Identification of a Hit Series of Antileishmanial Compounds through the Use of Mixture-Based Libraries

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S Supporting Information

ABSTRACT: From a screening campaign that included mixture-based libraries containing more than 6 million compounds, a lead series of bis-cyclic guanidines was identified as the most promising. Lead optimization resulted in the identification of potent ($IC_{50} < 500$ nM) and selective compounds within this series as well as potent and selective monoguanidines.

KEYWORDS: Leishmaniasis, screening, mixture-based libraries, lead optimization

Leishmaniasis is a disease caused by infection with *Leishmania* parasites, which is spread by the bite of phlebotomine sand flies. The most severe form of the disease is visceral leishmaniasis caused by the protozoan parasites *Leishmania donovani* and *Leishmania infantum*.^{1,2} People suffering from visceral leishmaniasis experience cachexia, cytopenia, fever, hepatosplenomegaly, and death if not treated.² It is estimated that there are 200,000 to 400,000 new cases of visceral leishmaniasis each year and >20,000 deaths a year due to the disease.³ Current therapeutics for patients with visceral leishmaniasis include liposomal amphotericin B (1), sodium stibogluconate (2), miltefosine (3), pentamidine (4), and paromomycin (5, Figure 1). All of these therapeutics are less than ideal through some combination of ease-of-use, efficacy, cost, safety, and drug resistance.⁴ While much effort is underway on the development of new therapeutics, there is a pressing need to continue to identify novel compounds for the treatment of this disease.

We have previously reported on the use of mixture-based libraries to rapidly identify and prioritize active scaffolds.^{5–7} Using this approach, we evaluated a scaffold-ranking library containing >6 million compounds in a *L. donovani* axenic amastigote assay as well as a high-content imaging screen utilizing macrophages infected with *L. donovani* amastigotes. As previously described in detail,^{5–7} a scaffold-ranking library is a collection of mixture samples where each mixture sample contains only compounds with the same core scaffold, and therefore, all of the compounds in a given mixture are close structural analogues. Additionally, each compound in a specific mixture sample is represented in approximately equal molar

concentration. From this initial screen, the bis-cyclic guanidine scaffold-ranking sample **Lib1** demonstrated potency in both of the aforementioned leishmaniasis assays with no sign of cytotoxicity in an uninfected macrophage counter screen.

Figure 1. Currently used antileishmaniasis agents.

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Table 1. Leishmaniasis Activity of R₁ Positional Scanning Study of Lib1^a

ID#	R ₁	a.a.	i.m.	ID#	R ₁	a.a.	i.m.	ID#	R ₁	a.a.	i.m.	ID#	R ₁	a.a.	i.m.
Lib1.001	H	0%	2.5	Lib1.012		0%	1.1	Lib1.023		10%	5.2	Lib1.033		0%	4.6
Lib1.002		69%	>10	Lib1.013		0%	0.9	Lib1.024		31%	>10	Lib1.034		61%	1.2
Lib1.003		94%	1.2	Lib1.014		99%	5.2	Lib1.025		91%	>10	Lib1.035		100%	1.2
Lib1.004		91%	>10	Lib1.015		66%	>10	Lib1.026		93%	0.9	Lib1.036		100%	>10
Lib1.005		42%	>10	Lib1.016		37%	1.3	Lib1.027		0%	2.5	Lib1.037		100%	2.4
Lib1.006		86%	3.7	Lib1.017		5%	>10	Lib1.028		14%	1.7	Lib1.038		100%	1.6
Lib1.007		81%	0.7	Lib1.018		23%	5.2	Lib1.029		61%	1.9	Lib1.039		98%	1.2
Lib1.008		87%	>10	Lib1.019		0%	5.1	Lib1.030		99%	3.6	Lib1.040		63%	>10
Lib1.009		14%	3.5	Lib1.020		4%	5.3	Lib1.031		100%	0.6	Lib1.041		99%	1.2
Lib1.010		0%	>10	Lib1.021		100%	1.3	Lib1.032		63%	2.9	Lib1.042		99%	0.5
Lib1.011		34%	>10	Lib1.022		0%	>10								

^aa.a. is the % inhibition at 5 μ M in the axenic amastigotes assay. i.m. is the IC₅₀ in μ M of the infected macrophage assay. The structure for this table is the same for each of the subsequent tables.

