

# Antigiardial Activity of Novel Guanidine Compounds

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From four focused compound libraries based on the known anticoccidial agent robenidine, 44 compounds total were synthesised and screened for anti-giardial activity. All active compounds were counter-screened for antibiotic and cytotoxic action. Of the analogues examined, 21 displayed  $IC_{50} < 5 \mu M$ , seven with  $IC_{50} < 1.0 \mu M$ . Most active were 2,2'-bis[4-(trifluoromethoxy)phenyl]methylene]carbonimidic dihydrazide hydrochloride (30), 2,2'-bis[4-(trifluoromethylsulfanyl)phenyl]methylene]carbonimidic dihydrazide

hydrochloride (32), and 2,2'-bis[(2-bromo-4,5-dimethoxyphenyl)methylene]carbonimidic dihydrazide hydrochloride (41) with  $IC_{50} = 0.2 \mu M$ . The maximal observed activity was a 5 h  $IC_{50}$  value of  $0.2 \mu M$  for 41. The clinically used metronidazole was inactive at this timepoint at a concentration of  $25 \mu M$ . Robenidine off-target effects at bacteria and cell line toxicity were removed. Analogue 41 was well tolerated in mice treated orally (100 mg/kg). Following 5 h treatment with 41, no *Giardia* regrowth was noted after 48 h.

## Introduction

*Giardia duodenalis*, a bi-nucleate protozoan pathogen, is the most common enteric human parasitic pathogen known, causing up to 1 billion human infections annually.<sup>[1–4]</sup> Infections are most common in developing nations, but are also prevalent in the developed world. *Giardia* has been added to the World Health Organization neglected diseases initiative.<sup>[5]</sup> *Giardia* infection occurs via cyst ingestion through a faecal-oral route or via contaminated food or water.<sup>[5,6]</sup> It results in a mal-absorptive gastrointestinal disease with acute, chronic and at times re-occurring symptoms including diarrhoea, bloating and abdominal cramping.<sup>[7]</sup> Persistent infection, especially in children and immunocompromised hosts, results in long term effects includ-

ing malnutrition, developmental delay and failure to thrive syndrome.<sup>[8]</sup>

Drug treatment most commonly uses the nitroimidazoles, nitrothiazole, nitrofurans, acridine, benzimidazole, and aminoglycoside compound classes.<sup>[8]</sup> The most frequently used nitroimidazoles, metronidazole (1) and tinidazole (2), show an 80–90% success; while albendazole (3), a benzimidazole, has a reported efficacy of 62–95%. Treatment failures with these drugs are common with side effects including genotoxicity, possible carcinogenesis, nausea, fatigue and general malaise.<sup>[8–10]</sup> Disturbingly, resistant organisms have been reported for all the commonly used drugs.<sup>[9–11]</sup> This combination of limitations highlights a pressing need for new treatments.

Current anti-giardial drug discovery efforts have focused in on the small molecule inhibition of a wide range of protein targets. These span pyruvate-ferredoxin oxidoreductase, nitroreductase 1 and thioredoxin reductase to activate nitroimidazoles,<sup>[12–14]</sup> NADH oxidase for activation of furazolidone,<sup>[15]</sup> auranofin inhibits thioredoxin reductase,<sup>[15]</sup> fumagillin inhibits methionine amino-peptidase,<sup>[16]</sup> orlistat inhibits gastric lipase,<sup>[17]</sup> proton-pump inhibitors have been investigated, with omeprazole inhibiting triosephosphate isomerase, a critical enzyme involved in glucose and glycogen metabolism,<sup>[18,19]</sup> disulfiram inhibits acetaldehyde dehydrogenase,<sup>[20,21]</sup> NBDHEX inhibits glutathione S-transferase.<sup>[22]</sup> Other potential targets include antioxidant and metabolic enzymes<sup>[23–29]</sup> and protein kinases, with the reduced number of core *Giardia* kinases suggesting limited redundancy, and thus potentially high efficacy.<sup>[30]</sup>

