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Salicylanilide Inhibitors of *Toxoplasma gondii*

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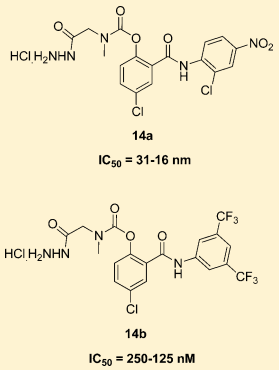
ABSTRACT: *Toxoplasma gondii* (*T. gondii*) is an apicomplexan parasite that can cause eye disease, brain disease, and death, especially in congenitally infected and immune-compromised people. Novel medicines effective against both active and latent forms of the parasite are greatly needed. The current study focused on the discovery of such medicines by exploring a family of potential inhibitors whose antiapicomplexan activity has not been previously reported. Initial screening efforts revealed that niclosamide, a drug approved for anthelmintic use, possessed promising activity in vitro against *T. gondii*. This observation inspired the evaluation of the activity of a series of salicylanilides and derivatives. Several inhibitors with activities in the nanomolar range with no appreciable in vitro toxicity to human cells were identified. An initial structure−activity relationship was explored. Four compounds were selected for evaluation in an in vivo model of infection, and two derivatives with potentially enhanced pharmacological parameters demonstrated the best activity profiles.



INTRODUCTION

The apicomplexan family of parasites, which includes members such as *Plasmodium*, *Babesia*, and *Toxoplasma*, are protozoa of great medical and economic significance.[1] *T. gondii* is one of the most successful parasites on earth, infecting all warm-blooded animals and one-third to one-half of the human population. This parasite can cause disease, toxoplasmosis, with eye and neurological damage, systemic illness, and death. Toxoplasmosis can be especially devastating in those infected congenitally, immune-compromised persons, or those with postnatally acquired infection.

Although the only definitive hosts of this obligate, intracellular parasite are members of the Felidae (cat) family, in humans and other intermediate hosts, *T. gondii* exists in two life stages: the rapidly proliferating tachyzoite form and the latent encysted bradyzoite form, which remains in the body for the duration of the lifetime of the host, maintaining the risk of recurrence.[2] There are currently no effective treatments against the bradyzoite form, and those medicines which target the tachyzoite form (pyrimethamine and sulfadiazine are the most effective) can be associated with toxicity and hypersensitivity.[3] Novel, nontoxic anti-*Toxoplasma* agents are greatly needed.

Niclosamide (5-chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide, **4**) is a well-established FDA-approved anthelminthic drug[4] whose activity in the tapeworm is thought to involve the uncoupling of oxidative phosphorylation.[5] It is not toxic at high concentrations when administered orally.[6] Earlier unpublished studies by our group had shown that niclosamide was a potential inhibitor of *T. gondii* (MIC_{50} 250−200 nM). Although niclosamide has the disadvantage of low solubility and low bioavailability,[7,8] its promising activity against *T. gondii* inspired the preparation and testing of a series of salicylanilides and derivatives in the anticipation of potentially improving potency and physicochemical and pharmacological properties. These were evaluated for activity against *T. gondii* tachyzoites and for toxicity toward host cells in vitro. Experiments were conducted to determine whether the observed activity was due to static or cidal effects. The most promising inhibitors that emerged from this study were the carbamate derivatives **14a** and **14b**, which possess an ionizable moiety appended to the salicylanilide core.

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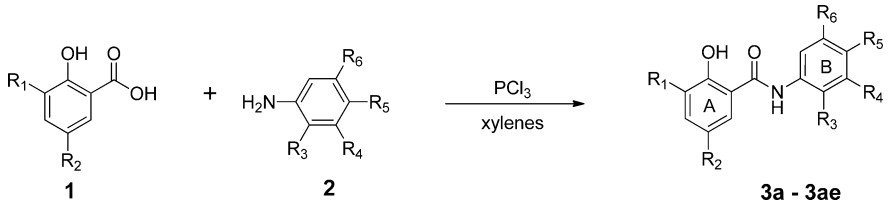
Scheme 1. Preparation of 3a−3 ae



As an apicomplexan parasite, *T. gondii* is often used as a model organism to study other members of this Order, such as *Plasmodium*, *Babesia*, and *Eimeria*. Because the inhibitory activity of the subject compounds against other apicomplexans is of great interest, selected compounds were also tested for efficacy against both drug-sensitive and drug-resistant strains of *Plasmodium falciparum*, the causative agent of the most virulent form of malaria in humans, and were found to be effective as described herein.

CHEMISTRY

Commercially available salicylic acids **1** were coupled with commercially available anilines **2** in hot xylenes in the presence of PCl_3 to furnish salicylanilides **3**[9] (Scheme 1). R_1−R_6 are defined in Table 1.

Reduction of niclosamide **4** with Zn dust in methanol and acetic acid followed by salt formation gave amino salicylanilide hydrochloride **5** (Scheme 2).

Simple ester or carbamate derivatives of **4** were obtained through treatment of **4** with various carbonyl chlorides **6** to provide acylated derivatives **7** (Scheme 3).

The fluorine-containing salicylanilide methyl ethers **10** were synthesized by HATU-mediated condensation of 5-fluoro-2-methoxybenzoic acid **8** with nitroanilines **9** (Scheme 4).

Sarcosine *tert*-butyl ester hydrochloride **11** was transformed into the free base and treated with phosgene in toluene to provide *tert*-butyl 2-((chlorocarbonyl)(methyl) amino)acetate **12**. We found that triphosgene and a solution of phosgene in toluene are essentially equivalent for this transformation. **12** reacted with **4** smoothly in warm pyridine under DMAP catalysis to furnish the expected carbamate ester. Sequential removal of the ester function by treatment with trifluoroacetic acid, condensation of the resulting carboxylic acid **13a** with *tert*-butyl carbazate using EDCI, and treatment of the resulting protected acid hydrazide with HCl in dioxane furnished the sarcosine hydrazide hydrochloride **14a**. In a similar fashion, salicylanilide **3j** was converted to the corresponding sarcosine hydrazide hydrochloride **14b** (Scheme 5).

IN VITRO BIOASSAYS

Parasite proliferation was monitored using stably transfected type I RH-YFP parasites, which constitutively express yellow fluorescent protein. Proliferation also was tested using a [^{3}H]-uracil incorporation assay, as uracil is incorporated into nucleic acids of *T. gondii* tachyzoites, but not mammalian cells, as they divide. Complementary challenge assays ensured that the observed fluorescence data was due to parasite inhibition and not to quenched fluorescence. Pyrimethamine and sulfadiazine were used as positive controls, and DMSO at a concentration of 0.1% was used as the negative control. Because *T. gondii* is an obligate parasite, compounds that are toxic to host cells will appear to inhibit parasite growth. Therefore, all test compounds were simultaneously evaluated for efficacy and toxicity against human cells.

Selected compounds exhibiting the ability to inhibit tachyzoite growth were evaluated to determine whether their activity was due to a static or cidal effect.

Compound activity against *P. falciparum*, a causative agent of malaria, was assessed using the Malaria SYBR Green I-Based Fluorescence (MSF) Assay. The assay is a microtiter plate drug sensitivity assay that uses the presence of malarial DNA as a measure of parasitic proliferation in the presence of antimalarial drugs or experimental compounds based on modifications of previously described methods by Plouffe et al.[10] and Johnson et al.[11] As the intercalation of SYBR Green I dye and its resulting fluorescence is relative to parasite growth, a test compound that inhibits the growth of the parasite will result in a lower fluorescence.

Selected compounds were examined for activity against two strains of *P. falciparum*: D6 (CDC/Sierra Leone), a drug-sensitive strain readily killed by chloroquine, and TM91-C235, a multidrug resistant strain resistant to chloroquine.

IN VIVO BIOASSAYS

Selected compounds were evaluated for in vivo efficacy against *T. gondii*. To assess toxic effects when administered orally, selected compounds were administered orally to mice daily for nine days at a dose of 100 mg/kg. At the end of the 10 days, the animals were evaluated for toxic effects.

Compounds **14a** and **14b** were tested for efficacy against *T. gondii* in the oocyst stage following per oral challenge in mice. Mice were infected by oral gavage with ME49 or TgGoatUS4 oocysts. Mice were treated with either 100 or 25 mg/kg of test substance. Diluent was DMSO (1.0%)/PEG 400 (5.0%)/0.5% CMC (94.0%), where PEG 400 is polyethylene glycol, average MW 400, 0.5% CMC = carboxymethylcellulose 0.5% in water.

RESULTS AND DISCUSSION

A limited medicinal chemistry effort was undertaken to probe the effects of the variation of ring substituents and the derivatization of the phenolic oxygen of the core salicylanilide structure. Salicylanilides **3a−3ae**, **4**, and **5** and derivatives **7a−7d**, **10a**, **10b**, **14 a**, and **14b** were tested for in vitro efficacy against *T. gondii* tachyzoites. It was decided that only those compounds possessing $MIC_{50} \leq 1$ μM would be considered active against *T. gondii*. The efficacy and corresponding cellular toxicity data appear in Table 1. Of the 39 compounds assayed, 16 (41%) had $MIC_{50} \leq 1$ μM, 12 (31%) had $MIC_{50} \leq 500$ nM, 6 (15%) had $MIC_{50} \leq 250$ nM, and 4 (10%) had $MIC_{50} \leq 125$ nM.

Initially, the phenolic functionality was maintained, and two alterations to the A (salicyl) ring were made while retaining the 2′-chloro-4′-nitro B (anilide) ring. Replacement of the 5-chloro of **4** with methyl (**3m**) decreased potency, while replacement with H (**3k**) eliminated activity altogether. Compounds with a

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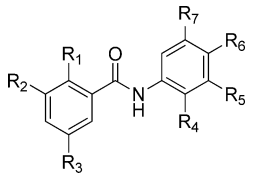
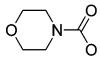
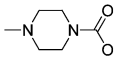
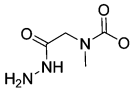
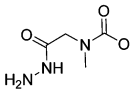
Table 1. Activity of Salicylanilides against *T. gondii*



Compound	R1	R2	R3	R4	R5	R6	R7	MIC_{50}	MIC_{90}
3a	OH	H	Cl	H	CH_3	H	H	>1 μM	>1 μM
3b	OH	H	Cl	H	Br	H	H	750-500 nM	1 μM-750 nM
3c	OH	H	Cl	H	CH_2CH_3	H	H	500-250 nM	750-500 nM
3d	OH	H	Cl	H	C≡CH	H	H	>1 μM	>1 μM
3e	OH	H	Cl	H	$CH{=}CH_2$	H	H	500-250 nM	1 μM-750 nM
3f	OH	H	Cl	H	CF_3	H	H	500-250 nM	500-250 nM
3g	OH	H	Cl	H	CN	H	H	>1 μM	>1 μM
3h	OH	H	Cl	H	F	H	H	570-500 nM	1 μM-750 nM
3i	OH	H	Cl	H	$C(CH_3)_3$	H	H	16-8 nM	31-16 nM
3j	OH	H	Cl	H	CF_3	H	CF_3	31-16 nM	250-125 nM
3k	OH	H	H	Cl	H	NO_2	H	>1 μM	>1 μM
3l	OH	H	Cl	H	CH_2Ph	H	H	1 μM-750 nM	1 μM-750 nM
3m	OH	H	CH_3	Cl	H	NO_2	H	500-250 nM	500-250 nM
3n	OH	H	Cl	H	Cl	H	Cl	500-250 nM	500-250 nM
3o	OH	H	Cl	H	F	H	F	>1 μM	>1 μM
3p	OH	H	Cl	F	H	Cl	H	750-500 nM	750-500 nM
3q	OH	H	Cl	OCH_3	OCH_3	OCH_3	H	>1 μM	>1 μM
3r	OH	H	Cl	Cl	H	H	CN	750-500 nM	1 μM-750 nM
3s	OH	H	Cl	H	CH_3	H	CH_3	>1 μM	>1 μM
3t	OH	H	Cl	H	C≡CH	F	H	>1 μM	>1 μM
3u	OH	H	Cl	H	OCH_2CH_3	H	H	>1 μM	>1 μM
3v	OH	H	Cl	H	OCH_3	OCH_3	H	>1 μM	>1 μM
3w	OH	H	Cl	H	$OCH(CH_3)_2$	H	H	>1 μM	>1 μM
3x	OH	H	Cl	H	OCH_3	H	CH_3	>1 μM	>1 μM
3y	OH	H	Cl	H	OPh	H	H	>1 μM	>1 μM
3z	OH	H	Cl	H	$OCH(CF_3)_2$	H	H	>1 μM	>1 μM
3aa	OH	I	I	H	Cl	H	H	>1 μM	>1 μM
3ab	OH	H	Cl	H	OCH2CH2O		H	>1 μM	>1 μM
3ac	OH	H	F	H	OCH_2CH_3	H	H	>1 μM	>1 μM
4	OH	H	Cl	Cl	H	NO_2	H	250-200 nM	250-200 nM
5	OH	H	Cl	Cl	H	NH_2	H	>1 μM	>1 μM
7a	PhC(=O)O	H	Cl	Cl	H	NO_2	H	125-61 nM	250-125 nM
7b		H	Cl	Cl	H	NO_2	H	>1 μM	>1 μM
7c		H	Cl	Cl	H	NO_2	H	>1 μM	>1 μM
7d		H	Cl	Cl	H	NO_2	H	750-500 nM	1 μM-750 nM
10a	CH_3O	H	F	H	CH_3	H	H	>1 μM	>1 μM
10b	CH_3O	H	F	H	OCH_3	H	H	>1 μM	>1 μM
14a		H	Cl	Cl	H	NO_2	H	31-16 nM	250-125 nM
14b		H	Cl	H	CF_3	H	CF_3	250-125 nM	250-125 nM

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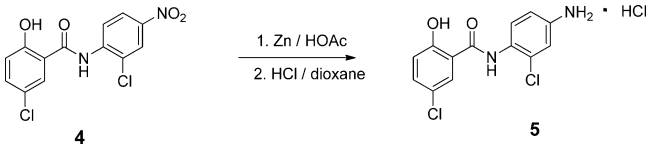
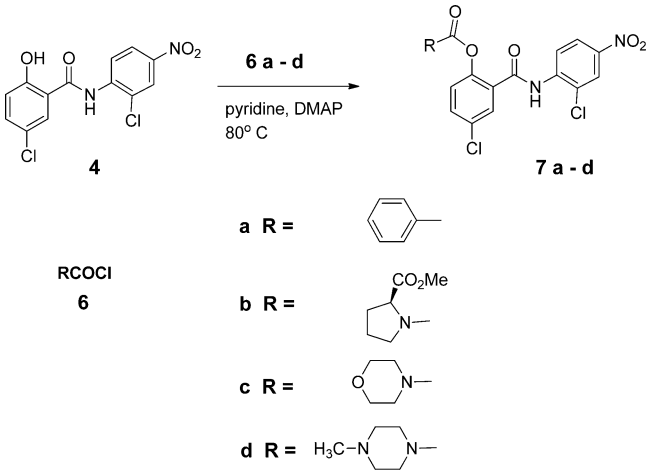
Scheme 2



Scheme 3



4-fluoro substituent (**3ac**, **10a**, and **10b**) or 3,5-diiodo substitution (**3aa**) demonstrated no activity. The decision was made to proceed with the study of 4-chloro A-ring analogues with a variety of B ring substituents.