A positional scanning library was then screened in order to develop a structure–activity relationship (SAR) profile around the core bis-cyclic guanidine scaffold. From this analysis, 27 individual compounds were selected as good candidates to validate the profile.

Upon confirmation of the SAR trends with the individual compounds, a lead optimization campaign was undertaken to further explore the SAR profile around the scaffold. These efforts resulted in the discovery of several new compounds possessing submicromolar IC₅₀s in the *L. donovani* infected macrophage assay. Among the hits is a monocyclic guanidine compound, **64**.

We initiated our study by screening 28 of our scaffold-ranking libraries at four doses (50, 25, 5, and 2.5 μ M) in an assay using axenic *L. donovani* amastigote strains, CR6 and CS1, as described in Wang et al.⁸ Only scaffold-ranking sample **Lib1** showed substantial activity at any of the doses tested, producing >90% inhibition at the 25 and 50 μ M doses.⁹

While the axenic amastigote assay allows for a quick assessment of a sample's intrinsic antileishmanial activity, it does have the weakness in that the effect of the host mammalian cell

cannot be taken into account. In order to assess the antileishmanial efficacy of the scaffold-ranking samples against intracellular parasites, a high content screening (HCS) assay with *L. donovani* infected J774 macrophages was utilized.¹⁰ In addition, a counter screen assay utilizing noninfected J774 macrophages was performed on the scaffold-ranking samples to assess their potential toxicity to the host mammalian cell. From these two assays, a selectivity index (SI) was calculated by dividing the IC₅₀ obtained from the noninfected macrophage by the IC₅₀ from the infected macrophage assay. From this infected macrophage screen, we found several scaffolds with attractive activity (submicromolar IC₅₀) and selectivity (>50 SI) profiles, one of which was **Lib1**. We thus turned our attention to exploring a positional scanning study on this scaffold.

Using our previously described synthetic methods,^{5–7} we screened a 110 sample array around **Lib1** (42 carboxylic acids $\times$ 42 carboxylic acids $\times$ 26 amino acids). In this manner, we were able to evaluate a total of 45,864 different bis-cyclic guanidine analogues, where each compound was replicated in three different samples. The results of these screens are shown in Tables 1–3. Although the libraries were evaluated at several

Table 2. R₂ Positional Scanning Study of Lib1^a

ID#	R ₂	a.a.	i.m.	ID#	R ₂	a.a.	i.m.
Lib1.043		91%	>10	Lib1.056		0%	>10
Lib1.044		99%	>10	Lib1.057		3%	>10
Lib1.045	H	84%	>10	Lib1.058		23%	5.3
Lib1.046		100%	1.2	Lib1.059		0%	5.4
Lib1.047		100%	5.1	Lib1.060		29%	>10
Lib1.048		0%	5.8	Lib1.061		56%	5.2
Lib1.049		7%	>10	Lib1.062		98%	5.3
Lib1.050		65%	>10	Lib1.063		99%	4.5
Lib1.051		0%	>10	Lib1.064		100%	5.1
Lib1.052		79%	>10	Lib1.065		95%	>10
Lib1.053		96%	>10	Lib1.066		26%	3.8
Lib1.054		99%	>10	Lib1.067		99%	>10
Lib1.055		97%	>10	Lib1.068		100%	0.6

^aa.a. is the % inhibition at 5 μ M in the axenic amastigotes assay. i.m. is the IC₅₀ in μ M of the infected macrophage assay.