Herein we report on the discovery and development of novel anti-giardial agents based on the anticoccidial agent, robenidine (4; Figure 1).<sup>[10,31]</sup>


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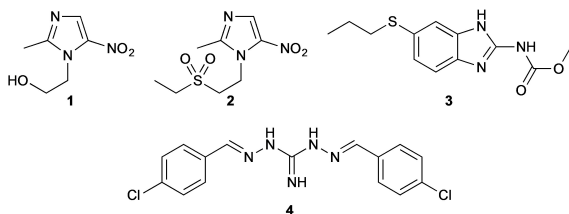
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## Results and Discussion

Preliminary screening of robenidine (**4**) against *Giardia* trophozoites *in vitro* revealed a 5 h  $IC_{50}$  of 0.9  $\mu\text{M}$  and a 24 h MIC (minimum inhibitory concentration) of 2.8  $\mu\text{M}$ . Clinically used metronidazole (**1**) was inactive at 25  $\mu\text{M}$  at 5 h, but showed a 24 h  $IC_{50}$  of 3.8  $\mu\text{M}$  (Table 1).<sup>[32–34]</sup>

While potent, robenidine (**4**) has off target antibacterial activity and cell line toxicity.<sup>[35]</sup> We sought to address these concerns through the synthesis and subsequent biological screening of focused compound libraries. Robenidine analogues were rapidly accessed through the condensation of a series of benzaldehydes and *N,N'*-diaminoguanidine hydrochloride (Scheme 1), afforded rapid access to the desired analogues (Table 1). Retention of the *N,N'*-diaminoguanidine core enabled the SAR activity of the pendent aromatic moieties to be examined in this initial study, as the nature of the robenidine anti-*Giardia* activity (what protein target) is unknown.

Limiting *Library 1* to mono-substituted halogenated benzaldehydes afforded nine analogues (**5**–**13**) in good to excellent yields. Of these, seven returned anti-*Giardia*  $IC_{50}$  values of <25  $\mu\text{M}$ ; three of which displayed no antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE) or *Escherichia coli* (**6**, **11** and **12**); with 4-Br **6** also showing no cytotoxicity against CaCo-2 or Vero cells at <10% cell death at 25  $\mu\text{M}$ . No activity against gram negative bacteria was noted (not shown). In this first library, brominated analogues (**6**, **9** and **11**) displayed the



**Figure 1.** Chemical structures of the clinically used metronidazole (**1**), tinidazole (**2**), albendazole (**3**) and lead robenidine (**4**).

highest levels of *Giardia* activity, but activity was only marginally enhanced relative to the corresponding Cl-analogues (**4**, **8** and **12**). Isosteric modification to 'F' saw moderate levels of activity (with 2-F **13**, inactive  $IC_{50}$  > 25  $\mu\text{M}$ ).

An extension of our structure activity relationship (SAR) evaluation saw the introduction of alkyl substituents with the synthesis (as per Scheme 1) and evaluation of **14**–**23**. The screening data for these *Library 2* analogues is presented in Table 2.

In each instance the alkyl substituted *Library 2* analogues displayed *Giardia* activity < 25  $\mu\text{M}$ . The highest levels of activity were observed with the more hydrophobic substituent with 4-Ph **17** ( $IC_{50}$  = 0.4  $\mu\text{M}$ ) more active than 2-Ph **18** ( $IC_{50}$  = 2.8  $\mu\text{M}$ ), while all other alkyl substituents (excepting the  $-\text{CH}_3$  analogues **14**–**16**) displayed excellent *Giardia* inhibition at 3.2–0.8  $\mu\text{M}$  (**22** and **19** respectively). Acetylenic **23** was well tolerated ( $IC_{50}$  = 1.2  $\mu\text{M}$ ). Given the high level of anti-*Giardia* activity, each compound was then evaluated for potential antibiotic activity with only 2-Ph **18** antibiotic inactive, and thus subjected to toxicity assessment with Vero and CaCo-2 cells. In this instance, **18** displayed toxicity at the 25  $\mu\text{M}$  concentration evaluated. Despite this finding, our data supports a possible dissection of the biological activity profile of this series of compounds, and we note the favourable anti-*Giardia* outcome with 2-disposed analogues (2-Br **11** and 2-Ph **18**).