Altering the electron-withdrawing character of the B ring substituents of **4** had a profound effect on activity. When a nitro group was replaced with an amino group at position 4′, (**5**) all activity was lost. Likewise, compounds possessing *O*-alkyl electron-donating substituents at 2′ or 4′ (**3q**, **3v**, **3ab**) were devoid of activity. It was surprising to note that replacement of the 2′ chloro with the more electronegative fluoro substituent, while simultaneously replacing the 4′ nitro with the less powerful electron withdrawing chloro group (**3p**), removed all activity.

A series of 3′monosubstituted compounds were examined. Various activities were observed, and it is clear that the nature of the substituent at this position has a profound effect. In this series, the activity range shows ^{t}Bu (**3i**) ≫ Et (**3c**) ≈ CH_2CH_2 (**3e**) ≈ CF_3 (**3f**) ≈ F (**3h**) > Br (**3b**) ≈ CH_2Ph (**3l**). All other 3′ substituents resulted in compounds with no activity. Clearly the introduction of 3′-alkoxy or aryloxy substitution resulted in no increase in activity and actually may even be detrimental. When compared to **3i**, the best in the 3′-monosubstituted series, both electronegative (halo, CF_3) and modestly electron-donating (alkyl) substitutions provided moderate activity.

The activity of a few of the 3′-monosubstituted salicylanilides prompted the evaluation of four compounds with substituents at both the 3′ and 5′ positions. Compounds **3o** (3′,5′-difluoro) and **3s** (3′,5′-dimethyl) were inactive, and **3n** (3′,5′-dichloro) showed slight activity. Interestingly, the 3′,5′-bis(trifluoromethyl) derivative **3j** displayed promising activity.

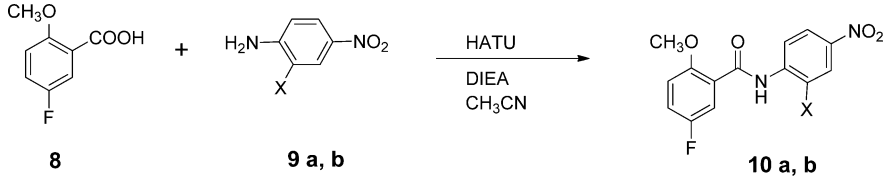
A cursory study of the effect of capping the phenol of **4** via acylation was undertaken. The acylated derivatives **7a**–**d** were prepared and tested. The carbamates **7b**, **7c**, and **7d** impart altered polarity and hydrogen bonding capabilities compared to **4**. **7d** showed modest activity, but **7c** and **7d** were devoid of activity. We were surprised to learn that benzoate ester **7a** showed an apparent increase in potency over **4**. In other studies, we found that **7a** and other carboxylic esters of **4** were hydrolytically labile when incubated in a mixture of THF and buffer (pH 7.4 or pH 8.5), returning **4** at rates dependent on the nature of the ester moiety (data not shown). Any differential activity of **7a** over **4** therefore may be due to altered solubility and permeability parameters which **7a** may possess and that eventual liberation of active **4** may be responsible for enhancing the observed activity. The intrinsic activity of intact **7a** cannot yet be ruled out, and this interesting phenomenon is currently under study. The carbamates **7b**, **7c**, and **7d** are much more stable against hydrolysis when incubated in a mixture of THF and buffer (pH 7.4 or pH 8.5) (data not shown) and are not expected to yield free **4** during bioassay. The fact that these derivatives have no activity suggests that either the increased steric demand of the carbamate groups, or the capping of the phenolic oxygen, renders these compounds inactive.

Two 5-fluorosalicylanilide methyl ethers (**10a** and **10b**) were synthesized, tested, and proved to be inactive. This series of salicylanilide ethers was not further pursued.

In an attempt to improve the activity of compound **4** and one of the most promising salicylanilide analogues **3j**, we prepared ionizable derivatives of each compound. The acid hydrazide salts **14a** and **14b** were designed to possess enhanced solubility and bioavailability relative to the parent structures. We were delighted to learn that **14a** and **14b** possess compelling in vitro and at least minimal in vivo activity against a highly virulent challenge. It is not yet clear whether the ionized derivatives have innate activity or whether these compounds provide, by virtue of increased solubility and permeability, a more effective delivery of the parent salicylanilides. It is possible that the sarcosine hydrazide moiety of these derivatives provides an initial enhancement of concentration by virtue of increased solubility and ultimately cleaves to liberate the active salicylanilide. Research is currently underway to elucidate this mode of enhancement.

The limited number of compounds studied allows a number of observations that suggest a primitive structure–activity relationship. It is appreciated that salicylanilides have been studied for decades, and that it is known that the electronic

Scheme 4



a X = Cl
b X = F

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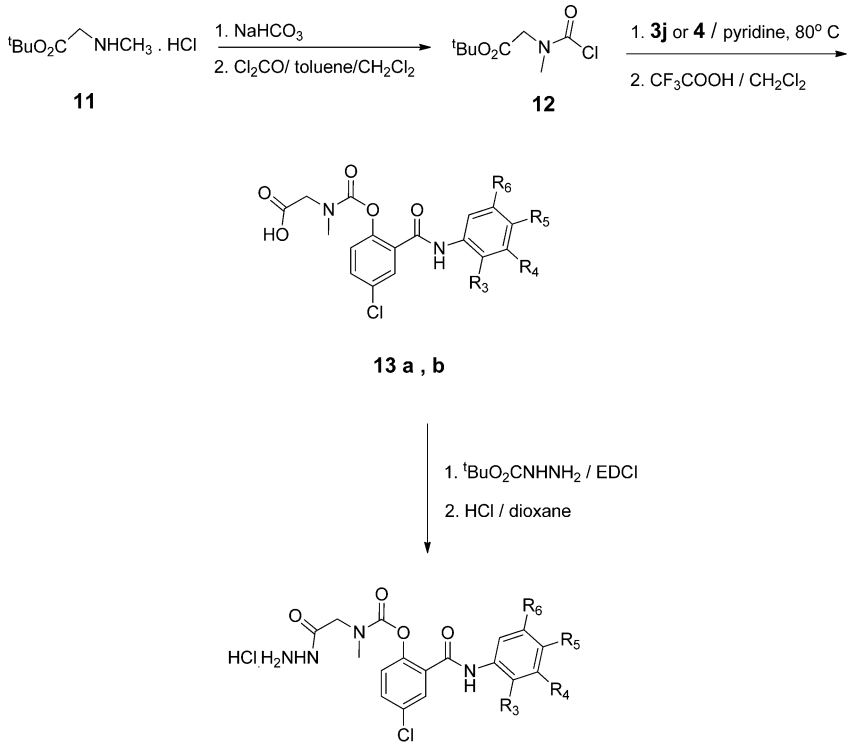
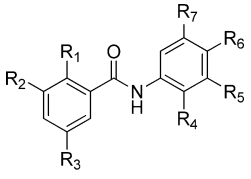
Scheme 5



environment of one aromatic ring strongly influences that of the other. The evaluation of effects on activity of rationally modifying a given substituent while holding the others constant was limited by the scope of the research project and the relative availability of starting materials. The main observation that emerged is that there is a strong interplay between all of the substituents and that a complex relationship of interaction across the amide bond exists. For example, whereas the activity of **4** (MIC_{50} = 250−200 nM, R6 = nitro) is destroyed in **5** (R6 = amino), **3i** (MIC_{50} = 16−8 nM, R6 = H, R5 = *t*-butyl), which possesses neither the strong electron-withdrawing group of **4** or the strong electron-donating group of **5** is active. Further research to elaborate on the observations and trends presented below is planned. The compounds studied are represented by generic structure **15**.



15

Observations of effects of substituents on activity and possible trends in Table 1 are summarized in Table 2.

These screening efforts revealed that six of the compounds were the most effective inhibitors. Of these, **3i**, **3j**, **7a**, **14a**, and **14b** were selected for further in vitro evaluation. Serial dilutions of these compounds to give additional test concentrations were made and studied to identify inhibitory IC_{50} and IC_{90} values. Graphical presentation of parasite inhibition is in Figure 1, while graphical display of toxicity to HFF cells is in Figure 2. The measured IC_{50} and IC_{90} ranges and the corresponding toxicity data are compared in Table 3.

Because the ideal antiparasitic agent would have cidal activity, it is of interest whether potential antiparasitic drugs exhibit a static (inhibition of growth and/or replication) or cidal (lethal) effect. To determine whether leading compounds in this study inhibited parasite proliferation by either a cidal or static mechanism, four were selected (**3i**, **3j**, **7a**, and **14a**) and applied at four to eight times MIC_{50} to parasites. In this assay, RH-YFP tachyzoites were treated with each compound at 1 μM under various dosing conditions:

- Condition A: Parasites were treated for four days, then compound was removed
- Condition B: Parasites were treated for 10 days, then compound was removed
- Condition C: Compound was refreshed at four days then removed at 10 days
- Condition D: Compound was maintained for the duration of the experiment

The four and 10 day time points were taken to reveal the impact of extended exposure of the parasites to the test substance. Compounds were refreshed at four days to examine whether compound degradation could contribute to an observed static effect. Parasite growth was assessed at days

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Table 2. Box 1

	observed substituent effects	trends
R1	• OH, Ph(CO)O, or ionized sarcosine hydrazide carbamates are tolerated • OH is not sufficient for activity • CH_3O, and neutral carbamates not tolerated	Appears that R1-tolerated substituents may be involved in intra- or intermolecular H-bond donors or acceptors. They may also impart electronic effects. The contributions of R1 may be muted by effects of other substituents.
R3	• Cl is necessary in some cases (e.g., **4** is active, **3m** is inactive) • Cl may contribute to activity (except for **3m**, all compounds with activity MIC_{50} < 500nM have R3 = Cl) • Cl is not sufficient for activity (**3a**, **3d**, **3g**, **3o**, **3q**, **3s**−**3z**, **3ab** are inactive) • CH_3 may be tolerated (**3m** is ∼half as active as **4**) • H is not tolerated in some cases (**4** is active, **3k** is inactive)	Appears that R3 substituents play a largely electronic role, but that the contributions of R3 to activity may be muted by effects of other substituents.
R4	• Cl may contribute to activity (the five most active compounds have R4 = Cl) • Cl is not sufficient for activity (**3k**, **5**, **7b**, and **7c** are inactive) • Cl is not necessary for activity (**3i** and **3j** are active) • H is tolerated in some cases	Appears that R4 substituents play a largely electronic role, but that the contributions of R4 to activity may be muted by effects of other substituents.
R5	When R1 = OH and R2 = R3 = R4 = H, the following observations are made: • *O*-alkyl and OPh are not tolerated • Alkyl substituents are tolerated and steric effects are important (**3i** is active, **3a** is inactive) • Alteration of the steric demands of a R5 substituent may provide improved activity (**3i** is much more active than **3l**) • CF_3, F, and Br provide modest activity	Appears that a combination of steric and electronic effects of R5 substituents contribute to activity.
R6	• NO_2 is necessary for the activity of **4** (**5** is inactive) • NO_2 is not sufficient for activity (**3k**, **7b**, and **7c** are inactive) • NO_2 effects are modulated by R3 (**3m** is about one-half as active as **4**; **3k** is inactive) • H is tolerated	Appears that the electron acceptor effects of R6, the electron donor effects of R1, and the nature of R3 contribute to the activity when all three substituents are present. Also appears that the effects of R6 are not necessary for activity when the appropriate R5 substituent is present.
R7	When R1 = OH and R2 = R3 = R4 = H, the following observations are made: • Dual substitution by electron acceptors Cl or CF_3 at R5 and R7 promote activity (**3j** and **3n** are active) • Dual substitution by CH_3 at R5 and R7 is inactive (3s is inactive)	Appears that combined electronic effects of R5 and R7 substituents contribute to activity.

