International Journal of Antimicrobial Agents 54 (2019) 496–501

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Short Communication

Development of a target-free high-throughput screening platform for the discovery of antileishmanial compounds

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ARTICLE INFO

Article history:
Received 25 April 2019
Accepted 10 July 2019

Editor: Prof. Julia Walochnik

Keywords:
Leishmaniasis
High-throughput screening
2,4-diaminoquinazoline
Drug discovery

ABSTRACT

Leishmania parasites are the causative agents of a wide spectrum of human diseases. The clinical manifestations of leishmaniasis range from self-healing skin lesions to fatality. The World Health Organization has classed leishmaniasis as a category 1 neglected tropical disease. Leishmaniasis represents a major international health challenge, affecting 12 million people per year and with nearly 310 million people at risk. The first-line chemotherapies used to treat leishmaniasis are intravenous pentavalent antimonials; however, these drugs are highly toxic. As the use of oral treatment options such as paromomycin and miltefosine has increased, the incidence of disease relapse has increased and drug resistance to antimonials has developed, emphasizing the importance of identifying new chemotherapies. A novel, target-free fluorometric high-throughput screen with an average Z-score of 0.73 +/- 0.13 has been developed to identify small molecules with antileishmanial activity. Screening of 10,000 small molecules from the ChemBridge DIVER-set^{TM} library cassette #5 yielded 210 compounds that killed 80% of parasites, resulting in a hit rate of 2.1%. One hundred and nine molecular scaffolds were represented within the hit compounds, and one scaffold that exhibited potent antileishmanial activity was 2,4-diaminoquinazoline. Host cell toxicity was determined prior to in-vitro infection of human THP-1 macrophages with *Leishmania donovani* mCherry expressing promastigotes; successful drug treatment was considered when the half maximal inhibitory concentration was <10 μM. BALB/c mice were infected with *Leishmania major* mCherry promastigotes and treated with small molecules that were successful during in-vitro infections. Several small molecules tested were as efficacious at resolving cutaneous leishmaniasis lesions in mice as known antimonial treatments.

1. Introduction

Leishmaniasis is caused by the protozoan parasites *Leishmania* spp., affecting over 12 million people per year with an additional 310 million people at risk of infection [1]. Leishmaniasis is endemic in over 98 countries [2], and results in an estimated 2.4 million disability-adjusted life years [1]. Leishmaniasis has a spectrum of clinical manifestations, ranging from lesions of the skin [cutaneous leishmaniasis (CL)] and mucus membranes to fatal visceral disease [visceral leishmaniasis (VL)] [3]. The majority of leishmaniasis deaths go unrecognized, and even with treatment access, VL may result in case-fatality rates of 10–20% [2]. Based on estimates, approximately 0.2–0.4 million cases of VL and 0.7–1.2 million cases of CL occur each year [2]. As one of 20 neglected tropical diseases, leishmaniasis does not receive adequate attention for the development of more effective treatments and prevention. Neglected tropical diseases account for 11% of the global disease burden, but only 1% of the new chemical entities approved between 2000 and 2011 were directed at these diseases [4].

The first-line treatment for all types of leishmaniasis is intravenous pentavalent antimonials [1,3]. These drugs were first introduced in the 1940s; however, antimonial compounds exhibit high cellular toxicity, require prolonged treatment, and the incidence of relapse is increasing [5,6]. More recently, other agents including amphotericin B (antifungal, repurposed), paromomycin (antibiotic,

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https://doi.org/10.1016/j.ijantimicag.2019.07.013
0924-8579/

repurposed), miltefosine (anticancer, repurposed) and sitamaquine (antimalarial, repurposed) have been implemented for use as treatment options. Like pentavalent antimonials, these compounds are associated with high cellular toxicity and are frequently accompanied by the emergence of drug resistance [7].

This study utilized a fluorometric high-throughput screen of small molecule libraries followed by combinatorial chemistry to identify new lead scaffolds. The robust 'hit-to-lead' platform was envisioned based on the best practices for development of antileishmanial compounds [8,9]. Preliminary screening of 10,000 compounds from the DIVERset-EXP™ library (ChemBridge, San Diego, CA, USA) was performed against axenic *Leishmania donovani* amastigotes constitutively expressing mCherry [10,11]. Active compounds [i.e. those with half maximal effective concentration (EC_{50}) values <10 μM] moved on to secondary screening against intracellular *L. donovani* and *Leishmania major* amastigotes in the human THP-1 macrophage cell line. Active compounds from these secondary screens were then tested in an in-vivo murine CL model.

Of the 10,000 compounds included in the primary screening, 210 compounds displayed effectiveness >80% of 50 μM miltefosine, while 61 compounds exhibited effectiveness equal to that of 50 μM miltefosine. Three scaffolds were represented multiple times in the active compounds identified from the preliminary screen. This paper reports on one of these scaffolds: 2,4-diaminoquinazoline (2,4-DAQ). Twenty-six of the 2,4-DAQ compounds assessed in secondary screening exhibited EC_{50} values between 1 and 10 μM against axenic *L. donovani* amastigotes with selectivity indices (SI) ranging from 1 to 122. Four of six 2,4-DAQ compounds tested in the in-vivo CL model were as effective as antimony tartrate.

2. Materials and methods

2.1. Parasite culture

L. donovani transgenic strain 1S2D (MHOM/SD/62/1S-CL2d) clone LdB constitutively expressing mCherry [11] was cultured in M199 supplemented with 10% fetal calf serum at 27°C and pH 7.4. Axenic amastigotes were differentiated as described previously [10].