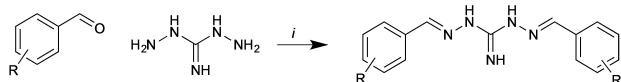
The introduction of a polar or heteroaromatic linked hydrophobic moieties with *Library 3* (**24**–**33**) was largely detrimental to anti-*Giardia* activity with only 4-OCF<sub>3</sub> **30**, 4-SCH<sub>3</sub> **31**, 4-SCF<sub>3</sub> **32** and 4-N(CH<sub>3</sub>)<sub>2</sub> **33** returning  $IC_{50}$  values < 2  $\mu\text{M}$  (0.2, 0.9, 0.2 and 1.1  $\mu\text{M}$ , respectively), but with concomitant activity against gram negative bacteria (Table 3). With the observed antibiotic activity, these analogues were not assessed for their toxicity profile.

Further library development explored the introduction of additional ring substituents with *Library 4*. The screening outcome for these analogues is presented in Table 4. As speed of action might be anticipated to influence uptake and efficacy of these agents, we explored the time to effective action, noting that essentially 100% eradication of the *Giardia* trophozoites

**Table 1.** Inhibition of *Giardia duodenalis* by *Library 1* Robenidine analogues possessing mono-halogenated aromatic rings (**4**–**13**).<sup>[a]</sup>

Compound	R	Anti- <i>Giardia</i> activity		Antibacterial activity (Y/N) <sup>[b]</sup>	Toxicity (Y/N) (% growth control) <sup>[c]</sup>	Selectivity ratio <sup>[d]</sup>
		MIC [ $\mu\text{M}$ ] (24 h)	$IC_{50}$ [ $\mu\text{M}$ ] (24 h)			
Metronidazole	–	8.3	3.8	N	N	> 6.6
Robenidine <b>4</b>	4-Cl	2.8	0.9	Y	Y (40.7 $\pm$ 22.6)	> 27.8
<b>5</b>	H	–	–	N	–	–
<b>6</b>	4-Br	8.3	1.7	N	N (97.1 $\pm$ 1.6)	> 14.7
<b>7</b>	4-F	12.5	6.52	Y	–	–
<b>8</b>	3-Cl	6.25	3.5	Y	–	–
<b>9</b>	3-Br	25	2.9	Y	–	–
<b>10</b>	3-F	> 25	> 25	Y	–	–
<b>11</b>	2-Br	25	0.8	N	Y (79.1 $\pm$ 2.3)	> 31.3
<b>12</b>	2-Cl	25	12.37	N	–	–
<b>13</b>	2-F	> 25	> 25	N	N <sup>[e]</sup> (93.9 $\pm$ 1.9)	–

[a] Toxicity (CaCo-2 and Vero cells) and antimicrobial assays performed at 25  $\mu\text{M}$ ; MIC: minimum inhibitory concentration; '–' not tested, as inhibitory activity at 25  $\mu\text{M}$  < 50%. [b] MSRA and VRE. [c] Percent CaCo-2 cell growth (25  $\mu\text{M}$ ); compounds are indicated as toxic if > 20% cells are affected. [d] Higher ratios indicate a more selective compound. [e] Toxicity assessed with Vero cells, not CaCo-2 cells.



**Scheme 1.** Reagents and conditions: (i) aldehyde (see Table 1 for detail), EtOH, reflux.

was achieved with some analogues in as little as 5 h. As such we determined the efficacy of *Library 4* at a 5 h timepoint.