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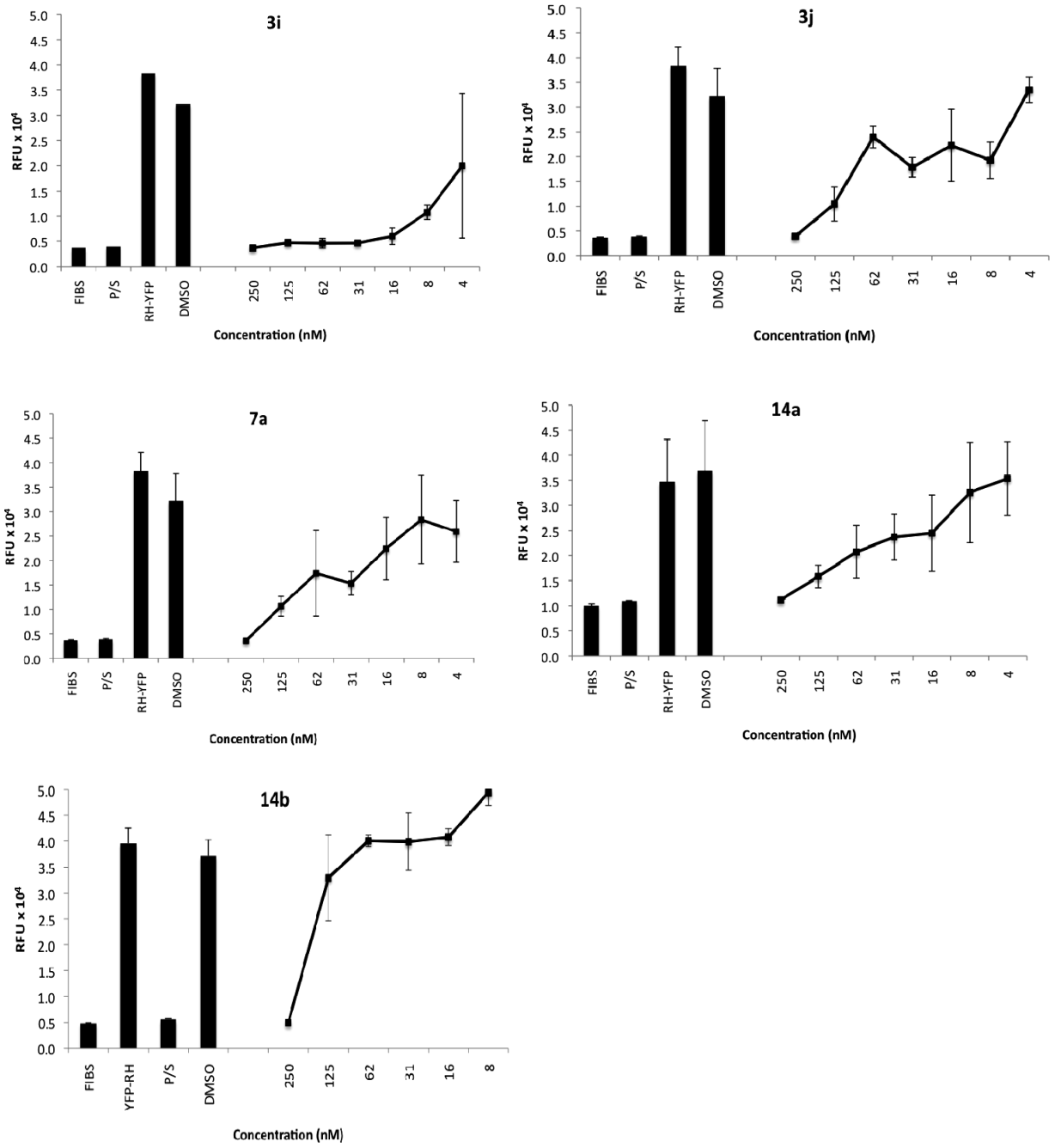

Figure 1. Inhibition of *T. gondii* RH-YFP fluorescence in the presence of compounds **3i**, **3j**, **7a**, **14a**, and **14b**. FIBS, host fibroblasts alone, not infected; P/S, infected control treated with pyrimethamine and sulfadiazine in combination; RH-YFP, untreated infected fibroblast control; 0.1% DMSO (vehicle) infected fibroblast control; [nM], concentration of inhibitor dissolved in 0.1% DMSO. Ordinate axes: relative fluorescence units.

11, 17, and 25. The growth data, as a function of RH-YFP fluoresence, is expressed in Figure 3.

Treatment with **3i** under condition A reveals that the parasite burden is roughly equivalent to untreated controls at day 11. At day 17, condition C dosing of **3i** also shows renewed growth. Application of **3i** under condition D demonstrates inhibition. These data suggest that **3i** is parasitostatic with 4 and 10 days of exposure to the compounds in vitro. In contrast, compounds **3j** and **7a** inhibited growth under all conditions employed in this experiment. No parasite growth observed after the removal of these compounds, even at day 25, suggesting that their activity is parasitocidal. Compounds **3j** and **7a** demonstrated a cidal effect after four days of treatment, while **14a** demonstrated a cidal effect after 10 days of treatment, comparable to treatment with the combination of pyrimethamine and sulfadiazine.

The effect of selected compounds on other apicomplexan parasites was also explored. Compounds **3i**, **3j**, **7a**, and **14a** were examined for activity against two strains of *P. falciparum*, a causative agent of malaria. One of these strains, D6 (CDC/Sierra Leone), is drug-sensitive and readily killed by chloroquine, while the second strain, TM91-C235, is multidrug resistant and shows resistance to chloroquine. The activity of these compounds was assessed using the Malaria SYBR Green I-Based Fluorescence (MSF) Assay. This microtiter plate drug sensitivity assay uses the presence of malarial DNA as a measure of parasitic proliferation. As shown in Table 4,

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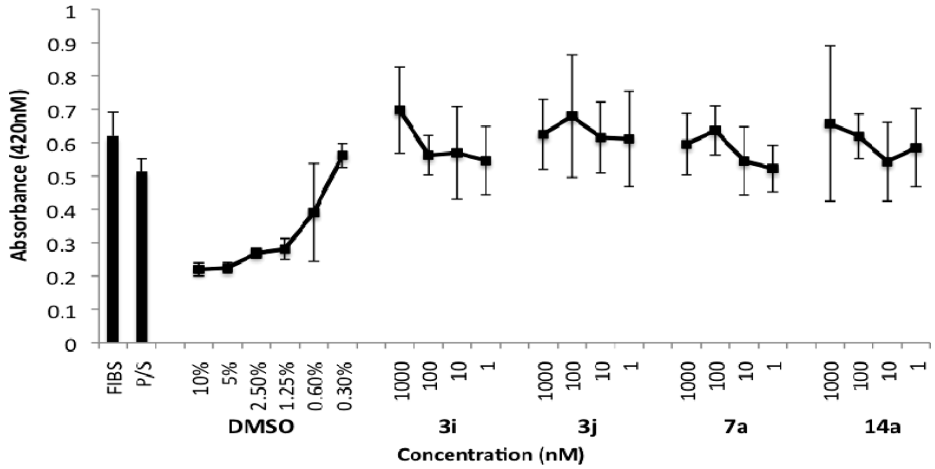

Figure 2. Effect of selected compounds on survival of HFF cells. Ordinate: optical density.

Table 3. Comparison of IC_{50} and IC_{90} Values of Lead Compounds Characterized Further in Vitro

compd	IC_{50} (nM)	IC_{90} (nM)	toxicity (nM)[a]
3i	16−8	31−16	>1000
3j	31−16	250−125	>1000
7a	125−61	250−125	>1000
14a	31−16	250−125	>1000
14b	250−125	250−125	>1000

[a]The HFF used as the host cells in the parasite assay also were used for the toxicity assay.

all compounds demonstrated activity against both *P. falciparum* strains, with **7a** the most effective (D6 chloroquine sensitive IC_{50} = 770 nM) and TM91-C235 chloroquine resistant IC_{50} = 690 nM). Compounds **7a**, **3j**, and **14a** were equally effective against the chloroquine-sensitive D6 and the multidrug resistant Thai strain, TM91-C235, while compound **3i** had a 2-fold higher IC_{50} against TM91-C235 (D6 IC_{50} = 3100 nM and TM91-C235 IC_{50} > 6500 nM). The lack of cross-resistance in compounds **7a**, **3j**, and **14a** is an encouraging finding for a novel scaffold and a valuable lead quality compound attribute given the rapid development of drug resistance against many antimalarials in the field. This initial finding is the basis for future research directed to the development of agents effective against *P. falciparum*.

The in vitro data for *T. gondii* prompted the selection of **3i**, **3j**, **7a**, **14a,** and **14b** for evaluation in mouse models of *T. gondii* infection. Initial difficulties were encountered with the formulation and preliminary safety studies of **3i**, **3j**, and **7a**, presumably due to limited aqueous solubility. Compound **3i**, although slightly more active as a static agent in vitro, was not further derivatized herein because it was not cidal and because of its poorer solubility. It was anticipated that **14a** and **14b**, by virtue of their polar, ionizable appended functionality, may possess improved physicochemical profiles. Consequently, **14a** and **14b** were chosen for evaluation in a mouse model of *T. gondii* oocyst infection.

To explore whether **14a** or **14b** exerted toxic effects when dosed orally, each compound was administered by gavage to mice daily for nine days at a dose of 100 mg/kg. At the beginning, throughout and at the end of the 10 days, all mice were alive and appeared healthy using well-being criteria including sleek fur, normal activity pattern, and movement, suggesting that neither compound had any observable toxic effect upon oral administration at the dosage studied. Thus, compounds **14a** and **14b** were tested for efficacy in a mouse model of *T. gondii* oocyst infection. The oocyst form of the parasite is excreted by cats and is often the form by which people and other animals become infected. This oocyst infection in mice is highly virulent and often fatal, making even limited survival following such a virulent challenge of importance. Mice were infected by oral gavage with ME49 or TgGoatUS4 oocysts. Mice were treated with either a high dose (100 mg/kg) or low dose (25 mg/kg) of **14a** or **14b** 1 mL suspension via oral gavage or were not treated. All uninfected mice dosed with compound alone remained asymptomatic, whereas all mice inoculated orally with oocysts of either strain died of acute toxoplasmosis 8−9 days post infection, and tachyzoites were found in smears of their mesenteric lymph nodes. Treatment with **14a** and **14b** increased survival by 1 day (Table 5, Figure 4).

Kaplan−Meier curves in Figure 4 permit direct comparison of the detailed data set in Table 5.

This is a highly virulent oocyst challenge lethal to all mouse strains tested so far. Although the protection is of modest biological significance, adding only a day of increased survival, it is protection against an otherwise lethal infection and was consistent across doses and strains of parasites and replicate experiments. These dosages of compounds also were not harmful.

In an attempt to discover the molecular target of selected compounds, insertional mutagenesis experiments were performed. The goal of this study was the identification of one or more genes which, when disrupted, confer resistance to the parasite, thus potentially identifying the gene product which, upon interaction with the active compound, inhibits the growth of the parasite. THdhxgTRP tachyzoites were successfully transfected with pLK47 vector plasmid to create parasites with random gene mutations. No parasite growth was observed after prolonged incubation in the presence of **3i**, **3j**, **7a**, or **14b** (data not shown). The value of this approach to elucidate molecular targets or target pathways of *T. gondii* inhibitors was recently demonstrated. In an unrelated study conducted in our laboratory, this methodology has successfully identified the *T. gondii* trafficking pathway inhibited by a series of *N*-benzoyl-2-hydroxybenzamides.[12] This insertional mutagenesis approach was used successfully in parallel studies (ref 12) which served as controls. One interpretation of the data reported herein is that the molecular target of the active inhibitors of the study may be essential. Specifically, the fact that it was not possible to identify

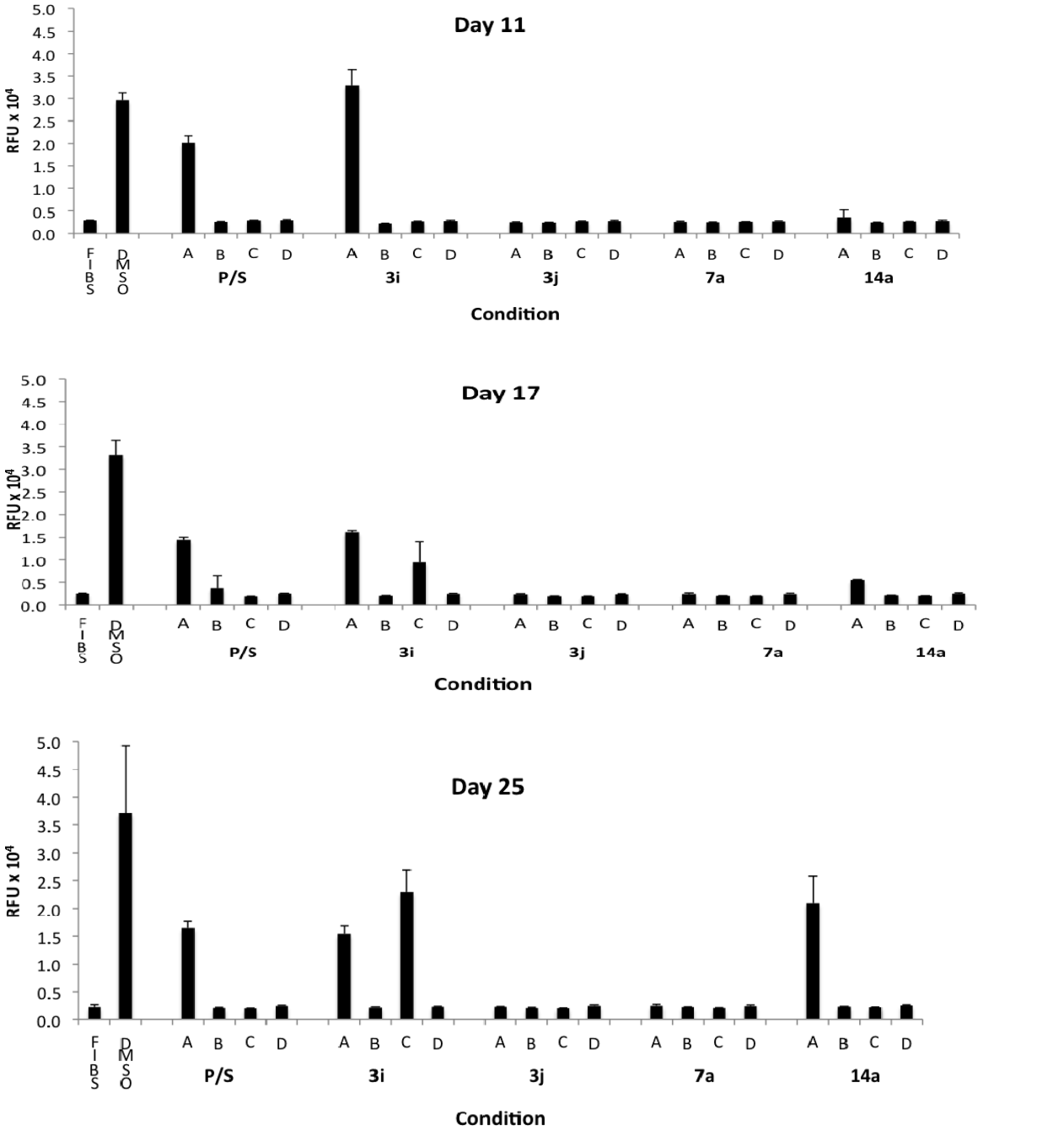

Figure 3. Effect of various dosing conditions on prolonged survival of RH-YFP tachyzoites, measured by inhibition of RH-YFP fluorescence. Abscissa: 4 days, condition A; 10 days, renewed at 4, condition B; 10 days, condition C; all time, condition D. Ordinate: relative flourescence units.

