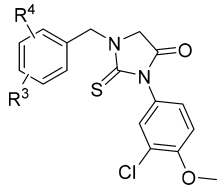
The breakthrough came with the 4-*N*,*N*-dimethylamino analogue (**26**), with an EC_{50} of 1500 nM, five times less active than **1**, but at least six times more potent than 4-methoxy analogue (**14**), which indicated a higher influence on activity from the *N*,*N*-dimethylamino group compared to the methoxy-group. 4-Iso-propyl **27**, 4-phenol **28**, 4-acetyl **29**, and 4-carboxylic **30** derivatives were all inactive. Substitution of chlorine in **1** with a methoxy group increased the potency of the 3,4-dimethoxy derivative **31** two-fold, while 2,4-dimethoxy derivative **32** showed a decrease in antitrypanosomal activity (EC_{50} of 161 and 637 nM, respectively), confirming the preferable substitution position as the para−meta combination. Addition of the third methoxy group at position 5 to compound **31** was detrimental to activity, as the corresponding compound **33** was completely inactive. By combining the best groups and positions mentioned above, we synthesized the 4-*N*,*N*-dimentylamino-3-chloro derivative **34** with *T. brucei* EC_{50} = 16 nM, 4-*N*,*N*-dimethylamino-3-methoxy analogue **36** with EC_{50} = 13 nM, and 4-*N*,*N*-dimethylamino-3-fluoro analogue **35** with EC_{50} = 125 nM. Switching the substitution position of dimethylamino and methoxy group in **36** led to a 30-fold drop in activity in compound **37**, which provided further support of the importance of para−meta positions for activity.

The next region that has been explored was the substitution on the benzyl ring in (3-chloro-4-methoxyphenyl)-2-thiohydantoin, starting with introduction of substituents at position 4 (Table 2). The 4-chloro derivative (**38**) was less potent than unsubstituted benzyl compound **1**. Compounds with activating or deactivating groups such as 4-methyl (**41**), 4-methoxy (**42**), 4-trifluoro (**43**), 4-cyano (**44**), 4-ethylcarboxylate (**45**), and 3-pyridyl analogue (**46**) had low or no activity. Improvement of antiparasitic potency was observed with the 4-fluoro derivative (**39**), which is seven times more potent against *T. brucei* parasites (EC_{50} = 52 nM) relative to **1**. Changing the position of the fluorine from para to meta (compound **40**) decreased potency 18-fold. Based on these results, we speculate that there is not enough space in the binding pocket for a large substituent near the para position and/or that there is specific hydrogen bonding to the fluorine at the para position. Nevertheless, we kept the 4-fluorine substitution in the benzyl ring and added substituents at the ortho and meta positions. The addition of the second fluorine at the meta position (**47**) decreased the potency 6-fold compared to **39**, while addition at the ortho position (**48**) retained the activity. The relative position of both fluorines was important as seen by inactive 2,5-difluoro analogue (**49**). Introducing a larger (compared with H or F) substituent like cyano (**50**) at the meta position eliminated the activity. The ortho position in compound **39** tolerated changes to the size and nature of the substituents, with 4-fluoro-2-chloro derivative (**51**) as the most active analogue with EC_{50} = 46 nM, and 4-fluoro-2-methoxy (**52**), 4-fluoro-2-nitro (**53**), 4-fluoro-2-trifluoromethyl (**54**) analogues were considered for further optimization to increase potency and stability.

To determine the importance of the 2-thiohydantoin core, we modified the 2-thiohydantoin cycle by conversion to hydantoin (S to O change) (**55**) or substitution of the glycine α-hydrogen with ethoxy (**56**), methyl (**57**), or carbonyl (**58**) groups (Figure 2). All changes to the 2-thiohydantoin core were highly detrimental to antitrypanozomal activity. Methylation of the α-benzyl position of **39** resulted in loss of activity for compound **59**. It was determined that the 2-thiohydantoin cycle should remain unmodified in further optimization. All compounds with a chiral center were prepared as racemates.

