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Phthalazinone Inhibitors of Inosine-5′-Monophosphate Dehydrogenase from *Cryptosporidium parvum*

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Abstract

Cryptosporidium parvum (*Cp*) is a potential biowarfare agent and major cause of diarrhea and malnutrition. This protozoan parasite relies on inosine 5′-monophosphate dehydrogenase (IMPDH) for the production of guanine nucleotides. A *Cp*IMPDH-selective N-aryl-3,4-dihydro-3-methyl-4-oxo-1-phthalazineacetamide inhibitor was previously identified in a high throughput screening campaign. Herein we report a structure-activity relationship study for the phthalazinone-based series that resulted in the discovery of benzofuranamide analogs that exhibit low nanomolar inhibition of *Cp*IMPDH. In addition, the antiparasitic activity of select analogs in a *Toxoplasma gondii* model of *C. parvum* infection is also presented.

Cryptosporidium species cause self-limiting gastrointestinal disease that can be chronic and fatal in immunosuppressed patients [1]. Oocysts from these organisms are resistant to common methods of water treatment, and therefore pose a potential biowarfare threat. The drugs currently approved for the treatment of cryptosporidiosis are largely ineffective, elevating the need for new chemotherapeutic agents to counter outbreaks. Genomic analysis of the most common human pathogens, *C. parvum* and *C. hominis,* revealed that these parasites have a streamlined purine salvage pathway that relies on IMP dehydrogenase

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(IMPDH) for the production of guanine nucleotides [2–4]. This enzyme catalyzes the oxidation of IMP to XMP with the production of NADH as outlined in Scheme 1[5]. Curiously, the *Cryptosporidium* IMPDH gene appears to have been obtained from an ε-proteobacterium via horizontal gene transfer [6], and is structurally distinct from the host ortholog (the *C. parvum* and *C. hominis* enzymes are identical and will be denoted as *Cp*IMPDH). Consequently, *Cp*IMPDH displays unique enzymatic properties compared with the host counterparts [7].

A previously reported high throughput screening (HTS) campaign identified several structurally distinct classes of selective *Cp*IMPDH inhibitors that bind to the NAD site [8, 9]. Structure-activity relationship studies of triazole [10], benzimidazole [9, 11] and urea [12] *Cp*IMPDH inhibitors, exemplified by **A110**, **C64**, **C97** and **P96**, respectively, have also now been reported (Figure 1). In addition, a co-crystal structure of the benzimidazole inhibitor **C64** with *Cp*IMPDH demonstrated a unique binding mode compared to inhibitors bound to human IMPDH [9]. The phthalazinone-based inhibitor **1** was also identified by HTS, with moderate potency (IC_{50} = 5.4 μM) and good selectivity (IC_{50} > 50 μM versus both human IMPDH1 and IMPDH2) [8]. Compound **1** (50 μM) did not display antiparasitic activity [8]. Herein we report a structure-activity relationship study of **1**, and the antiparasitic activity of select analogs in a *T. gondii* model of *C. parvum* infection.

Compound **1** and its analogs were prepared following a four-step sequence as depicted in Scheme 2 beginning with the Wittig olefination of phthalic anhydride, **2**, in the presence of carbethoxymethylidenetriphenylphosphorane in chloroform to produce (*E*)-ethyl-2-(3-oxoisobenzofuran-1(3H)-ylidene)acetate, **3**. Addition of hydrazines in refluxing ethanol generated the phthalazinone acetoesters **4**. Hydrolysis of the esters produced phthalazinone acetic acids **5** [13, 14]. Finally, coupling of the acetic acid moiety with various aromatic amines in DMSO afforded **6**.

The synthesized derivatives were evaluated for *Cp*IMPDH inhibition using an assay that monitored the production of NADH in the presence of varying inhibitor concentrations [10]. In order to evaluate the effects of non-specific binding, inhibition was also determined in the presence of 0.05% fatty acid free bovine serum albumin (BSA). None of the compounds inhibited human IMPDH2 (IC_{50} > 5 μM).