Of the 14 *Library 4* analogues developed, seven (**34**, **35**, **38**, **40–42** and **44**) returned anti-*Giardia* IC<sub>50</sub> values of ≤ 25 μM (Table 4 and ES†). The introduction of a second –Cl moiety (c.f. **34** vs **4**) was detrimental to activity, but the 3,4-difluoro **38** was active (IC<sub>50</sub> = 1.2 μM). However, the equivalent 2,5-difluoro **37** and pentafluoro **39** were inactive. Other variations in the phenyl ring substituent level and pattern were largely detrimental to activity (ES†). Of *Library 4*, only **41** was devoid of antibacterial activity and cell toxicity, and fortuitously this was the most active analogue with a 5 h IC<sub>50</sub> of 0.2 μM.

This combined with the lack of antibacterial activity and cell toxicity differentiates **41** from **4** as an anti-*Giardia* development

lead. To further investigate the validity of this compound series as leads in the development of potential anti-*Giardia* treatments, disubstituted **41** was examined in a mouse model of *Giardia* infection.

Our preliminary animal studies revealed no adverse effects with mice treated *per os* with 100 mg/kg **41** per day for 3 days, strongly suggesting that **41** is a viable lead development candidate. Moreover, in washout experiments, the removal of **4** and **41** after *Giardia* death did not result in *Giardia* regrowth after 48 hours (Figure 2).

## Conclusions

From the analogues noted herein, **6**, **11**, **13**, **40** and **41** were identified as potential anti-*Giardia* development candidates. Analogue **41** displays the most promising activity and safety profile of the analogues developed herein. All analogues showed the expected high levels of anti-*Giardia* activity.

The initial limitations of **4** as an anti-*Giardia* agent have been overcome through the development of **41**, with a 5-fold potency increase (5 h IC<sub>50</sub> = 0.2 μM), no antibacterial activity

**Table 2.** Inhibition *Giardia duodenalis* metabolism by *Library 2* Robenidine analogues possessing alkyl and aromatic substituents (**14–23**).<sup>[a]</sup>

Compound	R	Anti- <i>Giardia</i> activity		Antibacterial activity (Y/N) <sup>[b]</sup>	Toxicity (Y/N) (% growth control) <sup>[c]</sup>	Selectivity ratio <sup>[d]</sup>
		MIC [μM] (24 h)	IC <sub>50</sub> [μM] (24 h)			
Metronidazole	–	8.3	3.8	N	N	> 6.6
<b>14</b>	4-CH <sub>3</sub>	25	6.98–21.6	Y	–	–
<b>15</b>	3-CH <sub>3</sub>	6.25	4.9	Y	–	–
<b>16</b>	2-CH <sub>3</sub>	25	5.62–12.98	Y	–	–
<b>17</b>	4-Ph	2.8	0.4	Y	–	–
<b>18</b>	2-Ph	> 25	2.8	N	Y (75.4 ± 3.4)	> 8.9
<b>19</b>	4-(CH <sub>2</sub> ) <sub>2</sub> CH <sub>3</sub>	8.3	0.8	Y	–	–
<b>20</b>	4-(CH <sub>2</sub> ) <sub>3</sub> CH <sub>3</sub>	8.3	1.9	Y	–	–
<b>21</b>	4-CH(CH <sub>3</sub> ) <sub>2</sub>	6.25	1.9	Y	–	–
<b>22</b>	4-C(CH <sub>3</sub> ) <sub>3</sub>	8.3	3.2	Y	–	–
<b>23</b>	4-CCH	25	1.2	Y	–	–

[a] Toxicity (CaCo-2 and Vero cells) and antimicrobial assays performed at 25 μM; MIC: minimum inhibitory concentration; ‘–’ not tested, as inhibitory activity at 25 μM < 50%. [b] MSRA and VRE. [c] Percent CaCo-2 cell growth (25 μM); compounds are indicated as toxic if > 20% cells are affected. [d] Higher ratios indicate a more selective compound.