Based on the SAR described above, the next step was to combine the substituents with the highest influence on activity into one molecule in order to optimize the compounds' antitrypanosomal properties. We chose more than one pair of

Table 2. In Vitro Antitrypanosomal Evaluation of 3-Chloro-4-(dimethylamino)phenyl-2-thiohydantoin Derivatives (SAR of R_3−R_4)



compd	R^3	R^4	*T.brucei* EC_{50} (nM)[a]
1	H	H	346
38	4-Cl	H	853
39	4-F	H	52
40	3-F	H	968
41	4-Me	H	1553
42	4-OMe	H	>20000
43	4-CF_3	H	>20000
44	4-CN	H	17818
45	4−C(O)OEt	H	>20000
46	3-pyridyl	H	3000
47	4-F	3-F	313
48	4-F	2-F	63
49	5-F	2-F	9260
50	4-F	3-CN	>20000
51	4-F	2-Cl	46
52	4-F	2-OMe	191
53	4-F	2-NO_2	350
54	4-F	2-CF_3	924

[a]Concentration of compounds required to inhibit growth by 50% (EC_{50}) of *T. brucei brucei* strain BF427. Pentamidine isethionate was included as a control for all of the *T. brucei* EC_{50} assays with average ± SEM: 1.15 ± 0.09 nM (n = 32).

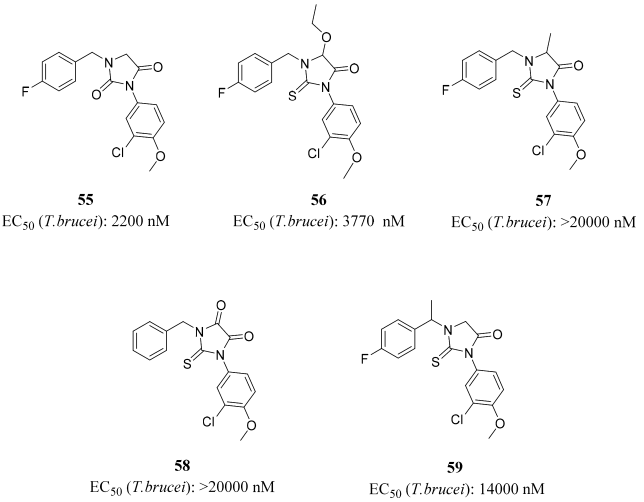


Figure 2. 2-Thiohydantoin ring modifications.

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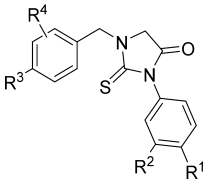
substituents (four for the phenyl ring and seven for the benzyl ring) in order to have a large pool to select the best compounds from.

For substituents R_1 and R_2 on the phenyl group, we selected 4-methoxy and 3-methoxy groups (**60**–**66**) and three 4-dimethylamino pairs: 4-dimethylamino and 3-chloro (**67**–**73**), 4-dimethylamino and 3-fluoro (**74**–**75**), and 4-dimethylamino and 3-methoxy (**76**–**82**) groups (Table 3). 4-Pyrrolidino-3-chloro analogues (**83**–**85**) were prepared with the aims discussed below. As for substituents R_3 and R_4 at the benzyl moiety, we selected the seven most active 4-fluoro analogues: 4-fluoro and 2-chloro, 4-fluoro with H at R_4, 4-fluoro and 2-fluoro, 4-fluoro and 2-methoxy, 4-fluoro and 3-fluoro, 4-fluoro and 2-nitro, and 4-fluoro and 2-trifluoromethyl groups (Table 3). The influence of each pair of substituents on the anti-*T. brucei* activity can be evaluated as follows (R^1 and R^2): (4-$N(CH_3)_2$ and 3-Cl) ≥ (4-$N(CH_3)_2$ and 3-OMe) > (4-pyrrolidino and 3-Cl) > (4-$N(CH_3)_2$ and 3-F) ≥ (4-OMe and 3-OMe). The R^3 and R^4 substituents are in the order (4-F and 2-Cl) > (4-F and H) ≥ (4-F and 2-F) > (4-F and 2-OMe) > (4-F and 3-F) > (4-F and 2-CF_3) ≥ (4-F and 2-NO_2) and are ordered in Table 3 accordingly. Out of 26 compounds, 23 had *T. brucei* EC_{50} values below 50 nM and showed no cytotoxicity up to 50 μM. The most active compounds **68** and **76**, with EC_{50} of 3 and 2 nM, respectively, were assessed for plasma protein binding, solubility, and metabolic stability against pooled mouse liver microsomes. They were also tested in mice for oral pharmacokinetics and brain concentrations (at 60 min postdose) to assess the brain to plasma ratios (Table 4).