concentrations, the best range of results was apparent at 5 μ M, and the percent inhibition at this concentration is shown.¹¹ This data allowed us to rank the relative likelihood that active individual compounds contain certain functionalities at a given position and to begin to augur certain trends. For example, based on the 5 μ M data, it is clear that the mixture defined with cyclohexylbutyl at the R₁ position (Table 1, sample Lib1.031) is more active (100%) than the one defined with isobutyl (0%) at the same R₁ position (Table 1, sample Lib1.022). This implies that it is more likely to find active individual compounds with cyclohexylbutyl defined at R₁ than with isobutyl at R₁. Across all three of these positions, oxygenated substituents dramatically reduced activity at the 5 μ M dose. Also, there appears to be little preference for stereochemical specificity at the R₂ position (Table 2), where diastereomeric mixtures (e.g., Lib1.046/Lib1.054 and Lib1.049/Lib1.057) demonstrated nearly identical activities. In addition to the axenic amastigote data in Tables 1–3, the IC₅₀ data from the HCS infected macrophage assay is also provided. The infected macrophage data does not correlate perfectly with the axenic amastigote data, which is consistent with what we observed in our initial scaffold-ranking studies. While both assays measure the ability of a sample to inhibit the growth of the amastigotes at a given

dose, the infected macrophage assay requires the samples to penetrate the macrophage first in order to get to the amastigote. Regardless, a number of the samples that produced sub-2.5 μ M IC₅₀ in the infected macrophage assay also produced a >80% response at the 5 μ M dose in the axenic amastigotes assay, demonstrating these samples' ability to inhibit the growth of the amastigotes in both assay formats.

In order to confirm some of the SAR trends that were observed in the positional scanning library data as well as to identify individual hit compounds with antileishmanial activity, a set of previously synthesized bis-cyclic guanidines were selected for testing.¹² The compounds were synthesized as previously described incorporating three different functionalities in each of the R₁, R₂, and R₃ positions resulting in 27 structural analogues (Table 4). These different functionalities, when incorporated into the positional scanning library, demonstrated a range of activities in the anti-*Leishmania* proliferation assays (Tables 1–3), therefore making them a good set to test the SAR, which is shown in Table 4. In general, when cyclohexylbutyl was fixed in the R₁ position (compounds 15 to 23), the compounds showed an increased response in the axenic amastigote assay (6 out of 9 showed >65% inhibition at 2.5 μ M) relative to the other compounds (2 out of 18). These results are consistent with the data generated from the positional scanning library data (Table 1) where Lib1.031 showed greater activity than Lib1.041 or Lib1.007. Of those nine individual compounds (15–23), the compounds where R₂ was R-2-naphthylmethyl were the inactive analogues (18–20). Again, this is consistent with the positional scanning data (Table 2) showing that Lib1.066 was much less active than Lib1.063 or Lib1.067. Using these trends as well as the range of activity in the infected macrophage and cytotoxicity assays, we initiated lead optimization activities.¹³

The first two candidates we selected for further exploration were 21 and 26, both of which showed submicromolar activity in the infected macrophage assay with dramatically different activities in the axenic amastigote assay. In both cases, we prepared a series of deletion analogues (in hopes of decreasing their lipophilicity) as well as inverted the stereocenter built into the initial scaffold (33 and 40, Table 5). In both cases, this scaffold change resulted in a significant decrease in the activity. For the series around 21, most of the deletion compounds resulted in a significant deleterious effect on the infected macrophage activity, with the sole exception being 34. However, the good activity and SI profiles for 42 and 43 suggested that we might be able to focus our attention on monosubstituted analogues. Toward this end, while biphenyl compound 46 was not particularly active, the truncated phenethyl compound 47 showed excellent activity and selectivity.

We also pursued additional studies around compounds 6 and 8, which differed only about the R₃ position, but showed considerable activity in the infected macrophage assay (Table 6). While we again observed some compounds with acceptable activity and SI profiles, we were pleased to note that monosubstituted analogue 50 was quite potent. We therefore synthesized and analyzed some analogues around this compound. Desfluoro analogue 55 gave an excellent profile and indicated a preference for meta-substitution (see 54, 56, and 57). Saturation of the ring revealed that 59 was another analogue with an excellent profile.