Inhibitor **1** exhibited an IC_{50} of 1.0 μM upon resynthesis. Gratifyingly, the activity was retained in the presence of 0.05% BSA (IC_{50} = 970 nM) as shown in Table 1. However, moving the electron donating group to the 2- or 3-position resulted in loss of activity (**7** and **10**). Replacing the 4-OMe with a variety of other electron donating substituents (**11** – **14**) was also detrimental. Likewise, replacing the phenyl with an amine (**8**) or alkyl group (**9**) or inserting a methylene between the benzene and amide nitrogen atom (**15**) was not tolerated. Replacement of the electron donating group at the 4-position with electron withdrawing groups improved potency. For example, the 4-chloro and 4-bromo analogs (**16** and **17**) demonstrated IC_{50} values of 230 and 130 nM, respectively. However, other electron withdrawing groups (**18** – **21**) generally were not as well tolerated. Also, transposition of the chlorine from the 4-position to the 3-position (**23**) resulted in loss of activity. Di-substitution at the 3,4-positions with chlorine and bromine atoms (**24** and **25**) resulted in increased potency. Furthermore, other 3,4-di-substituted analogs with either an electron-donating or an electron-withdrawing group at the 3-position were also active. Interestingly, in a number of cases substituents at the 3-position that alone were not tolerated when combined with electron withdrawing groups (especially Cl or Br) at the 4-position resulted in potent *Cp*IMPDH inhibitors (e.g. **26**, **27** and **37**). However, transposition of the substituents from the 3-position to the 2-position (**39** – **42**) resulted in poor inhibitors. In addition, several other di- and tri-substitutions (**43** – **46**) were also detrimental. Encouraged by the results of

3,4-disubstitution, the 2-naphthyl analog **47** was prepared and demonstrated an IC_{50} of 63 nM. Similar increases in *Cp*IMPDH activity by introduction of 3,4-disubstitution or incorporation of 2-naphthylene had also been observed in the structure-activity relationship studies resulting in **A110** and **C97** [10, 11]. Finally, analogs that lack methylation of the phthalazinone nitrogen atom (**48** and **49**) were found to be active indicating that this substituent is not necessary for *Cp*IMPDH inhibitory activity.

Inspired by the results with **47**, a variety of fused heterocycles were explored as replacements of the 2-naphthylene (Table 2). Although the 5-benzofuranyl analog **50** had reduced activity and the 5-benzoxazolyl analog **51** was inactive, the 2-methyl-5-benzofuranyl derivative **52** retained activity with an IC_{50} of 64 nM. Furthermore, incorporation of an additional ring (**53**) resulted in enhanced potency. Unsaturation of the ring further increased activity with **54** demonstrating an IC_{50} of 4 nM. However, the regioisomer **55** and two carbazoles **56** and **57** were not active.

Compounds with IC_{50} values less than 30 nM were candidates for evaluation of antiparasitic activity in a *Toxoplasma gondii* model of *C. parvum* infection [15]. In this model, the endogenous *T. gondii* IMPDH and hypoxanthine-guanine-xanthine phosphoribosyltransferase genes have been knocked out and the *Cp*IMPDH gene inserted to create *T. gondii*/*Cp*IMPDH, a model parasite that relies on *Cp*IMPDH for the production of guanine nucleotides. Both wild-type and *T. gondii*/*Cp*IMPDH were cultured in human foreskin fibroblasts immortalized with hTERT, so this assay also reports on host cell toxicity. Compounds **26**, **27**, **53** and **54** all displayed sub-micromolar activity against *T. gondii*/*Cp*IMPDH (Table 3). However, only **27** displayed selectivity • 30 versus the wild-type strain, strongly indicating that antiparasitic activity results from the inhibition of *Cp*IMPDH.

The inhibition of *Cp*IMPDH by compound **27** was characterized further. Whereas compound **1** is a mixed inhibitor of *Cp*IMPDH with respect to NAD^+ (K_{is} = 1.8 μM, K_{ii} = 7μM) [8], **27** is a pure noncompetitive inhibitor ($K_{is} = K_{ii} = 3.4 \pm 0.2$ nM; Figure 2).