**Table 3.** Inhibition *Giardia duodenalis* metabolism by *Library 3* Robenidine analogues possessing heteroatom-substituted aromatic rings (**24–33**).<sup>[a]</sup>

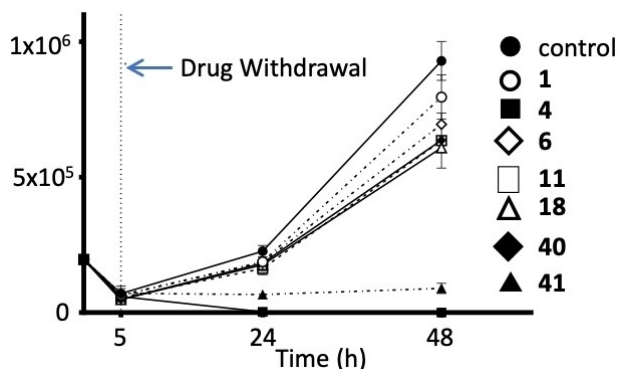
Compound	R	Anti- <i>Giardia</i> activity		Antibacterial activity (Y/N) <sup>[b]</sup>	Toxicity (Y/N) (% growth control) <sup>[c]</sup>	Selectivity ratio <sup>[d]</sup>
		MLC [μM] (24 h)	IC <sub>50</sub> [μM] (24 h)			
Metronidazole	–	8.3	3.8	N	N	> 6.6
<b>24</b>	4-OCH <sub>3</sub>	12.5	5.99	N	Y <sup>[e]</sup>	> 4.2
<b>25</b>	3-OCH <sub>3</sub>	> 25	> 25	Y	–	–
<b>26</b>	2-OCH <sub>3</sub>	> 25	> 25	Y	–	–
<b>27</b>	4-OH	> 25	> 25	Y	–	–
<b>28</b>	3-OH	> 25	> 25	Y	–	–
<b>29</b>	2-OH	> 25	> 25	Y	–	–
<b>30</b>	4-OCF <sub>3</sub>	2.8	0.2	Y	–	–
<b>31</b>	4-SCH <sub>3</sub>	8.3	0.9	Y	–	–
<b>32</b>	4-SCF <sub>3</sub>	2.8	0.2	Y	–	–
<b>33</b>	4-N(CH <sub>3</sub> ) <sub>2</sub>	25	1.1	Y	–	–

[a] Toxicity (CaCo-2 and Vero cells) and antimicrobial assays performed at 25 μM; MLC: minimum lethal concentration; ‘–’ not tested, as inhibitory activity at 25 μM < 50%. [b] MSRA and VRE. [c] Percent CaCo-2 cell growth (25 μM); compounds are indicated as toxic if > 20% cells are affected. [d] Higher ratios indicate a more selective compound. [e] Toxicity assessed with Vero cells, not CaCo-2 cells.

**Table 4.** Inhibition of *Giardia duodenalis* metabolism by Library 4 Robenidine analogues possessing di-, tri- and poly-substituted aromatic rings (34–47).<sup>[a]</sup>

Compound	R	Antigiardial activity		Antibacterial activity (Y/N) <sup>[b]</sup>	Toxicity (Y/N) (% growth control) <sup>[c]</sup>	Selectivity ratio <sup>[d]</sup>
		MLC [ $\mu\text{M}$ ] (24 h)	IC <sub>50</sub> [ $\mu\text{M}$ ] (5 h)			
34	2,4-Cl	3.13	2.49	N	Y <sup>[e]</sup> (55.8 ± 4.3)	> 10.0
35	3,5-Cl	25	11.6	N	–	–
36	2,6-Cl	–	–	N	–	–
37	2,5-F	–	–	N	–	–
38	3,4-F	8.3	1.2	Y	–	–
39	2,3,4,5,6-F	–	–	N	–	–
40	2-NHCOCH <sub>3</sub> , 4-Cl	25	0.8	N	Y (83.1 ± 4.4)	> 31.3
41	2-Br, 4,5-OCH <sub>3</sub>	2.8	0.2	N	N	> 125
42	3-Br, 4,5-OCH <sub>3</sub>	3.13	2.7	N	Y <sup>[e]</sup>	> 9.3
43	2-OH, 4-N(CH <sub>3</sub> ) <sub>2</sub>	–	–	Y	–	–
44	3,4-OCH <sub>3</sub>	> 25	24.9	N	–	–
45	3-NO <sub>2</sub> , 4-OH	–	–	N	–	–
46	3-OH, 4-OCH <sub>3</sub>	–	–	N	–	–
47	3-OCH <sub>3</sub> , 4-OH	> 25	> 25	Y	–	–