Table 4. Inhibition of *P. falciparum* D6 and C235 by Selected Compounds

compd	D6 IC_{50} (nM)	D6 R^2	C235 IC_{50} (nM)	C235 R^2
chloroquine	7		89	
3i	3100	0.93	>6000	0.67
3j	1400	0.96	1300	0.97
7a	770	0.97	690	0.97
14a	2700	0.95	2700	0.87

the molecular target with an insertional mutagenesis strategy, which has been successful in other work with other targets, indicates that it is likely that when this target is mutated it is lethal for the parasites (i.e., that the target is essential).

Table 5. Efficacy of Compounds 14a and 14b in B7 Mice Infected with *T. gondii* Me-49 or Tg-Goat-US4 Oocysts

compd	dose (mg/kg)	challenge	no. of mice	day of death
14a	100	none	5	none
14a	100	Me-49	5	8, 9, 9, 9, 9
14a	25	none	5	none
14a	25	Me-49	5	8, 9, 9, 9, 9
none	none	Me-49	5	8, 8, 8, 8, 8
14b	100	none	5	none
14b	100	TgGoatUS4	5	8, 9, 9, 9, 9
14b	25	none	5	none
14b	25	TgGoatUS4	5	8, 9, 9, 9, 9
none	none	TgGoatUS4	5	8, 8, 8, 8, 8

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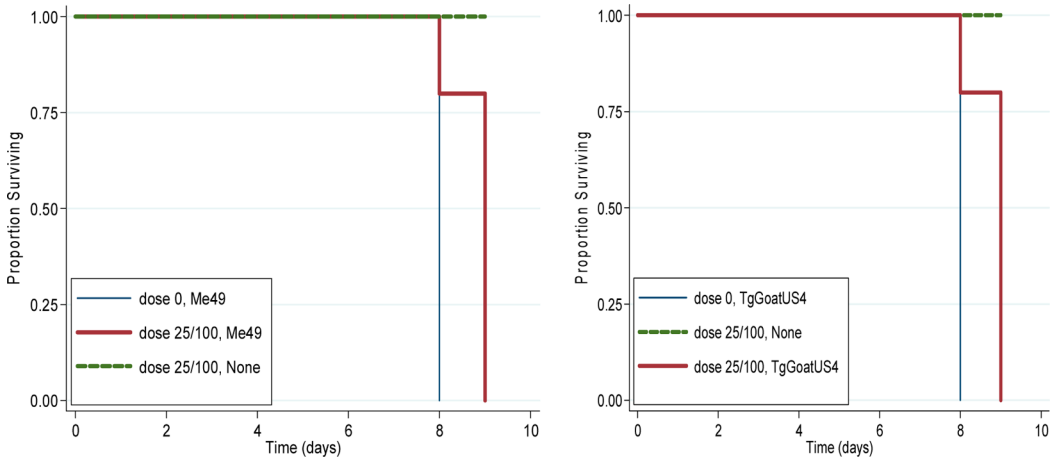

Figure 4. Effect of compound **14a** (left panel) on survival in a mouse model following challenge with *T. gondii* oocysts of the Me-49 strain. On day 8, $p < 0.014$. $N = 5$ per group. Doses 25 and 100 mg/kg (dose 25/100) are shown with a single line as both have the same results. Effect of compound **14b** (right panel) on survival in a mouse model following challenge with *T. gondii* oocysts of the TgGoatUS4 strain. On day 8, $p < 0.014$. $N = 5$ per group. Doses 25 and 100 mg/kg (dose 25/100) are shown with a single line as both have the same results.

The cidal activity of the lead compounds also indicates this is the case and that it is not possible to identify the target by prolonged culture of the parasite in the presence of the concentration of the inhibitor we utilized. Future approaches to target and interacting molecule identification might also include prolonged growth at subinhibitory concentrations to try to identify a resistant mutant followed by sequencing of that mutant. Expression profiling by determining transcriptome of treated parasites early after their treatment to identify the signature of pathways perturbed by the compounds using the connectivity map might also be useful. Also, proteomics with a similar approach, and trying pull down experiments with candidate proteins from *T. gondii* that are homologues of known targets of niclosamide, determining compound effects on these targets, and whether overexpression of the targets could rescue inhibition of parasite growth are other approaches to target identification which could be used in the future.

As *T. gondii* and *P. falciparum* are both members of the same phylum *Apicomplexa*, it is gratifying that the activity profile of the inhibitors is similar for both parasites. It will be of interest in the future to characterize utility of these compounds as agents to treat toxoplasmosis and malaria and perhaps diseases caused by other apicomplexan parasites as well. When molecular targets are identified in the future, it will be of interest to determine whether selectivity or broader utility for multiple apicomplexan parasites with similar targets will be the strengths of this new family of inhibitors. Discoveries of safe and highly cidal leads as potent as the compounds we have created for diseases such as toxoplasmosis and malaria that are worldwide scourges and harm many children are rare.

CONCLUSIONS

Herein, the inhibitory properties of the antihelminthic drug niclosamide inspired the synthesis and evaluation of a series of salicylanilides. The initial in vitro screen yielded five promising agents, compounds **3i**, **3j**, **7a**, **14a**, and **14b**, which were active at low nanomolar concentrations and were not toxic to human host cells. Compound **3i** had a static effect on parasite proliferation, which resumed after drug pressure was removed, while the activity of compounds **3j**, **7a**, and **14a** was cidal. Compounds **14a** and **14b** compounds were effective at prolonging very slightly survival of mice infected with *T. gondii* oocysts and showed no signs of toxicity. Diluent alone had no effect. It will next be important to explore the activity of these compounds against the latent encysted bradyzoite life stage.

3i, **3j**, **7a**, and **14a** were examined for activity against two drug-resistant strains of *P. falciparum*, the causative agent of the most virulent form of malaria in humans. All compounds demonstrated activity against *P. falciparum*, with **7a** as the most effective.

The next steps in the exploration of this series will include detailed investigation of structure−activity relationships, optimizing potency and pharmacodynamic properties, and the identification of the molecular target of this family of compounds. The failure to create drug-resistant insertional mutants in this study suggests the possibility that the target of the compounds studied may be essential. This is a promising characteristic for novel medicines given the risk of emergence of drug-resistant strains. Identification of the molecular target of these compounds would enhance the optimization of activity through structural modification.

EXPERIMENTAL SECTION

Chemistry. *Synthesis of Potential Inhibitors.* Unless otherwise stated, all solvents and reagents were used as received from vendors. ^{1}H NMR spectra were measured at either 400 MHz (Varian) or 500 MHz (Varian Inova AS500) in DMSO-d_6 or $CDCl_3$. HPLC-MS analyses were carried out with a Shimadzu LCMS 2020 using a Phenomenex CHO-8463 C18 column (50 mm × 3.0 mm) with a gradient of 10% acetonitrile:90% water (0.1% formic acid) to 100% acetonitrile (0.1% formic acid) over 5 min. Retention times (T_R) are reported in minutes (min). Mass spectra (ESI) are reported in positive (m/z^+) and/or negative (m/z^-) mode. The calculated exact mass is denoted as EM. Unless otherwise stated, all compounds were obtained at ≥95% purity (HPLC-MS). Niclosamide (compound **4**) was purchased from Sigma-Aldrich (St. Louis, MO). Reactants and reagents were used as received from the vendor except as noted.

General Method of Salicylanilide Synthesis.[9] A suspension of the salicylic acid derivative and aniline in xylenes (0.1−0.5 M) was warmed to reflux, and then a solution of phosphorus trichloride in CH_2Cl_2 or xylenes was introduced dropwise. When the reaction was complete, as determined by TLC or HPLC-MS, the reaction mixture was rapidly transferred while hot by pipet, cannulation, or decanting to a beaker and allowed to cool under rapid stirring. This action removed tarry residue which may accumulate on the reaction vessel walls during the reaction. Typically, the product crystallized from the reaction

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solvent as it cooled or was induced to crystallize upon the slow addition of hexanes when the temperature of the reaction solvent reached 75−80 °C.

*N-(3-Methylphenyl)-5-chloro-2-hydroxybenzamide (**3a**).* A boiling solution of 5-chlorosalicylic acid (0.72 g, 4.17 mmol) in xylenes (10 mL) was treated dropwise with a 2.0 M solution of PCl_3 in CH_2Cl_2 (0.83 mL, 1.67 mmol). After 2 h, the reaction solution was transferred via pipet to a beaker and was allowed to cool to room temperature under rapid stirring. The product separated as off-white crystals. The crude product was recrystallized from EtOAc. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.291 (s, 3H), 6.942 (d, J = 7.2 Hz, 1H), 6.988 (d, J = 8.8 Hz, 1H), 7.227 (M, 1H), 7.425−7.508 (M, 3H), 7.943 (d, J = 2.24 Hz, 1H), 10.322 (s, 1H). HPLC T_R 2.70 min; m/z^+ 261.95 $[M + H]^+$; m/z^- 259.85, $[M - H]^-$ (EM = 261.06).

*N-(3-Bromophenyl)-5-chloro-2-hydroxybenzamide (**3b**).* Using the method described for compound **3a**, 5-chlorosalicylic acid (0.57 g, 3.30 mmol) reacted with 3-bromoaniline (0.36 mL, 3.30 mmol) and 2 M PCl_3 in CH_2Cl_2 (0.66 mL, 1.32 mmol) in xylenes (8 mL). The crude product was recrystallized from EtOAc/hexanes. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.024 (m, 2H), 7.354 (m, 3H), 7.474-(m, 1H), 7.660 (m, 1H), 7.895 (m, 1H), 8.056 (s, 1H), 10.482 (s, 1H). HPLC T_R 2.84 min; m/z^+ 327.85 $[M + H]^+$; m/z^- 325.75 $[M - H]^-$ (EM = 324.95).

*N-(3-Ethylphenyl)-5-chloro-2-hydroxybenzamide (**3c**).* Using the method described for compound **3a**, 5-chlorosalicylic acid (0.63 g, 3.65 mmol) reacted with 3-ethylaniline (0.45 mL, 3.65 mmol) and 2 M PCl_3 in CH_2Cl_2 (0.73 mL, 1.45 mmol) in xylenes (9 mL). The crude product was recrystallized from EtOAc/hexanes. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.198 (t, J = 7.6 Hz, 3H), 2.618 (q, J = 7.6 Hz, 2H), 7.012 (m, 2H) 7.254 (m, 1H), 7.457 (m, 3H), 7.983 (m, 1H), 10.357 (s, 1H). HPLC T_R 2.86 min; m/z^+ 275.95 $[M + H]^+$; m/z^- 273.80 $[M - H]^-$ (EM = 275.07).

*N-(3-Ethynylphenyl)-5-chloro-2-hydroxybenzamide (**3d**).* Using the method described for compound **3a**, 5-chlorosalicylic acid (0.75 g, 4.35 mmol) reacted with 3-ethynyllaniline (0.45 mL, 4.35 mmol) and 2 M PCl_3 in CH_2Cl_2 (0.87 mL, 1.74 mmol) in xylenes (19 mL). The crude product was recrystallized from 2-methyl-1-propanol. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.191 (s, 1H), 6.997 (d, J = 8.8 Hz, 1H), 7.231 (d, J = 7.6 Hz, 1H), 7.366 (dd, J = 8.0, 8.0 Hz, 1H), 7.442 (dd, J = 8.8, 2.8 Hz, 1H), 7.692 (m, 1H), 7.889 (m, 2H), 10.423 (s, 1H). HPLC T_R 2.67 min; m/z^+ 271.90 $[M + H]^+$; m/z^- 269.80 $[M - H]^-$ (EM = 271.04).

*N-(3-Vinylphenyl)-5-chloro-2-hydroxybenzamide (**3e**).* Using the method described for compound **3a**, 5-chlorosalicylic acid (0.56 g, 3.24 mmol) reacted with 3-vinyllaniline (0.37 mL, 3.24 mmol) and 2 M PCl_3 in CH_2Cl_2 (0.65 mL, 1.30 mmol) in xylenes (10 mL). The crude product was recrystallized from EtOH. ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.305 (dd, J = 0.8, 11.6 Hz, 1H), 5.825 (dd J = 0.8, 17.6 Hz, 1H), 6.750 (dd, J = 11.6, 17.6 Hz, 1H) 7.024 (d, J = 8.8 Hz, 1H), 7.272 (d, J = 7.6 Hz, 1H), 7.360 (dd, J = 7.6, 8.0 Hz, 1H), 7.478 (dd, J = 2.8, 8.8 Hz, 1H), 7.618 (d, J = 8.0 Hz, 1H), 7.804 (s, 1H), 7.972 (d, J = 2.8, 1H), 10.416 (s, 1H). HPLC T_R 2.77 min; m/z^+ 273.90 $[M + H]^+$; m/z^- 271.80 $[M - H]^-$ (EM = 273.06).

*N-(3-(Triflouromethyl)phenyl)-5-chloro-2-hydroxybenzamide (**3f**).* Using the method described for compound **3a**, 5-chlorosalicylic acid (2.19 g, 12.69 mmol) reacted with 3-trifluoromethylaniline (1.58 mL, 12.69 mmol) and 2 M PCl_3 in CH_2Cl_2 (2.54 mL, 5.08 mmol) in xylenes (32 mL). The crude product was recrystallized from EtOH. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.035 (d, J = 9.0 Hz, 1H), 7.488 (m, 2H), 7.619 (dd, J = 8.0, 8.0 Hz, 1H), 7.934 (m, 2H), 8.209 (s, 1H), 10.624 (s, 1H). HPLC T_R 2.843 min; m/z^+ 315.90 $[M + H]^+$; m/z^- 628.80 $[2M - H]^-$, 314.80 $[M - H]^-$ (EM = 315.03).

*N-(3-Cyanophenyl)-5-chloro-2-hydroxybenzamide (**3g**).* Using the method described for compound **3a**, 5-chlorosalicylic acid (2.06 g, 11.94 mmol) reacted with 3-amino benzonitrile (1.41 g, 11.94 mmol) and 2 M PCl_3 in CH_2Cl_2 (2.39 mL, 4.78 mmol) in xylenes (30 mL). The crude product was recrystallized from EtOH. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.012 (dd, J = 1.4, 8.5 Hz, 1H), 7.459 (dd, J = 1.8, 8.5 Hz, 1H), 7.580 (m, 2H), 7.856 (m, 1H), 7.916 (m, 1H), 8.182 (s, 1H), 10.588 (s, 1H). HPLC T_R 2.479 min.; m/z^+ = 313.95 $[M + CH_3CN + H]^+$, 272.90 $[M + H]^+$; m/z^- = 542.95 $[2M - H]^-$, 270.85 $[M - H]^-$ (EM = 272.04).