Compound **27** displayed good stability in mouse liver microsomes ($T_{1/2}$= 79 min). This compound was advanced into the IL12 knockout mouse model of *C. parvum* infection [16–19]. Disappointingly, no antiparasitic activity was observed with once per day oral dosing of **27** (250 mg/kg) for seven days. It may be that alternative dosing regimes or modifications in formulations could improve efficacy in vivo. Additional optimization of pharmacokinetic properties may also be necessary for this compound series in order to achieve in vivo efficacy.

In conclusion, a SAR study of phthalazinone-based *Cp*IMPDH inhibitors revealed that expansion of the aniline ring could increase inhibitory activity, while maintaining selectivity relative to the human orthologs. The phthalazinone-based *Cp*IMPDH inhibitors described herein could serve as lead compounds for the development of effective treatments of cryptosporidiosis as well as useful molecular probes for studying *Cryptosporidium* parasites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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data for these entire compounds were maintained using ChemAxon, http://www.chemaxon.com/. JRM would like to thank Ms. Nina McNair for technical support. CRJ would like to thank Drs. Sivapriya Kirubakaran and Jihan Khan for insightful and helpful suggestions.

References and Notes

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19. The anticryptosporidial activity of **27** was assessed in the IL-12 knockout mouse model, which resembles the acute human disease. Mice were inoculated with 1,000 oocysts and treated by gavage with vehicle (10%DMSO/corn oil), vehicle plus 250 mg/kg **27**, or 2000 mg/kg paromomycin starting 4 hrs post infection. Treatment was given once daily for 7 days and mice sacrificed on day 8 (peak infection). Parasite load was quantified by FACS assays by the presence of the oocysts in the feces at days 0, 4 and 7. Fecal pellets from the mice were collected and homogenized in adjusted volumes of 2.5% potassium dichromate. Aliquots (200 μl) of vortexed samples were processed over micro-scale sucrose gradients. The oocyst-containing fraction was collected and washed. Purified oocysts were incubated with a fluorescein-labeled oocyst-specific monoclonal antibody (OW5O-FITC) for 20 min. Samples were adjusted to 600 μl and assayed assayed with a 102-s sampling interval (100 μl)l) using logical gating of forward/side scatter and OW5O-FITC fluorescence signal on a Becton Dickinson FACScan flow cytometer. Flow cytometry data were evaluated by analysis of variance (Microsoft Excel; Microsoft Corporation, Redmond, WA).

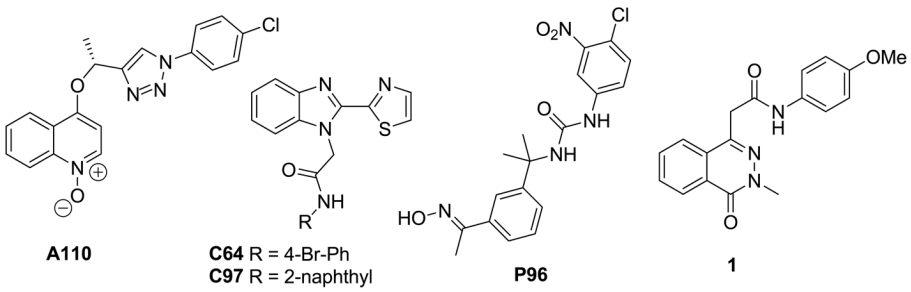


Figure 1.
Optimized *Cp*IMPDH inhibitors **A110**, **C64** and **C97** and HST identified *Cp*IMPDH inhibitor **1**.