Encouraged by the identification of multiple monosubstituted analogues with submicromolar activity and low cytotoxicity,

Table 3. R₃ Positional Scanning Study of Lib1^a

ID#	R ₃	a.a.	i.m.	ID#	R ₃	a.a.	i.m.	ID#	R ₃	a.a.	i.m.	ID#	R ₃	a.a.	i.m.
Lib1.069	H	0%	5.4	Lib1.080		13%	>10	Lib1.091		7%	0.9	Lib1.101		0%	1.4
Lib1.070		38%	>10	Lib1.081		21%	1.3	Lib1.092		7%	5.1	Lib1.102		9%	1.2
Lib1.071		63%	3.6	Lib1.082		86%	>10	Lib1.093		58%	1.3	Lib1.103		91%	1.3
Lib1.072		88%	0.6	Lib1.083		3%	1.2	Lib1.094		79%	1.8	Lib1.104		94%	1.3
Lib1.073		11%	1.3	Lib1.084		19%	1.3	Lib1.095		10%	2.7	Lib1.105		11%	>10
Lib1.074		11%	1.3	Lib1.085		1%	1.8	Lib1.096		21%	1.7	Lib1.106		97%	3.5
Lib1.075		25%	1.4	Lib1.086		13%	4.8	Lib1.097		15%	1.4	Lib1.107		42%	>10
Lib1.076		57%	0.4	Lib1.087		2%	1.3	Lib1.098		92%	>10	Lib1.108		11%	>10
Lib1.077		11%	6.1	Lib1.088		12%	0.7	Lib1.099		100%	4.9	Lib1.109		90%	0.7
Lib1.078		9%	7	Lib1.089		99%	5.1	Lib1.100		15%	1.2	Lib1.110		62%	2.7
Lib1.079		12%	>10	Lib1.090		0%	0.7								

^aa.a. is the % inhibition at 5 μ M in the axenic amastigotes assay. i.m. is the IC₅₀ in μ M of the infected macrophage assay.

Table 4. Individually Synthesized Compound Activity^a

#	R ₁	R ₂	R ₃	a.a. [5 μ M]	a.a. [2.5 μ M]	i.m.IC ₅₀ (μ M)	cytotox IC ₅₀ (μ M)	SI
6	A	D	G	26%	9%	0.6	48	81
7	A	D	B	43%	0%	>5	50	10
8	A	D	H	100%	47%	0.3	28	89
9	A	E	G	100%	37%	3.5	50	14
10	A	E	B	75%	6%	1.8	50	28
11	A	E	H	0%	0%	1.3	50	39
12	A	F	S	6%	0%	>5	50	10
13	A	F	B	94%	0%	1.3	26	14
14	A	F	H	97%	35%	>5	46	9
15	B	D	G	98%	68%	1.3	16	12

Table 4. continued

#	R ₁	R ₂	R ₃	a.a. [5 μ M]	a.a. [2.5 μ M]	i.m.IC ₅₀ (μ M)	cytotox IC ₅₀ (μ M)	SI
16	B	D	B	99%	71%	>5	15	3
17	B	D	H	98%	78%	5	12	2
18	B	E	G	97%	6%	1.3	50	38
19	B	E	B	0%	0%	0.9	50	56
20	B	E	H	95%	15%	>5	34	7
21	B	F	G	95%	97%	0.7	25	36
22	B	F	B	95%	77%	>5	24	5
23	B	F	H	95%	70%	0.3	20	69
24	C	D	G	94%	96%	>5	32	6
25	C	D	B	8%	11%	0.6	27	41
26	C	D	H	0%	0%	0.9	22	25
27	C	E	G	0%	0%	0.6	36	57
28	C	E	B	99%	99%	2.6	32	13
29	C	E	H	0%	0%	1.7	41	25
30	C	F	G	85%	14%	2.5	26	11
31	C	F	B	28%	0%	>5	23	5
32	C	F	H	57%	14%	0.9	17	20

^aa.a. is the % inhibition in the axenic amastigotes assay. i.m. is the infected macrophage assay. cytotox is the cytotoxicity in J774 macrophage cells. SI is the selectivity index (cytotox/i.m.).

we wanted to determine the necessity of having two guanidine units. We were further struck by the prospect that the butyl substitution at R₂ could serve as a surrogate for the