*N-(3-Fluorophenyl)-5-chloro-2-hydroxybenzamide (**3h**).* Using the method described for compound **3a**, 5-chlorosalicylic acid (2.42 g, 14.02 mmol) reacted with 3-amino benzonitrile (1.35 mL, 14.02 mmol) and 2 M PCl_3 in CH_2Cl_2 (2.80 mL, 5.61 mmol) in xylenes (30 mL). The crude product was recrystallized from 2-methyl-1-propanol. ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.995 (m, 2H), 7.433 (m, 3H), 7.706 (m, 1H), 7.897 (d, J = 2.8 Hz, 1H), 10.519 (s, 1H), 11.641 (s, 1H). HPLC T_R 2.639 min; m/z^+ 265.90 $[M + H]^+$; m/z^- 263.85 (EM = 265.03).

*N-(3-tert-Butylphenyl)-5-chloro-2-hydroxybenzamide (**3i**).* Using the method described for compound **3a**, 5-chlorosalicylic acid (2.04 g, 11.82 mmol) reacted with 3-*tert*-butylaniline (1.76 g, 11.82 mmol) and 2 M PCl_3 in CH_2Cl_2 (2.336 mL, 4.73 mmol) in xylenes (30 mL). At completion of reaction, the hot xylenes solvent was decanted, cooled to room temperature, and then diluted with hexanes (30 mL). This was stored at 4 °C for 30 h, during which time an off-white crystalline solid separated. The product was recrystallized from EtOAc/hexanes to give a mixture of the title compound (89.9% and an unidentified impurity (10.1%). ^{1}H NMR of the major component (400 MHz, DMSO-d_6) δ 1.280 (S, 9H), 7.172 (dq, J = 8.0, 0.8 Hz, 1H), 7.283 (dd, J = 8.0, 0.8 Hz, 1H), 7.455 (dd, J = 8.8, 2.6 Hz, 1H), 7.560 (dd, J = 8.0, 0.8 Hz, 1H), 7.679 (M, 1H), 7.982 (d, J = 2.6 Hz, 1H), 10.345 (S, 1H), 11.903 (S, 1H). HPLC T_R 3.095 min; m/z^+ 303.95 $[M + H]^+$; m/z^- = 301.85 $[M - H]^-$ (EM = 303.10).

*N-(3,5-Bis(triflouromethyl)phenyl-5-chloro-2-hydroxybenzamide (**3j**).* Using the method described for compound **3a**, 5-chlorosalicylic acid (0.94 g, 5.48 mmol) reacted with 3,5-bis(trifluoromethyl)aniline (0.85 mL, 5.48 mmol) and 2 M PCl_3 in CH_2Cl_2 (1.10 mL, 2.19 mmol) in xylenes (15 mL). At completion of reaction, the hot xylenes solvent was decanted, cooled to room temperature, and then diluted with hexanes (50 mL). This was stirred at room temperature for 14 h, during which time pure product separated as white crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.048 (d, J = 8.7 Hz, 1H), 7.493 (dd, J = 9.0, 2.7 Hz, 1H), 7.845 (M, 2H), 8.449 (S, 2H), 10.851 (S, 1H), 11.427 (S, 1H). HPLC T_R 3.118 min; m/z^- 381.80 (EM = 383.01).

*N-(2-Chloro-4-nitrophenyl)-2-hydroxybenzamide (**3k**).* Using the method described for compound **3a**, salicylic acid (1.03 g, 7.46 mmol) reacted with 2-chloro-5-nitroaniline (1.29 g, 7.46 mmol) and 2 M PCl_3 in CH_2Cl_2 (1.50 mL, 2.98 mmol) in xylenes (20 mL). The crude product was recrystallized from EtOH. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.055 (M, 1H), 7.495 (M, 1H) 8.053 (dd, J = 8.0, 1.5 Hz, 1H) 8.283 (dd, J = 9.0, 2.5 Hz, 1H), 8.415 (d, J = 2.5 Hz, 1H), 8.854 (d, J = 9.0 Hz, 1H). HPLC T_R 2.482 min; m/z^+ 292.95 $[M + H]^+$; m/z^- 290.80 $[M - H]^+$ (EM = 292.03).

*N-(3-Benzyl phenyl)-5-chloro-2-hydroxybenzamide (**3l**).* Using the method described for compound **3a**, 5-chlorosalicylic acid (0.81 g, 4.69 mmol) reacted with 3-benzylaniline (0.86 g, 4.69 mmol) and 2 M PCl_3 in CH_2Cl_2 (0.94 mL, 1.88 mmol) in xylenes (15 mL). The crude product was recrystallized from toluene. ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.951 (S, 2H), 7.017 (m, 2H), 7.248 (m, 6H), 7.460 (dd, J = 8.5, 2.5 Hz, 1H), 7.550 (m, 2H), 7.948 (d, J = 2.0 Hz, 1H) 10.363 (s, 1H), 11.846 (s, 1H), HPLC T_R 3.017 min; m/z^+ 337.95 $[M+H]^+$; m/z^- 335.85 (EM = 337.09).

*N-(2-Chloro-5-nitrophenyl)-5-methyl-2-hydroxybenzamide (**3m**).* Using the method described for compound **3a**, 5-methylsalicylic acid (0.77 g, 5.06 mmol) reacted with 2-chloro-4-nitroaniline (0.87 g, 5.06 mmol) and 2 M PCl_3 in CH_2Cl_2 (1.01 mL0, 2.02 mmol)in xylenes (18 mL). The desired product (64.5% pure) was found to contain 5.5% of an unidentified contaminant after collection upon cooling of the reaction solvent. ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.261 (s, 3H), 6.949 (d, J = 8.5 Hz, 1H), 7.272 (dd, J = 8.5, 2.0 Hz, 1H), 7.812 (d, J = 2.0 Hz, 1H), 8.257 (dd, J = 9.0, 2.5, 1H), 8.384 (d, J = 2.5, 1H), 8.824 (d, J = 9.0 Hz, 1H). HPLC T_R 2.628 min; m/z^+ 306.95 $[M + 1]^+$; m/z^- 304.85 $[M - H]^-$ (EM = 306.04).

*N-(2,4-Dichlorophenyl)-5-chloro-2-hydroxybenzamide (**3n**).* A suspension of 5-chlorosalicylic acid (1.73 g, 10.0 mmol) and 2,4-dichloraniline (1.62 g, 10.0 mmol) in xylenes (50 mL) was heated to reflux and a solution of PCl_3 (0.35 mL, 4.0 mmol) in xylenes (5.0 mL)

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was introduced in a dropwise manner. After 90 min, the reaction mixture was transferred to a beaker via pipet and was allowed to cool to room temperature under rapid stirring. The crude product was recrystallized from EtOAc. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.075 (dd, J = 8.8, 1.2 Hz, 1H), 7.473 (m, 2H), 7.693 (dd, J = 2.4, 1.2 Hz, 1H), 7.982 (dd, J = 2.8, 1.2 Hz, 1H), 8.457 (dd, J = 8.8, 1.2 Hz, 1H), 10.925 (s, 1H), 12.241 (s, 1H). HPLC T_R 2.889 min; m/z^+ 315.85 $[M + H]^+$; m/z^- 313.70 $[M - H]^-$ (EM = 314.96).

*N-(2,4-Difluorophenyl)-5-chloro-2-hydroxybenzamide (**3o**).* Using the method described for compound **3n**, 5-chlorosalicylic acid (1.73 g, 10.0 mmol) and 2,4-difluoroaniline (1.29 g, 10.0 mmol) reacted in refluxing xylenes (25 mL) in the presence of PCl_3 (0.35 mL, 4.0 mmol). The crude product was recrystallized from EtOH. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.045 (d, J = 9.0 Hz, 1H), 7.143 (M, 1H), 7.410 (M, 1H), 7.498 (dd, J = 9.0, 2.5 Hz, 1H), 7.973 (d, J = 2.5 Hz, 1H), 8.116 (M, 1H), 10.624 (s, 1H), 12.139 (s, 1H). HPLC T_R 2.523 min; m/z^+ 283.90 $[M + H]^+$; m/z^- 281.75 $[M - H]^-$ (EM = 283.02).

*5-Chloro-N-(4-chloro-2-fluorophenyl)-2-hydroxybenzamide (**3p**).* Using the method described for compound **3n**, 5-chlorosalicylic acid (1.73 g, 10.0 mmol) and 2,4-difluoroaniline (1.46 g, 10.0 mmol) reacted in refluxing xylenes (25 mL) in the presence of PCl_3 (0.35 mL, 4.0 mmol). The crude product was recrystallized from EtOAc. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.057 (d, J = 8.8 Hz, 1H), 7.335 (dd, J = 8.8, 1.2 Hz), 7.508 (dd, J = 8.8, 2.8 Hz, 1H), 7.577 (dd, J = 10.4, 2.0 Hz, 1H), 7.959 (d, J = 2.8 Hz, 1H), 8.245 (dd, J = 8.8, 8.8 Hz), 10.704 (s, 1H), 12.166 (s, 1H). HPLC T_R 2.756 min; m/z^+ 299.90 $[M + H]^+$; m/z^- 297.75 $[M - H]^-$ (EM = 298.99).

*5-Chloro-2-hydroxy-N-(3,4,5-trimethoxyphenyl)benzamide (**3q**).* Using the method described for compound **3n**, 5-chlorosalicylic acid (1.73 g, 10.0 mmol) and 3,4,5-trimethoxaniline (1.83 g, 10.0 mmol) reacted in refluxing xylenes (20 mL) in the presence of PCl_3 (0.35 mL, 4.0 mmol). The crude product was dissolved in 100 mL of boiling toluene:heptane (1:1 v/v), and the resulting solution was decanted from a dark insoluble residue. White crystalline product separated upon cooling. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.88−11.84 (s, 1H), 10.33−10.29 (s, 1H), 7.99−7.95 (d, J = 2.7 Hz, 1H), 7.51−7.45 (dd, J = 8.8, 2.6 Hz, 1H), 7.15−7.11 (s, 2H), 7.05−6.99 (d, J = 8.8 Hz, 1H), 3.81−3.77 (s, 6H), 3.68−3.64 (s, 3H), 3.35−3.31 (s, 3H). HPLC T_R 2.400 min; m/z^+ 337.95 $[M + H]^+$; m/z^- 335.85 $[M - H]^-$ (EM = 337.07).

*5-Chloro-N-(2-chloro-5-cyanophenyl)-2-hydroxybenzamide (**3r**).* Using the method described for compound **3n**, 5-chlorosalicylic acid (0.86 g, 5.0 mmol) and 3-amino-4-chlorobenzonitrile (0.76 g, 5.0 mmol) reacted in refluxing xylenes (10 mL) in the presence of PCl_3 (0.18 mL, 2.0 mmol). The crude product was recrystallized from EtOH/water to provide a tan-colored solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.053 (d, J = 8.5 Hz), 7.345 (dd, J = 8.5, 3.0 Hz), 7.407 (dd, J = 8.5, 2.5 Hz), 7.598 (d, J = 8.5 Hz), 8.079 (d, J = 3.0 Hz), 8.979 (s, J = 2.5 Hz), 11.105 (s, 1H), 11.700 (s, 1H.) HPLC T_R 2.551 min; m/z^+ 306.90 $[M + H]^+$; m/z^- = 304.75 $[M - H]^-$ (EM = 306.00).

*5-Chloro-N-(3,5-dimethylphenyl)-2-hydroxybenzamide (**3s**).* Using the method described for compound **3a**, 5-methylsalicylic acid (1.04 g, 6.03 mmol) reacted with 3,5-dimethylaniline and 2 M PCl_3 in CH_2Cl_2 (1.21 mL, 2.42 mmol) and 2 M in refluxing xylenes (15 mL). Pure white crystalline product resulted. ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.260 (s, 6H), 6.780 (s, 1H), 7.015 (M, 2H), 7.315 (s, 2H), 7.451 (dd, J = 9.0, 2.5 Hz, 1H), 7.965 (d, J = 2.5 Hz, 1H), 10.265 (s, 1H). HPLC T_R 2.848 min; m/z^+ 275.95, m/z^- 273.80 (EM = 275.07).

*5-Chloro-N-(3-cyano-4-fluorophenyl)-2-hydroxybenzamide (**3t**).* Using the method described for compound **3n**, 5-chlorosalicylic acid (1.73 g, 10.0 mmol) and 3-ethynyl-4-fluoroaniline (1.36 g, 10.0 mmol) reacted in refluxing xylenes (25 mL) in the presence of PCl_3 (0.35 mL, 4.0 mmol). The crude product was recrystallized from EtOAc/hexanes. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.57 (s, 1H), 10.64 (s, 1H), 8.24 (m, 1H), 8.03 (m, 1H), 7.87 (m, 1H), 7.64 (m, 1H), 7.48 (m, 1H), 7.04 (m, 1H). HPLC T_R 2.546 min; m/z^+ 290.85 $[M + H]^+$; m/z^- 288.80 $[M - H]^-$ (EM = 289.03).

*5-Chloro-N-(3-ethoxyphenyl)-2-hydroxybenzamide (**3u**).* Using the method described for compound **3n**, 5-chlorosalicylic acid (1.73 g, 10.0 mmol) and 3-ethoxyaniline (1.37 g, 10.0 mmol) reacted in refluxing xylenes (25 mL) in the presence of PCl_3 (0.35 mL, 4.0 mmol). The crude product was purified by column chromatography (silica gel, 35 EtOAc:65 hexanes). ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.03−11.61 (s, 1H), 10.62−10.19 (s, 1H), 8.09−7.86 (d, J = 2.7 Hz, 1H), 7.61−6.51 (m, 6H), 4.47−3.72 (q, J = 7.0 Hz, 2H), 1.69−0.97 (t, J = 7.0 Hz, 3H). HPLC T_R 2.729 min; m/z^+ 307.90 $[M + H]^+$; m/z^- 305.85 $[M - H]^-$ (EM = 307.06).