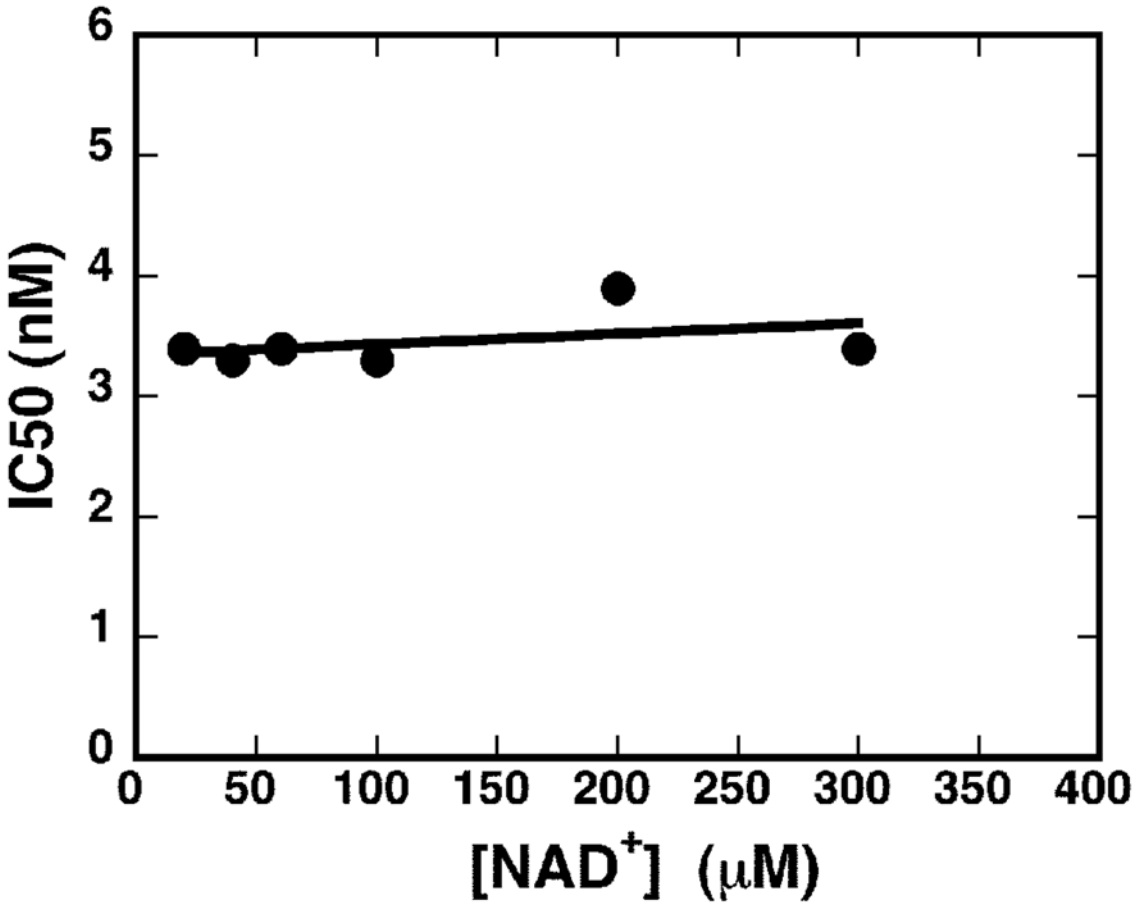
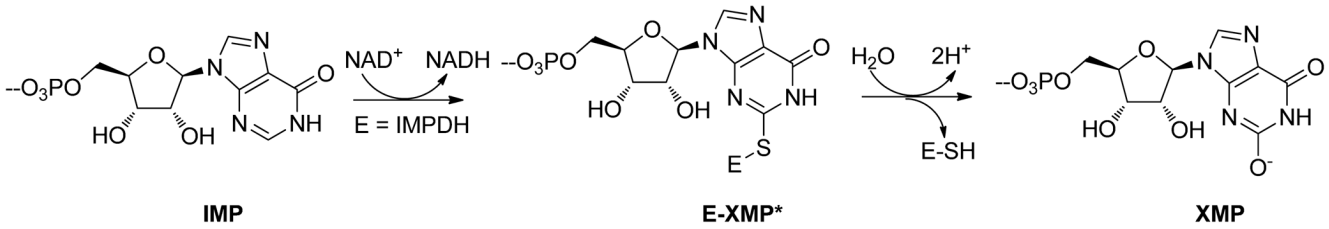
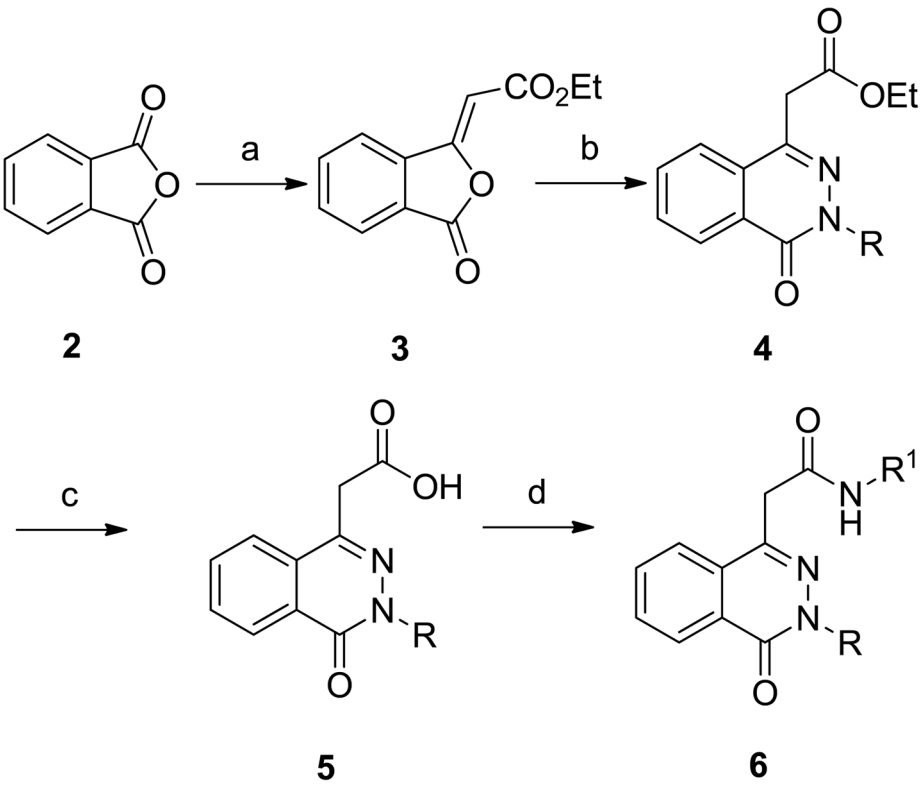