Table 3. In Vitro Activity and Cytotoxicity for Select 2-Thiohydantoins



60-66 R^1=R^2=OMe
67-73 R^1=$N(Me)_2$, R^2=Cl
74-75 R^1=$N(Me)_2$, R^2 =F
76-82 R^1=$N(Me)_2$, R^2=OMe
83-85 R^1=Pyrrolidine, R^2= Cl

compd	R^1	R^2	R^3	R^4	*T. brucei* EC_{50} (nM)[a]	cytotoxicity CC_{50} (× 10^3 nM)[b]
60	OMe	OMe	F	2-Cl	14	36.8
61			F	H	49	>50
62			F	2-F	33	>50
63			F	2-OMe	107	>50
64			F	3-F	115	>50
65			F	2-NO_2	555	>50
66			F	2-CF_3	37	>50
67	$N(Me)_2$	Cl	F	2-Cl	2	>50
68			F	H	3	39
69			F	2-F	4	>50
70			F	2-OMe	11	12.6
71			F	3-F	13	>50
72			F	2-NO_2	19	>50
73			F	2-CF_3	22	>50
74	$N(Me)_2$	F	F	2-Cl	15	>50
75			F	2-F	28	>50
76	$N(Me)_2$	OMe	F	2-Cl	2	>50
77			F	H	7	>50
78			F	2-F	6	>50
79			F	2-OMe	11	39.6
80			F	3-F	28	>50
81			F	2-NO_2	44	>50
82			F	2-CF_3	28	>50
83	PYR	Cl	F	2-Cl	9	>50
84			F	H	27	>50
85			F	2-F	10	>50

[a]Concentration of compound required to inhibit growth by 50% (EC_{50}) of *T. brucei brucei* strain BF427. Pentamidine isethionate was included as a control for all of the *T. brucei* EC_{50} assays with average ± SEM: 1.15 ± 0.09 nM (n = 32). [b]Concentration of compound required to inhibit growth by 50% (CC_{50}) of human lymphoblasts (CRL-8155). Quinacrine was included as a control for all CRL-8155 assays, with an average ± SEM: 2.34 ± 0.31 μM (n = 5).

Table 4. Activity, CYP Inhibition, Metabolic Stability, Oral Pharmacokinetics, Brain Penetration, Protein Binding, and Solubility for Compounds 68 and 76

compd	**68**	**76**
T. brucei EC_{50} (nM)[a]	3.2	1.9
CYP3A4 IC_{50} (nM)[b]	12798	7760
microsome $T_{1/2}$ (min)[c]	15	11
oral PK C_{max} (μM)[d]	2.1 ± 0.1	2.5 ± 0.3
oral PK AUC (min·μmol/L)[d]	1376 ± 187	750 ± 130
brain/plasma ratio[e]	0.74 ± 0.30	1.68 ± 0.35
protein binding (%)[f]	88.8 ± 1.5	96.3 ± 0.5
solubility at pH 7.4 (μM)[g]	2.1	4.1