[a] Toxicity (CaCo-2 and Vero cells) and antimicrobial assays performed at 25  $\mu\text{M}$ ; MLC: minimum lethal concentration; '–' not tested, as inhibitory activity at 25  $\mu\text{M}$  < 50%. [b] MSRA and VRE. [c] Percent CaCo-2 cell growth (25  $\mu\text{M}$ ); compounds are indicated as toxic if > 10% cells are affected. [d] Higher ratios indicate a more selective compound. [e] Toxicity assessed with Vero cells, not CaCo-2 cells.



**Figure 2.** Growth recovery of *Giardia duodenalis* after exposure to selected analogues for 5 h. –●– control (no compound); –○– metronidazole (1); –v– Robenidine (4); –v–(40); –◇–(6); –△– (18); –□– (11); –◊– (41) Cell numbers were determined 24 and 48 h post compound removal.

and no observed cytotoxicity in the systems examined. This activity also contrasts the clinically used metronidazole with known antibacterial activity, slow onset of activity (24 h vs 5 h) and 20-fold lower anti-giardial activity. Indeed, 41 shows marked impact on *Giardia* trophozoites after only 1–2 h (data not shown).

As yet the drug target of these analogues remains undetermined. However, in our antibiotic studies with related compounds we observed that robenidine analogues affected the bacterial cell wall.<sup>[31,32,36–39]</sup> It is possible that a similar mechanism, against the trophozoite cell wall is in play. This would be in keeping with our electron microscopy observations of gross morphological changes to the dorsal cytoplasmic membrane of trophozoites, including membrane rupture. These effects would adversely affect the ability of the *Giardia* trophozoite to attach to membrane, interrupting a crucial stage in the parasite lifecycle, with the observed parasite killing.<sup>[31,36]</sup>

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## Conflicts of interest

S.W.P. is director of Neoculi Pty. Ltd., who are seeking to develop these analogues for use in at-risk species. The authors declare no other conflicts of interest.

## Data Availability Statement

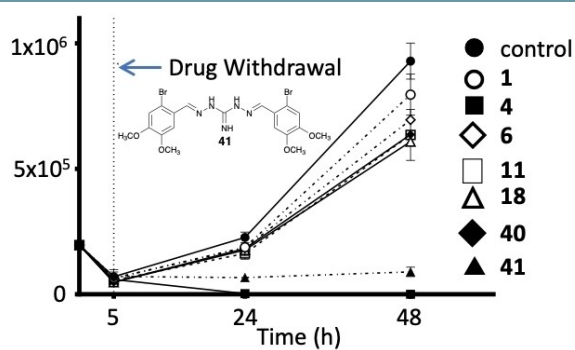
The data that support the findings of this study are available in the supplementary material of this article.

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## RESEARCH ARTICLE



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### Antigiardial Activity of Novel Guanidine Compounds

**Rapid and complete killing of *Giardia* trophozoites:** From four focused compound libraries based on the known anticoccidial agent robenidine, 44 compounds were synthesised and screened for anti*giardial* activity. All

active compounds were counter-screened for antibiotic and cytotoxic action. Of the analogues examined, 21 displayed  $IC_{50}$  values less than  $5 \mu M$ , seven with  $IC_{50}$  values less than  $1.0 \mu M$ .