Figure 2.
Inhibition of *Cp*IMPDH by **27**.



Scheme 1.
IMPDH catalyzed conversion of **IMP** to **XMP** via intermediate **E-XMP***.

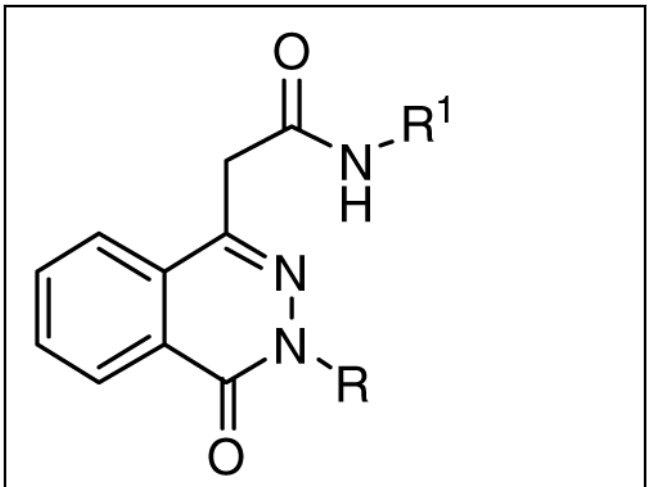


Scheme 2.