[a]Concentration of compound required to inhibit growth by 50% (EC_{50}) of *T. brucei brucei* strain BF427. Pentamidine isethionate was included as a control for all of the *T. brucei* EC_{50} assays with average ± SEM: 1.15 ± 0.09 nM (n = 32). [b]Concentration to inhibit CYP3A4 by 50%. Ketoconazole was run as a control with a value of 29.6 nM, which aligned with other assays (25.62 ± 3.48 nM (n = 29)). [c]Compound half-life time in mouse microsomes. Testosterone ($T_{1/2}$ = 3.97 min) and dextromethorphan HBr ($T_{1/2}$ = 9.51 min) were included as controls and fell into acceptable ranges. Average ± SEM of testosterone, 4.6 ± 0.6 min (n = 47), and dextromethorphan HBr, 10.2 ± 0.9 min (n = 51) [d]Average values ± SEM of three mice each given a single dose at 50 mg/kg by oral gavage. [e]Average values ± SEM of three mice each given a single ip dose at 5 mg/kg. [f]Protein binding was assessed for each compound in triplicate at 2 μM solution in 50% mouse plasma. Propranolol was included as a binding control with 91.7 ± 1.0% total binding. [g]Solubility was assessed in phosphate buffered saline.

With rather moderate metabolic stability, high brain plasma ratios, a reasonable PK profile, and low protein binding, compounds **68** and **76** were selected for in vivo mouse efficacy experiments. In a model of the acute phase of HAT, five mice were infected with *T. brucei rhodesiense* at day 0 and were given the test compounds for 4 days, beginning 2 days postinfection. Compounds **68** and **76** attained 5/5 cures, as determined by the absence of detectable parasitemia in any of the treated mice for 60 days postinfection (Figure 3). All mice that received only vehicle showed high parasitemia on the last day of dosing, with all concentrations >1.5 × 10^7 parasites/mL of blood, and were euthanized.

The 100% cure rate in the murine model demonstrates that in vitro activity of these compounds translates to an in vivo model, clearing parasites from mice blood and lymphatic systems.

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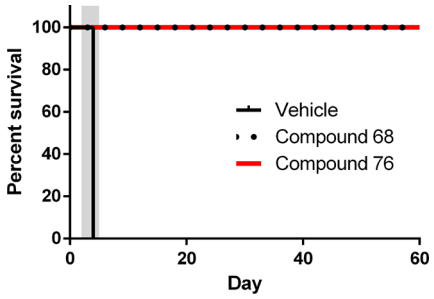

Figure 3. Mouse efficacy model of acute *T. brucei* infection. All mice were infected with *T. brucei rhodesiense* STIB900 on day 0. Groups of five mice each were treated with compound **68** and compound **76** (50 mg/kg by oral gavage b.i.d.) or vehicle from days 2−5 (gray-shaded area). Mice were monitored for parasitemia in tail-blood samples through day 60 postinfection.

In summary, we have discovered 1-benzyl-3-aryl-2-thiohydantoins as potent antitrypanosomal agents. Extensive SAR studies identified 4-fluorobenzyl/4-fluoro-2-chloro benzyl and 4-dimethylamino-3-chloro/(methoxy)phenyl substituents at 2-thiohydantion core with the highest improvement in antiparasitic activity. Compounds **68** and **76** completely cleared parasites from mice in an acute *T. brucei* infection model. Investigation of more thiohydantion analogues is needed to find compounds optimized for metabolic stability, oral PK properties, and brain exposure, at which point we can advance the scaffold to efficacy studies in a chronic *T. brucei* infection model, where the parasites have crossed the blood−brain barrier.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00230.

Biochemical assays, pharmacokinetic and in vivo experiments, experimental procedures for the synthesis of compounds **68** and **76**, general experimental procedure and characterization of compounds **1** and **14**−**85** (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: gelb@chem.washington.edu (M.H.G.).
*E-mail: fbuckner@uw.edu (F.S.B.).

ORCID

J. Robert Gillespie: 0000-0002-5650-0333
Michael H. Gelb: 0000-0001-7000-5219

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

All rodent experiments were done in compliance with the University of Washington Institutional Animal Care and Use Committee (IACUC) approved protocol.
The authors declare no competing financial interest.

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■ ABBREVIATIONS

HAT, Human African Trypanosomiasis; SAR, structure−activity relationship; WHO, World Health Organization; PYR, pyrrolidine; SEM, standard error of the mean

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