*5-Chloro-N-(3,4-dimethoxyphenyl)-2-hydroxybenzamide (**3v**).* Using the method described for compound **3n**, 5-chlorosalicylic acid (1.73 g, 10.0 mmol) and 3,4-dimethoxyaniline (1.53 g, 10.0 mmol) reacted in refluxing xylenes (25 mL) in the presence of PCl_3 (0.35 mL, 4.0 mmol). The crude product was purified by column chromatography (silica gel, 35 EtOAc:65 hexanes) then recrystallized from EtOAc. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.04−12.00 (s, 1H), 10.33−10.29 (s, 1H), 8.03−7.99 (d, J = 2.6 Hz, 1H), 7.51−7.45 (dd, J = 8.8, 2.7 Hz, 1H), 7.41−7.37 (d, J = 2.4 Hz, 1H), 7.28−7.22 (dd, J = 8.7, 2.4 Hz, 1H), 7.04 (d, J = 8.8 Hz, 1H), 6.94 (d, J = 8.8 Hz, 1H). NMR HPLC T_R 2.359 min; m/z^+ 307.90 $[M + H]^+$; m/z^- 305.85 $[M - H]^-$ (EM = 307.06).

*5-Chloro-2-hydroxy-N-(3-isopropoxyphenyl)benzamide (**3w**).* Using the method described for compound **3n**, 5-chlorosalicylic acid (1.73 g, 10.0 mmol) and 3-isopropoxyaniline (1.51 g, 10.0 mmol) reacted in refluxing xylenes (25 mL) in the presence of PCl_3 (0.35 mL, 4.0 mmol). The crude product was purified by column chromatography (silica gel, 1 EtOAc:1 hexanes). ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.23−11.64 (s, 1H), 10.87−10.01 (s, 1H), 7.96−7.92 (d, J = 2.7 Hz, 1H), 7.50−7.43 (dd, J = 8.8, 2.7 Hz, 1H), 7.39−7.34 (t, J = 2.2 Hz, 1H), 7.27−7.18 (m, 2H), 7.03−6.98 (d, J = 8.8 Hz, 1H), 6.72−6.67 (ddd, J = 7.8, 2.5, 1.4 Hz, 1H), 4.71−4.43 (hept, J = 6.0 Hz, 1H), 1.34−1.13 (d, J = 6.0 Hz, 6H). HPLC T_R 2.843 min; m/z^+ 305.95 $[M + H]^+$; m/z^- 303.85 $[M - H]^-$ (EM = 305.08).

*5-Chloro-2-hydroxy-N-(5-methoxy-2-methylphenyl)benzamide (**3x**).* Using the method described for compound **3n**, 5-chlorosalicylic acid (0.645 g, 3.74 mmol) and 5-methoxy-2-methylaniline (0.513 g, 3.74 mmol) reacted in refluxing xylenes (15 mL) in the presence of PCl_3 (0.13 mL, 1.50 mmol). The crude product was recrystallized from EtOH/H_2O. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.331 (s, 1H), 3.742 (s, 1H), 6.708 (dd, J = 8.4, 1.2, Hz), 7.053 (d, J = 8.4 Hz, 1H), 7.175 (d, J = 8.4 Hz, 1H), 7.188 (m, 1H), J = 7.617 (s, 1H), 8.018 (s, 1H). HPLC T_R 2.510 min; m/z^+ 291.90 $[M + H]^+$; m/z^- 289.85 $[M - H]^-$ (EM = 291.07).

*5-Chloro-2-hydroxy-N-(3-phenoxyphenyl)benzamide (**3y**).* Using the method described for compound **3n**, 5-chlorosalicylic acid (0.919 g, 5.33 mmol) and 3-phenoxyaniline (0.987 g, 5.33 mmol) reacted in refluxing xylenes (20 mL) in the presence of PCl_3 (0.19 mL, 2.13 mmol). The crude product was recrystallized from EtOAc/hexanes. ^{1}H NMR (500 MHz, $CDCL_3$) δ 6.858 (m, 1H), 6.982 (d, J = 8.8 Hz, 1H), 7.057 (d, J = 7.6 Hz, 2H), 7.151 (dd, J = 7.6, 7.2 Hz, 1H), 7.355 (m, 6H), 7.462 (d, J = 2.4 Hz, 1H), 7.794 (s, 1H), 11.776 (s, 1H). HPLC T_R 2.999 min; m/z^+ 339.95 $[M + H]^+$; m/z^- 337.85 $[M - H]^-$ (EM = 339.07).

*5-Chloro-N-(3-(difluoromethoxy)phenyl)-2-hydroxybenzamide (**3z**).* Using the method described for compound **3n**, 5-chlorosalicylic acid (0.86 g, 4.98 mmol) and 3-difluoromethoxyaniline (0.0.79 g, 4.98 mmol) reacted in refluxing xylenes (15 mL) in the presence of PCl_3 (0.17 mL, 1.99 mmol). The crude product was recrystallized from EtOAc. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.82−11.49 (s, 1H), 10.63−10.27 (s, 1H), 7.92−7.87 (d, J = 2.7 Hz, 1H), 7.69−7.65 (t, J = 2.2 Hz, 1H), 7.56−7.51 (ddd, J = 8.2, 2.0, 1.0 Hz, 1H), 7.49−7.45 (dd, J = 8.8, 2.7 Hz, 1H), 7.44−7.39 (t, J = 8.2 Hz, 1H), 7.39−7.06 (t, J = 74.0 Hz, 1H), 7.04−6.99 (d, J = 8.8 Hz, 1H), 6.98−6.93 (dd, J = 8.1, 2.3 Hz, 1H). NMR HPLC T_R 2.665 min; m/z^+ 313.85 $[M + H]^+$; m/z^- 311.80 $[M - H]^-$ (EM = 313.03).

*N-(3-Chlorophenyl)-2-hydroxy-3,5-diiodobenzamide (**3aa**).* A mixture of 5-chlorosalicylic acid (0.573 g, 1.47 mmol) and 2-hydroxy-3,5-diiodobenzoic acid (0.154 mL, 1.47 mmol) in xylenes (15 mL) was brought to reflux whereupon all solids dissolved. A solution of PCl_3 (0.051 mL, 0.588 mmol) in xylenes (5 mL) was introduced in a dropwise fashion. After 1 h, the reaction mixture was allowed to cool to

40 °C and was decanted into rapidly stirring hexanes (40 mL) at rt. White crystals separated, and after 1 h the product was collected by filtration, washed well with hexanes, and air-dried. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.87−12.83 (s, 1H), 10.72−10.68 (s, 1H), 8.38−8.34 (d, J = 2.0 Hz, 1H), 8.27−8.22 (d, J = 1.9 Hz, 1H), 7.86−7.81 (t, J = 2.1 Hz, 1H), 7.67−7.61 (ddd, J = 8.2, 2.1, 1.0 Hz, 1H), 7.47−7.40 (t, J = 8.1 Hz, 1H), 7.29−7.23 (ddd, J = 8.1, 2.1, 0.9 Hz, 1H). HPLC T_R 3.351 min; m/z^+ 499.70 $[M + H]^+$; m/z^- 497.65 $[M - H]^-$ (EM = 498.83).

*N-(2,3-Dihydrobenzo[b][1,4]dioxin-6-yl)-2-hydroxybenzamide (**3ab**).* A mixture of 5-chlorosalicylic acid (1.73 g, 10.0 mmol) and 3,4-methylenedioxyaniline (1.51 g, 10.0 mmol) in xylenes (20 mL) was brought to reflux, whereupon all solids dissolved. A solution of PCl_3 (0.35 mL, 4.0 mmol) in xylenes (5 mL) was introduced in a dropwise fashion. After 1 h, the reaction solution was decanted from a dark residue which had accumulated in the reaction vessel. After cooling, decolorizing carbon (0.25 g) was introduced, and the mixture was heated at 80 °C for 30 min. Decolorizing carbon was removed by filtration through a filter aid. The solution was washed with water, dried over $MgSO_4$, and concentrated. Pure product was obtained by column chromatography (silica gel, 35 EtOAc:65 hexanes. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.99−11.90 (s, 1H), 10.30−10.22 (s, 1H), 8.01−7.94 (d, J = 2.7 Hz, 1H), 7.50−7.43 (dd, J = 8.8, 2.7 Hz, 1H), 7.34−7.29 (d, J = 2.5 Hz, 1H), 7.14−7.08 (dd, J = 8.7, 2.5 Hz, 1H), 7.04−6.98 (d, J = 8.8 Hz, 1H), 6.89−6.82 (d, J = 8.7 Hz, 1H), 4.29−4.21 (qd, J = 3.7, 2.0 Hz, 4H). HPLC T_R 2.482 min; m/z^+ 305.90 $[M + H]^+$; m/z^- 303.80 $[M - H]^-$ (EM = 305.05).

Preparation of *N*-(3-ethoxyphenyl)-5-fluoro-2-hydroxybenzamide (**3ac**). 5-Fluorosalicylic acid (0.312 g, 2.0 mmol), 3-ethoxyaniline (0.274 g, 2.0 mmol), PCl_3 (0.174 mL, 2.0 mmol), and xylenes (15 mL) were placed in a sealed tube and heated to 150 °C for 2 h. Upon cooling to room temperature, the reaction solution was diluted with EtOAc and washed successively with saturated $NaHCO_3$, water, and brine and then dried ($MgSO_4$). Solvents were evaporated, and the residue was purified by preparative TLC (silica gel, 35 EtOAc:65 hexanes). Significant decomposition was observed during the chromatography. Pure product (0.027 g, 4.9% yield) was obtained. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.59 (s, 1H), 10.36 (s, 1H), 7.74 (dd, J = 9.7, 3.2 Hz, 1H), 7.39 (t, J = 2.1 Hz, 1H), 7.28 (m, 3H), 7.00 (dd, J = 9.0, 4.6 Hz, 1H), 6.71 (ddd, J = 7.8, 2.5, 1.4 Hz, 1H), 4.03 (q, J = 6.9 Hz, 2H), 1.34 (t, J = 7.0 Hz, 3H). HPLC T_R 2.532 min; m/z^+ 275.95 $[M + H]^+$; m/z^- 549.00 $[2M - H]^-$, 273.80 $[M - H]^-$ (EM = 275.10).

*N-(4-Amino-2-chlorophenyl)-5-chloro-2-hydroxybenzamide Hydrochloride (**5**).* A slurry of niclosamide (**4**, 1.435 g, 4.38 mmol) in 1MeOH: 1 HOAc (20 mL) was treated with portions of Zn dust (3.0 g total) over 3 h at rt. A mild exotherm was observed. As the reaction progressed, precipitates caused a thickening of the reaction mixture. MeOH was added from time to time to facilitate magnetic stirring. The reaction mixture was filtered through a plug of Celite and concentrated to an off-white residue. This was partitioned between EtOAc and saturated $NaHCO_3$ (copious precipitate appeared in the aqueous phase). Water was added to the mixture to facilitate phase separation. The EtOAc phase was washed with brine, dried ($MgSO_4$), and concentrated to a tan solid. This was recrystallized from EtOAc/hexanes to provide tan crystals (≥99% pure by LC-MS). The aniline product was dissolved in EtOAc by warming a suspension (0.2 M) to 50 °C, and HCl in dioxane (4M, 2 equiv) was added in a dropwise manner. The resulting slurry was diluted with 1 volume of EtOAc, cooled to room temperature, and stirred for an additional 10 min. The product was collected by filtration and washed well with EtOAc. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.37 (s, 1H), 10.81 (t, J = 2.0 Hz, 1H), 8.26 (m, 1H), 7.99 (d, J = 2.8 Hz, 1H), 7.51 (dd, J = 8.8, 2.8 Hz, 1H), 7.38 (m, 1H), 7.16 (m, 2H). HPLC T_R 1.125 min; m/z^+ 339.85 $[M + CH_3CN + 2]^+$, 337.90 $[M + CH_3CN + H]^+$, 298.85 $[M + 2]^+$ 296.85 $[M + H]^+$; m/z^- 296.80 $[M + 2 - H]^-$ 294.80 $[M - H]^-$ (EM = 296.01).

*4-Chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl Benzoate (**7a**).* Benzoyl chloride (0.49 mL, 4.27 mmol) was added dropwise to a suspension of niclosamide (1.27 g, 3.88 mmol) in a solution of 4-dimethylaminopyridine (DMAP, 30 mg) in pyridine (15 mL) at rt. The suspension was warmed to 80 °C, whereupon all solids dissolved. Reaction continued at this temperature for 2 h. The cooled reaction mixture was diluted with EtOAc (100 mL) and was washed successively with 1N HCl until the aqueous wash was acidic (about pH 1) to litmus. The EtOAc phase was washed with brine, dried ($MgSO_4$), and concentrated to an off-white solid. The crude product was recrystallized from EtOAc/hexanes. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.56 (s, 1H), 8.31 (m, 1H), 8.25 (m, 1H), 8.08 (m, 2H), 7.90 (m, 2H), 7.25 (m, 1H), 7.54 (m, 3 H).). HPLC T_R 2.990 min; m/z^+ 430.95 $[M + H]^+$; m/z^- 428.80 $[M - H]^-$ (EM = 430.01).