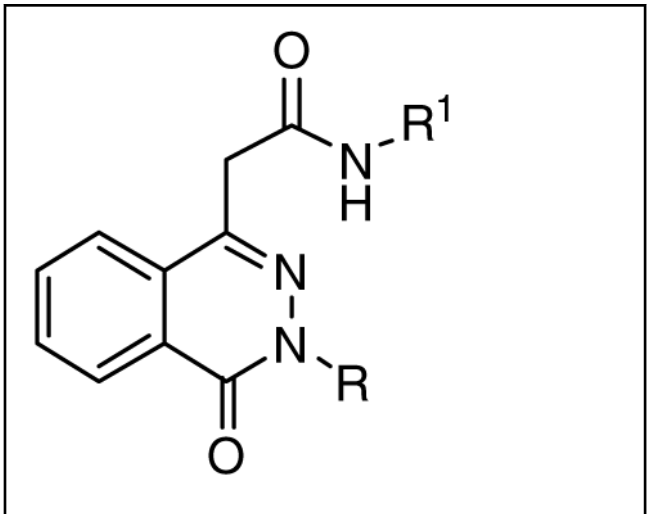
General procedure for the synthesis of phthalazinone derivatives of **1**. a) Ph_3PCHCO_2Et, $CHCl_3$, reflux, 16 h. b) NH_2NH_2 or NH_2NHMe, EtOH, reflux, 3 h. c) 3 M NaOH/THF, reflux, 2 h followed by acidification. d) R^1-NH_2, EDC, HOBt, DIPEA, DMSO, 3–5 h.

Table 1
SAR of anilide and nathylide phthalazinone inhibitors

Assays as described in [10].



Compound	R	R^1	(−)-BSA (nM)	(+)-BSA (nM)
1	Me	4-OMePh	1000 ± 200	930 ± 180
7	Me	2-OMePh	>5000	ND
8	Me	NH_2	>5000	ND
9	Me	t-Bu	>5000	ND
10	Me	3-OMePh	>5000	ND
11	Me	4-OEtPh	>5000	ND
12	Me	4-OCF_3Ph	>5000	ND
13	Me	4-MePh	>5000	ND
14	Me	4-i-PrPh	>5000	ND
15	Me	CH_2(4-OMePh)	>5000	ND
16	Me	4-ClPh	230 ± 50	280 ± 50
17	Me	4-BrPh	130 ± 30	130 ± 20
18	Me	4-FPh	>5000	ND
19	Me	4-CNPh	950 ± 90	940 ± 90
20	Me	4-CF_3Ph	>5000	ND
21	Me	4-SO_2MePh	>5000	ND
22	Me	3-CF_3Ph	>5000	ND
23	Me	3-ClPh	>5000	ND
24	Me	3,4-di-ClPh	55 ± 6	95 ± 2
25	Me	3-Cl-4-BrPh	30 ± 1	75 ± 2
26	Me	3-CF_3-4-BrPh	24 ± 2	48 ± 4
27	Me	3-CF_3-4-ClPh	13 ± 1	25 ± 5