Table 5. Analogues of 21 and 26^a

ID	*	R ₁	R ₂	R ₃	i.m. IC ₅₀ (μM)	cytotox IC ₅₀ (μM)	SI
21	S				0.7	25	36
33	R				8.9	50	6
34	S			H	1.8	27	16
35	S		H		3.5	6	2
36	S	H			2.5	50	20
37	S		H	H	5.2	24	5
38	S	H	H		10	13	1
39	S	H	H	H	4.8	50	10
26	S				0.9	22	25
40	R				3.8	21	6
41	S			H	4.2	13	3
42	S		H		0.7	23	33
43	S	H			1.2	50	43
44	S		H	H	3.5	50	14
45	S	H		H	10	50	5
46	S	H	H		4.9	50	10
47	S	H	H		0.6	20	36

^ai.m. is the infected macrophage assay. Cytotox is the cytotoxicity in J774 macrophage cells. SI is the selectivity index (cytotox/i.m.).

scaffold linker (Figure 2). In this regard, we could consider the R₁ and R₃ substituents together. Toward this end, we synthesized a series of monoguanidine analogues with and without the butyl substituent (Table 7), which clearly showed that the butyl group improved the activity. Furthermore, the optimal activity of 47 was closely reflected in analogue 64, which showed an excellent SI and was more potent in our infected macrophage assay as miltefosine (3), a clinically used antileishmanial agent that is used as our standard.

In summary, using our scaffold ranking and positional scanning libraries, we were able to identify a series of guanidine-based compounds that possessed potent antileishmanial activity in multiple assays as well as selectivity over human macrophages, demonstrating that activity is unlikely to arise through simple promiscuous interactions. These compounds are structurally distinct from any of the currently used antileishmaniasis agents, as shown in Figure 1, and thus represent a novel opportunity for lead optimization. We were subsequently able

Table 6. Analogs of 6–8^a

ID	*	R ₁	R ₂	R ₃	i.m. IC ₅₀ (μM)	cytotox IC ₅₀ (μM)	SI
6	S				0.6	48	81
7	S				5.0	50	10
8	S				0.3	28	88
48	R				0.6	50	79
49	S		H		2.5	9	4
50	S		H	H	0.9	7	9
51	S			H	3.5	11	3
52	S	H			1.3	22	17
53	S	H		H	5.0	50	10
54	S		H	H	3.5	50	14
55	S		H	H	0.9	50	56
56	S		H	H	3.9	22	6
57	S		H	H	1.2	16	13
58	S		H	H	1.8	19	10
59	S		H	H	0.6	17	28
60	S		H	H	5.4	14	3
61	S		H	H	4.4	6	1

^ai.m. is the infected macrophage assay. Cytotox is the cytotoxicity in J774 macrophage cells. SI is the selectivity index (cytotox/i.m.).

to use these libraries to focus in on compounds such as 64 that retain not only excellent activity (an IC₅₀ of 1.13 μM in the infected macrophage assay when run in quadruplicate with a standard deviation of 0.81 μM) and selectivity (an SI of 23 over J774 macrophages) but also a reasonable molecular weight (245 g/mol) and physicochemical properties (e.g., cLogP = 3.26, tPSA = 39). Work toward the further optimization of these compounds with optimal pharmacokinetic and pharmacodynamics properties as well as attempts to ascertain their

Figure 2. Similarity of R₁ and R₃.

Table 7. Monoguanidines^a

ID	R ₁	R ₂	i. m. IC ₅₀ (μM)	cytotox IC ₅₀ (μM)	SI
62			2.4	15	6
63		H	4.8	15	3
64			1.1	25	23
65		H	10	8	1
66			4.7	12	3
67			0.6	6	10

^ai.m. is the infected macrophage assay. Cytotox is the cytotoxicity in J774 macrophage cells. SI is the selectivity index (cytotox/i.m.).

target or mechanism of action is underway and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00045.

Experimental details for the preparation and characterization of all compounds and how the assays were performed (PDF)

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Notes

The authors declare no competing financial interest.

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