*(S)-1-(4-Chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl) 2-Methyl Pyrrolidine-1,2-dicarboxylate (**7b**).* (Step A) (*S*)-Methyl 1-(chlorocarbonyl)pyrrolidine-2-carboxylate. L-Proline methyl ester hydrochloride (2.62 g, 15.82 mmol) was partitioned between saturated $NaHCO_3$ (50 mL) and CH_2Cl_2 (50 mL). The CH_2Cl_2 phase was dried (MgSO4) and concentrated to an oil. This was dissolved in THF (20 mL), and DIEA (3.03 mL, 17.40 mmol) was added. The resulting solution was added dropwise to a solution of phosgene in toluene (12.5%, 24.0 mL, 20.88 mmol) previously cooled to 0 °C. The reaction mixture was allowed to warm to room temperature over 1 h, then was filtered and concentrated to provide an oil. This was used in the next step without purification. (Step B) (*S*)-1-(4-Chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl) 2-methyl pyrrolidine-1,2-dicarboxylate. A solution of (*S*)-methyl 1-(chlorocarbonyl)pyrrolidine-2-carboxylate (from step A, 0.397 g, 2.07 mmol) and DMAP (10 mg) in $CHCl_3$ (15 mL) was added to a slurry of niclosamide (645 mg, 1.973 mmol) in pyridine (15 mL). This was warmed to 80 °C (all solids dissolved) and maintained 1 h. The cooled solution was diluted with EtOAc (100 mL). The resulting mixture was washed successively with 1N HCl until the aqueous wash was acidic (about pH 1) to litmus. The EtOAc solution was then washed with brine, dried ($MgSO_4$), and concentrated to an off-white solid. The crude product was recrystallized from EtOAc/hexanes. The ^{1}H NMR spectrum is complex and reveals the existence of rotational isomers, presumably due to restricted rotation about the pyrolidine-1-carbamoyl bond. Analysis of HPLC and MS data reveals a single compound in ≥95% purity. ^{1}HNMR (300 MHz, DMSO-d_6) δ 1.823−2.003 (m, 3H, pyrrolidine C4 methylene and pyrrolidine C3 syn-H), 2.213 (m, 1H, pyrrolidine C3 anti-H), 3.450 (m, 1H, pyrrolidine C5), 3.599 (m, 1H, pyrrolidine C5), 3.600 (s, 1.5 H, methyl ester), 3.621 (s, 1.5 H, methyl ester), 4.252 (m, 0.5 H, pyrrolidine methine), 4.541 (m, 0.5H, pyrrolidine methine), 7.108 (d, J = 8.5 Hz, 0.33 H, H3), 7.216 (d, J = 8.5 Hz, 0.33 H, H3), 7.361 (d, J = 9 Hz, 0.33H, H3), 7.545 (m, 0.33H, H4), 7.665 (m, 0.66H, H4), 7.763 (m, 0.66H, H6), 7.974 (m, 0.33H, H6), 8.133 (m, 0.66H, H6′), 8.287 (m, 1H, H5′), 8.302 (m, 0.33H, H3′), 8.539 (m, 0.33H, H3′), 8.446 (m, 0.33H, H3′), 8.822 (d, J = 9.5 Hz, 0.33H, H6′), 10.313 (s, 0.33H, NH), 10.385 (s, 0.33H, NH), 11.367 (br s, 0.17H, NH), 12.519 (br s, 0.17H, NH). HPLC T_R 2.743 min; m/z^+ 482.00 $[M + H]^+$; m/z^- 479.90 $[M - H]^-$; (EM = 481.04). Note: The optical purity of this material was not determined.

*4-Chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl Morpholine-4-carboxylate (**7c**).* 4-Morpholine carbonyl chloride (0.353 mL, 3.08 mmol) was added dropwise to a suspension of niclosamide **4** (0.503 g, 1.54 mmol) in pyridine (8.0 mL) containing DMAP (10 mg). This was raised to reflux (all solids dissolved) and maintained 3 h. The hot solution was admitted via pipet dropwise into rapidly stirring distilled water (100 mL), whereupon a fine white solid precipitated. This mixture was stirred at room temperature for 2 h. The crude product was collected by filtration, washed well with water (100 mL) and 1N HCl (200 mL), and then dried under a stream of room air for 2 h. The product was then dried in a vacuum oven (28″ Hg, 50 °C) for 60 h. The off-white product was recrystallized from EtOAc to give white crystals. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.60 (m, 2H), 8.45 (m, 1H), 8.33 (m, 1H), 7.96 (m, 1H), 7.62 (m, 1H), 7.24 (m, 1H), 3.78−3.87 (m, 8H). HPLC T_R 2.569 min; m/z^+ 440.00 $[M + H]^+$; m/z^- 437.85 $[M - H]^-$ (EM = 439.03).

*4-Chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl 4-Methylpiperazine-1-carboxylate (**7d**).* 4-Methyl-1-piperazinecarbonyl chloride (0.603 g, 3.02 mmol) was added to a suspension of niclosamide **4** (0.495 g, 1.51 mmol) in pyridine (8.0 mL) containing DMAP (10 mg). The mixture was raised to reflux for 1 h. The hot solution

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was introduced by pipet to rapidly stirring water at rt. After 1 h, the solids were collected by filtration and added to 50 mL of rapidly stirred 1N HCl. This was stirred for 30 min, and then the crude product was collected by filtration and dried in a vacuum oven (28″ Hg, 50 °C) for 18 h, then recrystallized from EtOH. A small portion of the product was converted to the free base by partitioning between EtOAc and saturated $NaHCO_3$. TLC (SiO_2, 5 MeOH:95 $CHCl_3$) demonstrated a single component, R_f = 0.37. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.91 (s, 1H), 8.81 (d, J = 9.2 Hz), 8.34 (d, J = 2.4 Hz), 8.22 (dd, J = 9.2, 2.4 Hz), 7.87 (d, J = 2.8 Hz), 7.51 (dd, J = 2.8, 8.4 Hz), 7.13 (d, J = 8.4 Hz), 3.68 (m, 2H), 3.56 (m, 2H), 2.39 (m, 4H), 2.28 (s, 3H). HPLC T_R 1.664; m/z^+ 452.95 $[M + H]^+$; m/z^- 450.90 $[M - H]^-$ (EM = 452.07).

*N-(2-Chloro-4-nitrophenyl)-5-fluoro-2-methoxybenzamide (**10a**).* A solution of 5-fluoro-2-methoxybenzoic acid (0.340 g, 2.0 mmol), 2-chloro-4-nitroaniline (0.173 g, 1.0 mmol), and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU, 0.76 g, 2.0 mmol) in MeCN (4.0 mL) was treated with DIEA (0.10 mL), and the resulting solution was stirred at rt for 16 h. EtOAc (10 mL) was added, and the organic phase was washed with 50% 1N HCl in saturated NaCl and then brine, dried over $MgSO_4$, and concentrated to afford a yellow solid. This was recrystallized from EtOAc:hexanes. ^{1}H NMR (500 MHz, DMSO-d_6) δ 13.78 (s, 1H), 12.88 (s, 1H), 8.76 (dd, J = 4.4, 1.4 Hz, 1H), 8.53 (dd, J = 8.4, 1.4 Hz, 1H), 7.51 (dd, J = 8.4, 4.4 Hz, 1H), 7.37 (m, 2H), 7.13 (dd, J = 9.1, 4.3 Hz, 1H), 3.79 (s, 3H). HPLC T_R 2.176 min; m/z^+ 288.90 $[M - Cl]^+$ (EM = 324.03).

*5-Fluoro-N-(2-fluoro-4-nitrophenyl)-2-methoxybenzamide (**10b**).* A solution of 5-fluoro-2-methoxybenzoic acid (0.510 g, 3.0 mmol), 2-fluoro-4-nitroaniline (0.156 g, 1.0 mmol), and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU, 1.140 g, 3.0 mmol) in MeCN (6.0 mL) was treated with DIEA (0.150 mL), and the resulting solution was stirred at room temperature for 2 h. The reaction mixture was added to rapidly stirring water, and the resulting precipitate was washed well with water and then dissolved in EtOAc, dried over $MgSO_4$, and concentrated to a yellow solid. The crude product was recrystallized from EtOAc/hexanes. ^{1}H NMR (500 MHz, DMSO-d_6) δ13.78 (s, 1H), 12.89 (s, 1H), 8.76 (dd, J = 4.4, 1.4 Hz, 1H), 8.53 (dd, J = 8.4, 1.4 Hz, 1H), 7.51 (dd, J = 8.4, 4.4 Hz, 1H), 7.40 (dd, J = 8.8, 3.3 Hz, 1H), 7.35 (ddd, J = 9.1, 8.1, 3.3 Hz, 1H), 3.79 (s, 3H). HPLC T_R 2.190 min; m/z^+ 288.90 $[M - F]^+$; m/z^- 292.85 $[M - CH_3]^-$ (EM = 308.06).

*tert-Butyl 2-((Chlorocarbonyl)(methyl)amino)acetate (**12**).* A solution of sarcosine *tert*-butyl ester hydrochloride **11** (4.214 g, 23.20 mmol) in CH_2Cl_2 (40 mL) was shaken with saturated $NaHCO_3$ in a separatory funnel. The organic phase was dried over $MgSO_4$ and concentrated to a clear oil (2.298 g, 68% yield). A solution of phosgene in toluene (20%, 10.9 mL, 23.75 mmol) was cooled to −25 °C, and a solution of sarcosine *tert*-butyl ester (2.298 g, 15.78 mmol) and DIEA (5.5 mL, 31.66 mmol) in CH_2Cl_2 (10 mL) was introduced in a dropwise fashion. The solution was allowed to warm to room temperature over 1 h and was then washed with 1N HCl (50 mL) and EtOAc sufficient to form two layers was added. The organic phase was washed with water and then brine and dried over $MgSO_4$. This solution was used without further manipulation.

*2-(((4-Chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenoxy)-carbonyl) (Methyl)amino)acetic Acid (**13a**).* (Step A) *tert*-Butyl 2-(((4-chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenoxy)carbonyl)-(methyl)amino)acetate. A solution of **12** (15.78 mmol) in EtOAc (ca. 0.5M) was introduced dropwise to a refluxing solution of niclosamide (2.56 g, 7.92 mmol) in pyridine (80 mL containing DMAP (50 mg). After 30 min, 100 mL of solvent was distilled from the reaction mixture. The remaining reaction mixture was cooled to room temperature, diluted with EtOAc (50 mL), and washed successively with 1N HCl until the aqueous wash was acidic (about pH 1) to litmus. The organic phase was washed with brine, dried ($MgSO_4$), and concentrated to an off-white solid. This was used without further purification. (Step B) 2-(((4-Chloro-2-((2-chloro-4-nitrophenyl) carbamoyl) phenoxy) carbonyl) (methyl)amino) acetic acid. The solid from step A (542 mg, 1.09 mmol) was dissolved in CH_2Cl_2 (15 mL), and CF_3COOH (15 mL) was added. After 16 h, the reaction solution was concentrated to an oily residue. This was dissolved in $CHCl_3$ (25 mL) and concentrated. The $CHCl_3$ chase was repeated to leave a sinterable foam. This was layered with 35 EtOAc:65 hexanes (50 mL) and warmed to 45 °C under rapid stirring for 30 min. Hexane (30 mL) was added, and the stirring mixture was allowed to cool to room temperature over 20 min. The product was collected by filtration. The ^{1}H NMR spectrum was complex and revealed the existence of rotational isomers, presumably due to restricted rotation about the sarcosine-1-carbamoyl bond. Analysis of HPLC and MS data reveals a single compound in ≥95% purity. T_R 2.382 min; m/z^+ 441.85 $[M + H]^+$; m/z^- 324.75 $[M - \text{sarcosine} - H]^-$ (EM = 441.01).

*4-Chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl (2-Hydrazinyl-2-oxoethyl)(methyl) Carbamate Hydrochloride (**13b**).* (Step A) *tert*-Butyl 2-(2-(((4-chloro-2-((2-chloro-4-nitrophenyl) carbamoyl)-phenoxy)carbonyl)(methyl)amino)acetyl)hydrazinecarboxylate. *tert*-Butyl carbazate (417 mg, 3.16 mmol) was added to a solution of **13a** (1.27 g, 2.87 mmol) in THF (8.7 mL) with stirring at rt. A solution of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (660 mg, 3.44 mmol) in CH_2Cl_2 (17.4 mL) was introduced dropwise at rt. The homogeneous solution was stirred at room temperature for 14 h, then concentrated. The residue was partitioned between EtOAc and 1 N HCl. The EtOAc phase was washed with water and then with brine, dried ($MgSO_4$), and concentrated to an off-white solid 1.20 g (75.1%). This was used without further manipulation. (Step B) 4-Chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl (2-hydrazinyl-2-oxoethyl)(methyl)carbamate hydrochloride. The product from step A (2.52 g, 5.70 mmol) was dissolved in a solution of HCl in dioxane (4.0 M, 10 mL), and the solution was stirred at room temperature for 30 min, then concentrated to an off-white solid which was washed well with EtOAc and then hexanes and dried under a stream of air to provide a white solid (2.50 g, 89%). ^{1}H NMR spectrum was complex and revealed the existence of two rotational isomers, presumably due to restricted rotation about the sarcosine-1-carbamoyl bond. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.09 (s, 1H), 10.45 (s, 1H), 10.40 (s, 1H), 8.41 (dd, J = 2.3, 1.5 Hz, 1H), 8.31−8.25 (m, 1H), 8.10 (m, 1H), 7.80 (dd, J = 6.2, 2.6 Hz, 1H), 7.71−7.65 (m, 1H), 7.36 (d, J = 8.7 Hz, 1H), 7.26 (d, J = 8.7 Hz, 1H), 4.21 (s, 1H), 4.05 (s, 1H), 3.07 (s, 1.5H), 2.92 (s, 1.5H). HPLC T_R 2.03 min; m/z^+ 455.95 $[M + H]^+$; m/z^- 453.95 $[M - H]^-$ (EM = 308.06).

*2-(((2-((2,4-Bis(trifluoromethyl)phenyl)carbamoyl)-4 Chlorophenoxy)carbonyl) (Methyl) amino) Acetic Acid (**14a**).* (Step A) *tert*-Butyl 2-(((2-((2,4-bis(trifluoromethyl)phenyl)carbamoyl)-4-chlorophenoxy)carbonyl)(methyl)amino)acetate. *N*-(3,5-Bis(trifluoromethyl)phenyl-5-chloro-2-hydroxybenzamide (**3j**, 6.47 g, 16.86 mmol) was added to a solution of **12** (4.20 g, 20.22 mmol) in pyridine (35 mL), and the mixture was warmed to 80 °C for 3 h. The reaction solution was cooled to room temperature, diluted with EtOAc (200 mL), and washed four times with 1N HCl (final wash ph about 1 to litmus), once with brine, and dried ($MgSO_4$). Concentration afforded an off-white solid, which was triturated with hexanes to provide the product (7.01 g, 75%), which was used without further purification. (Step B) 2-(((2-((2,4-Bis(trifluoromethyl)phenyl)-carbamoyl)-4-chlorophenoxy)carbonyl)(methyl)amino) acetic acid. The product from step A (5.43 g, 9.70 mmol) was dissolved in CH_2Cl_2 (15 mL), and CF_3COOH (7.5 mL) was added. The reaction mixture was stirred at room temperature for 14 h, and then the reaction mixture was concentrated and the residue was chased twice with $CHCl_3$ (25 mL). The ^{1}H NMR spectrum was complex and revealed the existence of rotational isomers, presumably due to restricted rotation about the sarcosine-1-carbamoyl bond. Analysis of HPLC (T_R = 2.65 min) and MS data (m/z^+ 499.05, $[M + H]^+$, m/z^- 381.80, $[M - C_4H_6NO]^-$) revealed a single compound in ≥95% purity.