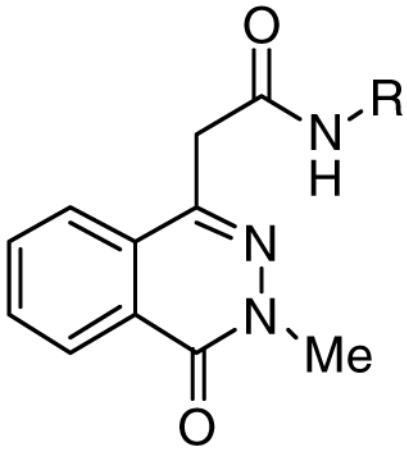


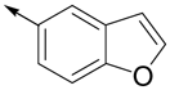
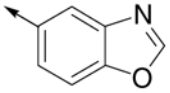
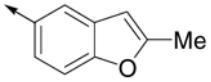
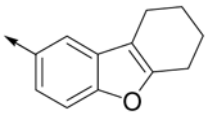
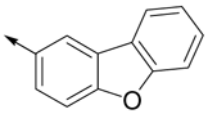
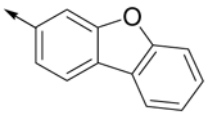
Compound	R	R^1	(−)-BSA (nM)	(+)-BSA (nM)
28	Me	3-CF_3-4-CNPh	54 ± 5	52 ± 6
29	Me	3-Cl-4-CNPh	290 ± 40	470 ± 120
30	Me	3-CF_3-4-FPh	150 ± 20	150 ± 30
31	Me	3-CF_3-4-OMePh	>5000	ND
32	Me	3-CF_3-4-NH_2Ph	>5000	ND
33	Me	3-F-4-ClPh	200 ± 30	240 ± 30
34	Me	3-Me-4-ClPh	79 ± 9	96 ± 19
35	Me	3-Me-4-BrPh	70 ± 10	140 ± 10
36	Me	3-Me-4-CNPh	400 ± 100	460 ± 90
37	Me	3-OMe-4-ClPh	33 ± 1	40 ± 7
38	Me	3,4-di-CNPh	>2500	>2500
39	Me	2-CF_3-4-ClPh	>5000	ND
40	Me	2-CF_3-4-BrPh	>5000	ND
41	Me	2-F-4-BrPh	1100 ± 200	1700 ± 100
42	Me	2,4-BrPh	>5000	ND
43	Me	2,4-di-ClPh	>5000	ND
44	Me	3,5-di-ClPh	>5000	ND
45	Me	3,4,5-tri-ClPh	>5000	ND
46	Me	3,4,5-tri-FPh	>5000	ND
47	Me	2-naphthyl	63 ± 3	140 ± 20
48	H	3-CF_3-4-ClPh	40 ± 1	61 ± 1
49	H	3-CF_3-4-BrPh	32 ± 6	130 ± 8

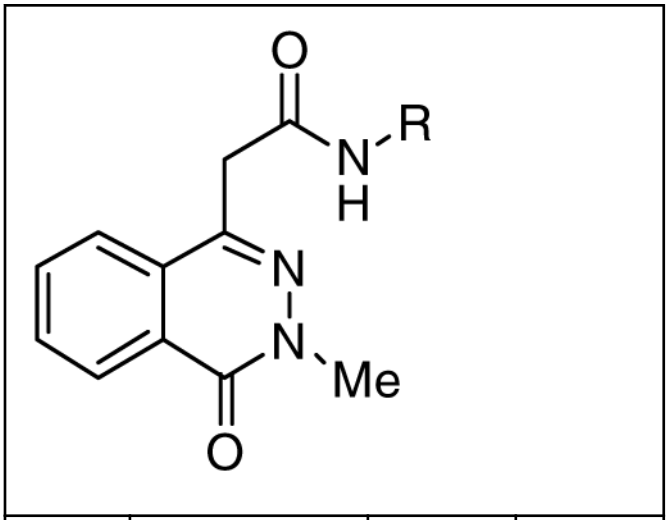
All values are average of three independent determinations unless otherwise noted (* two determinations). ND, not determined.

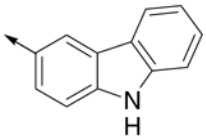
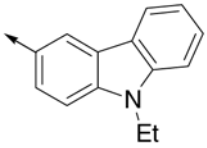
Table 2
SAR of heterotricyclic anilide phthalazinone inhibitors

Assays as described in [10].



Compound	R	(−)-BSA (nM)	(+)-BSA (nM)
50		93 ± 15	85 ± 12
51		>5000	ND
52		64 ± 11	72 ± 15
53		20 ± 5	150 ± 20
54		4 ± 1	30 ± 10
55		>5000	ND



Compound	R	(−)-BSA (nM)	(+)-BSA (nM)
56		>5000	ND
57		>5000	ND

All values are average of three independent determinations unless otherwise stated. ND, not determined.

Table 3

Antiparasitic activity

Assays as described in [15].

Compound	EC_{50}[a] (μM)		Selectivity [b]
	Toxo/WT	Toxo/*Cp*IMPDH	
26	4 ± 3 *	0.3 ± 0.2	14
27	23 ± 4	0.4 ± 0.3	60
53	1 ± 1 *	0.5 ± 0.2	2
54	3 ± 1	0.4 ± 0.1	7

Values are the average and standard deviations of three independent determinations unless otherwise stated

* denotes average and range of two determinations).

[a] *T. gondii* RH (Toxo/WT) should be resistant to *Cp*IMPDH inhibitors while *T. gondii*/*Cp*IMPDH (Toxo/*Cp*IMPDH) should be sensitive.

[b] Selectivity = EC_{50}(Toxo/WT)/EC_{50}(Toxo/*Cp*IMPDH).