*2-((2,4-Bis(trifluoromethyl)phenyl)carbamoyl)-4-chlorophenyl(2-hydrazinyl-2-oxoethyl) (Methyl)carbamate Hydrochloride (**14b**).* (Step A) *tert*-Butyl 2-(2-(((2-((2,4-bis (trifluoromethyl) phenyl)-carbamoyl)-4-chlorophenoxy) carbonyl) (methyl)amino) acetyl) hydrazine carboxylate. A solution of 2-(((2-((2,4-bis(trifluoromethyl)phenyl)-carbamoyl)-4-chlorophenoxy)carbonyl) (methyl)amino)acetic acid (**14a**, 540 mg,1.08 mmol) in CH_2Cl_2 (5.0 mL) was treated with *tert*-butyl

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carbazate (172 mg, 1.30 mmol) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (249 mg, 1.30 mmol). The solution was stirred at room temperature for 4 h, then concentrated, and the residue was dissolved in EtOAc (15 mL), washed with 1N HCl and then with brine, dried over $MgSO_4$, and concentrated to a scinterable foam. The ^1H NMR spectrum was complex and revealed the existence of rotational isomers, presumably due to restricted rotation about the sarcosine-1-carbamoyl bond. Analysis of HPLC (T_R = 2.81 min) and MS data (m/z^+ 634.90 $[M + Na]^+$, m/z^- 610.85, $[M - H]^-$) reveals a single compound in ≥95% purity. (Step B) 2-((2,4-Bis(trifluoromethyl)phenyl)-carbamoyl)-4-chlorophenyl (2-hydrazinyl-2-oxoethyl)(methyl) carbamate hydrochloride (**14b**, 2.263 g, 3.69 mmol) was dissolved in a solution of HCl in dioxane (15 mL). After stirring 2 h at room temperature, the reaction mixture was concentrated and the residue was chased twice with 15 mL portions of $CHCl_3$, providing a scinterable foam (1.90 g, 94%). ^1H NMR (500 MHz, DMSO-d_6) δ 11.03 (s, 1H), 10.95 (s, 1H), 9.15 (s, 1H), 9.08 (s, 1H), 8.36 (s, 4H), 7.84 (s, 2H), 7.80 (dd, J = 5.2, 2.7 Hz, 2H), 7.70−7.64 (m, 1H), 7.38 (d, J = 8.7 Hz, 1H), 7.26 (d, J = 8.7 Hz, 1H), 4.17 (q, J = 18.2, 13.8 Hz, 3H), 3.97 (s, 2H), 3.79 (s, 2H), 3.00 (s, 3H), 2.83 (s, 3H). HPLC T_R 2.38 min; m/z^+ 512.90 $[M + H]^+$; m/z^- 510.80 $[M - H]^-$ (EM = 512.07).

Biology. *In Vitro Evaluation of Inhibition of T. gondii Tachyzoites.* Test compounds were dissolved in DMSO to make a 10 mM solution, and subsequently diluted with IMDM-C to the concentrations used in bioassay. In the in vitro experiments, DMSO concentration was not greater than 0.1% unless otherwise specified. RH-YFP parasites, which stably express yellow fluorescent protein, were used. Tachyzoites were extracted from HFF cells by double passage through a 25 gauge[12] needle, centrifuged for 15 min at 1500 rpm, and resuspended in IMDM-C. Confluent monolayers of HFF cells were infected with parasites in 96-well plates (Falcon 96 Optilux Flat-bottom) with 3500 parasites in 100 μL per well. One hour after inoculation, test compounds and control media were added for a final volume of 200 μL per well. Parasite proliferation was assessed using $[^3H]$-uracil incorporation[13] or YFP fluorescence assay.

T. gondii Parasite and Cell Culture. Human foreskin fibroblast (HFF) cells were maintained in confluent monolayers in Iscoves's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum, 1% GlutaMAX, and 1% penicillin−streptomycin−fungizone (IMDM-C). *T. gondii* tachyzoites were cultivated in HFF monolayers.[12,13] Parasites and cells were maintained at 33 or 37 °C and 5% CO_2. The strains of parasite used in this study include RH, RH-YFP, Me49 strain,[14] andTgGoatUS4 isolate.[15]

[^{3}H]-Uracil and [^{3}H]-Thymidine Incorporation Assays. First, 25 μL of 0.1mCi/mL $[^3H]$-uracil or $[^3H]$-thymidine was added to each well 24 h before reading plates. Then at harvesting, contents of the wells were transferred onto a 96-well UniFilter GF/C filter plates using a Filtermate 196 cell harvester (Packard). $[^3H]$-Uracil and $[^3H]$-thymidine incorporation was measured using a Microplate scintillation luminescence counter (Packard).[12,13]

YFP Fluorescence Assay. After 72 h of initiation of the in vitro challenge assay, parasite proliferation was assessed by reading fluorescence of YFP parasites with a Synergy H4 Hybrid Reader (BioTek) and Gen5 1.10 software, using a bottom optics positions, excitation wavelength of 514 nm, and emission wavelength of 540 nm.[12]

In Vitro Toxicity Assay. HFF cells were grown to ∼30% confluence in 96-well plates. Inhibitory compounds and control media were added to wells in concentrations equal to those being tested in challenge assays. After 72 h, $[^3H]$-thymidine incorporation assay was conducted to assess cell growth.[12,13] Alternatively, toxicity was assessed using WST-1 cell proliferation reagent (Roche). Confluent HFF cells were treated with inhibitory and control compounds. On the final day of experiment, 10 μL of WST-1 reagent was added to each well. Plates were incubated for 1 h in the dark at 37 °C, and absorbance was measured using Synergy H4 hybrid reader (BioTek) fluorometer at 420 nM.[12]

Antiplasmodial SYBR Green I-Based Fluorescence (MSF) Assay. D6 (CDC/Sierra Leone) and TM91-C235 (WRAIR, Thailand) laboratory strains of *P. falciparum* were used for each drug sensitivity assessment. The parasite strains were maintained continuously in long-term cultures as previously described in Johnson et al,[11] and *P. falciparum* strains in late-ring or early trophozoite stages were cultured in predosed 384-well microtiter drug assay plates in 38 μL culture volume per well at a starting parasitemia of 0.3% and a hematocrit of 2%. Predosed, sterile, 384-well black optical bottom microtiter drug plates for use in the MSF assay were produced using a Tecan EVO Freedom liquid handling system (Tecan US, Durham, NC). Dose response plates were produced at a final concentration ranging from 0.5−10000 ng/mL in quadruplicate (12 2-fold serial dilutions of each test compound or chloroquine control in DMSO). Each run was validated by a batch control plate with chloroquine (Sigma-Aldrich Co., catalogue no. C6628) at a final concentration of 2000 ng/mL. The cultures were incubated for 72 h at 37 °C, 5% CO_2, 5% O_2, and 90% N_2. Lysis buffer (38 μL per well), consisting of 20 mM Tris HCl, 5 mM EDTA, 1.6% Triton X, 0.016% saponin, and SYBR green I dye at a 20× concentration (Invitrogen, catalogue no. S-7567), was then added to the assay plates utilizing the Tecan EVO Freedom system for a final SYBR Green concentration of 10×. Plates were incubated in the dark at room temperature in the dark for 24 h. Compound activity was assessed by examining for the relative fluorescence units (RFU) per well using the Tecan Genios Plus (Tecan US, Inc., Durham, NC). GraphPad Prism (GraphPad Software Inc., San Diego, CA) using the nonlinear regression (sigmoidal dose−response/variable slope) equation was used to determine IC_{50} values.

Determination of Static or Cidal Effects. RH-YFP tachyzoites were treated with each compound at 1 μM under one of four conditions: (a) parasites were treated for four days, then compound was removed; (b) parasites were treated for 10 days, then compound was removed; (c) compound was refreshed at four days and removed at 10 days; or (d) compound was maintained for the duration of the experiment. The 4 and 10 day time points were taken to reveal the impact of extended exposure of the parasites to the test substance. Compounds were refreshed at four days to examine whether compound degradation could contribute to an observed static effect. Parasite growth was assessed at 11, 17, and 25 days.

In Vivo Toxicity and Oocyst Assays. HLA B07 transgenic mice were produced at Pharmexa-Epimmune (San Diego, CA, USA) and bred at the University of Chicago. All studies were conducted with Institutional Animal Care and Use Committee at the USDA, the University of Chicago, and the University of Strathclyde.

Infection of Mice with T. gondii Oocysts. Oocysts were obtained by feeding infected tissues of Swiss Webster mice to cats, sporulated in 2% sulfuric acid on a shaker for one week, and stored at 4 °C until used (Dubey 2010). Oocysts were counted in a disposable hemocytometer and diluted 10-fold from 10^{-1} to 10^{-7} to reach an end point of ≅1 oocyst. All 10-fold dilutions were made in 50 mL tubes with 2% sulfuric acid (5 mL aliquot + 45 mL sulfuric acid), and dilutions were stored at 4 °C to avoid variability in inocula preparations. For inoculation of mice, oocysts from the designated dilution were neutralized with 3.3% sodium hydroxide with neutral red as indicator (approximately the same volume as the inoculum). The resultant mixture was inoculated orally into five mice for each dilution (unless indicated otherwise) via a gastric needle with a blunt bulb (22 gauge, 50 mm long, Cadence Science catalogue no. 7920), without washing to avoid variability of the inocula during washing procedure. All orally inoculated mice were housed in autoclavable rodent cages with biohazard signs to incinerate bedding and food for 10 days to avoid spread of *T. gondii* because some oocysts pass unencysted in mouse feces.[16]

Bioassay of T. gondii in Mice. Mice were observed daily for the duration of the experiment. All mice were examined for *T. gondii* infection. Impression smears of tissues (usually mesenteric lymph nodes or lungs) were examined microscopically for tachyzoites. Survivors were bled 6−8 weeks later, and 1:25 dilution of their sera were tested for *T. gondii* antibodies using the (MAT) as described.[17] The last infective dilution was considered to have one viable organism. The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues. Seroconversion at 6 weeks was considered as indication of the presence of live parasites in the inocula. However, brains of all mice that survived 6 weeks were examined for tissue cysts, irrespective of serological results. With the strains of *T. gondii* used here, tissue cysts are found in all seropositive mice.

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*Evaluation of Efficacy of **14a** in a Mouse Model of T.gondii Oocyst Infection.* Five groups of B7 female mice weighing approximately 25 g were used. Group 1 received only **14a** (100 mg/kg, 2.5 mg/mL, 1.0 mL) by oral gavage. Group 2 received only **14a** (25 mg/kg, 0.5 mg/mL, 1.0 mL) by oral gavage. Group 3 received **14a** (100 mg/kg, 2.5 mg/mL, 1.0 mL) and 100 ME49 oocysts by oral gavage. Group 4 received **14a** (25 mg/kg, 0.5 mg/mL, 1.0 mL) and 100 ME49 oocysts by oral gavage. Group 5 received only 100 ME49 oocysts by oral gavage.

*Evaluation of Efficacy of **14b** in a Mouse Model of T.gondii Oocyst Infection.* Five groups of B7 female mice weighing approximately 25 g were used. Group 1 received only **14b** (100 mg/kg, 2.5 mg/mL, 1.0 mL) by oral gavage. Group 2 received only **14b** (25 mg/kg, 0.5 mg/mL, 1.0 mL) by oral gavage. Group 3 received **14b** (100 mg/kg, 2.5 mg/mL, 1.0 mL) and 100 TgGoatUS4 oocysts by oral gavage. Group 4 received **14a** (25 mg/kg, 0.5 mg/mL, 1.0 mL) and 100 TgGoatUS4 oocysts by oral gavage. Group 5 received only 100 TgGoatUS4 oocysts by oral gavage.

Insertional Mutagenesis Experiments. THdhxgTRP tachyzoites were transfected with pLK47 vector plasmid.[12] Parasites were extracted from HFF cells and resuspended in 1 mL of Cytomix electroporation buffer solution (120 mM KCl, 150 μM $CaCl_2$, 5 mM K_2HPO_4, 5 mM KH_2PO_4, 25 mM HEPES, 2 mM EDTA, and 5 mM $MgCl_2$ in sterile H_2O). Equivalent amounts of plasmid DNA and Cytomix solution containing 10×10^7 parasites were combined for a total volume of 400 μL and electroporated using BioRad electroporator at 1.5k, 25fF, and 100 Ω. This resulted in a random insertion of the plasmid in the parasite genome and random disruption of a gene. Successfully transfected parasites were selected by month-long treatment with mycophenolic acid (25 μg/mL) and xanthine (50 μg/mL). After one month, the mixed mutant population was exposed to **3i**, **3j**, **7a**, or **14b** to select for those mutants whose gene disruption conferred resistance to the drug. No parasite growth was noted after six weeks of exposure to mycophenolic acid, xanthine, and any of the four test compounds.

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Notes

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ABBREVIATIONS USED

CDC, Centers for Disease Control and Prevention; DIEA, diisopropylethylamine; DMAP, dimethylaminopyridine; DMSO, dimethylsulfoxide; ESI, electrospray interface; EtOAc, ethyl acetate; FDA, Food and Drug Administration; FIBS, fibroblasts; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HFF, human foreskin fibroblasts; IC, inhibitory concentration; IC_{50}, half-maximal inhibitory concentration; IC_{90}, concentration necessary to achieve 90% inhibition; IMDM-C, Iscove's Modified Dulbecco's Media; LC-MS, high performance liquid chromatography−mass spectral analysis; *m*/*z*, mass to charge ratio; MAT, modified agglutination test; MeCN, acetonitrile; MIC, minimum inhibitory concentration; MSF, Malaria SYBR Green I-Based Fluorescence Assay; OD, optical density; *P. falciparum*, *Plasmodium falciparum*; P/S, pyrimethamine and sulfadiazine in combination; RH-YFP, tachyzoites expressing YFP; SYBR, SYBR Green Dye I; *T. gondii*, *Toxoplasmosis gondii*; T_r. chromatographic retention time; WST-1, water-soluble tetrazolium salt 1; YFP, yellow fluorescent protein

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