2.2. High-throughput screen

Transgenic *L. donovani* axenic amastigotes at a density of 1×10^6/mL were seeded in 384-well plates. Ten thousand compounds from the DIVERset library cassette #5 (ChemBridge) were screened against amastigotes at 50 μM, and after an incubation period of 72 h, fluorescence was measured (518 nm excitation/605 nm emission) using a FlexStation 3 Bench Top Multi-Mode Reader (Molecular Devices, San Jose, CA, USA). The screen was performed in duplicate.

2.3. Secondary screens

2.3.1. Cytotoxicity

THP-1 (human acute monocytic leukemia derived) host macrophages were cultivated in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics at 37°C and 5% CO_2. THP-1 cells were incubated with phorbol 12-myristate 7-acetate for 48 h to differentiate into mature macrophages. Differentiated macrophages were seeded in 96-well plates at 1×10^6/mL and incubated for 48 h in the presence of decreasing drug concentrations starting at 50 μM along with appropriate solvent controls. Cell viability assays were conducted using CellTiter-Blue (Promega, Madison, WI, USA), based on resazurin reduction. After the incubation period, CellTiter-Blue reagent was added to cells for 4 h at 37°C and fluorescence was measured (555 nm excitation/580 nm emission) using a FlexStation 3 Bench Top Multi-Mode Reader (Molecular Devices). Each assay was performed in triplicate and the screen was performed in duplicate. The 50% cytotoxic concentration (CC_{50}) values were calculated by non-linear regression analysis using GraphPad Prism Version 5.0 for Windows.

2.3.2. Axenic amastigotes

Axenic amastigotes were differentiated as described previously [10]; briefly, transgenic *L. donovani* promastigotes expressing mCherry were differentiated by pH and temperature shift. Amastigotes were seeded in 96-well plates at 1×10^6/mL and incubated for 48 h in the presence of decreasing drug concentrations starting at 50 μM along with appropriate solvent controls. Fluorescence was monitored (518 nm excitation/605 nm emission) over 48 h using a FlexStation 3 Bench Top Multi-Mode Reader (Molecular Devices). Each assay was performed in triplicate and the screen was performed in duplicate. The half maximal inhibitory concentration (IC_{50}) values were calculated by non-linear regression analysis using GraphPad Prism Version 5.0 for Windows. SI values were calculated as the THP-1 CC_{50} value divided by the IC_{50} value.

2.3.3. Intramacrophage

Transgenic *L. donovani* promastigotes constitutively expressing mCherry and THP-1 macrophages were cultured as described above. Metacyclic promastigotes were isolated using a Ficoll-400 density gradient as described previously [12]. Differentiated macrophages were exposed to parasites for 4 h at a multiplicity of infection of 10 parasites:1 macrophage, then washed thoroughly with phosphate buffered saline. After 4 h, macrophages were incubated overnight at 37°C and 5% CO_2. Macrophages were transferred to 96-well plates at 1×10^6/mL and exposed to decreasing drug concentrations starting at 50 μM. Fluorescence was monitored (518 nm excitation/605 nm emission) over 48 h using a FlexStation 3 Bench Top Multi-Mode Reader (Molecular Devices). Plates were kept at 27°C overnight, so surviving amastigotes transformed readily back into promastigotes. Each assay was performed in triplicate and the screen was performed in triplicate. The IC_{50} values were calculated by non-linear regression analysis using GraphPad Prism Version 5.0 for Windows. SI values were calculated as the CC_{50} value divided by the IC_{50} value.

2.4. ADME/in-vitro DMPK

The most potent hits from the initial screen were re-evaluated for their pharmacokinetic properties in standard in-vitro ADME assays, performed at Q^2 Solutions (Morrisville, NC, USA).

2.5. In-vivo cutaneous leishmaniasis murine model

Groups of BALB/c mice were injected in the left footpad with 30 μL of 1×10^6 transgenic *L. major* [strain FVI (MHOM/JL/80/Friedlin)] stationary promastigotes constitutively expressing mCherry. After 14 days, mice received a single daily dose of 30 μL of compound (0.110 μg/kg) intralesionally; treatment lasted for 2 weeks (5 days treatment, 2 days rest). Mice were imaged using an IVIS Lumina System (PerkinElmer, Waltham, MA, USA) with fluorescence as a proxy for parasite load [11].

3. Results

3.1. High-throughput screen

We used transgenic axenic amastigotes expressing a red fluorescent protein (mCherry) [11,13] in a 384-well plate format with miltefosine as the control to calculate the Z-score [14]. The average Z-score for this assay is 0.73 +/- 0.13 and the average signal window is 22.41 +/- 11.35.

Table 1
Results from high-throughput screen of DIVERset™ library cassette #5

DIVERset™ library cassette #5	Effective	Total
# Compounds present in library		10,000
Effectiveness >80%	210	10,000
Effectiveness >90%	116	10,000
Effectiveness equal to 50 μM miltefosine	61	10,000
# Scaffolds (one compound)	76	
# Enriched scaffolds (two compounds or more)	33	
2,4-DAQ		
# 2,4-DAQ present in library (10k)		188
Effectiveness >80%	30	188
Effectiveness >50%	96	188
Effectiveness >30%	163	188
IC_{50} ≤10 μM (axenic amastigotes)	26	26
CC_{50} >10 μM (THP-1 macrophage)	4	26
IC_{50} ≤10 μM (in-vitro infection model)	8	9
Relatively effective (in-vivo CL model)	3	6

2,4-DAQ, 2,4-diaminoquinazoline; CC_{50} IC_{50}, half maximal inhibitory concentration; CL, cutaneous leishmaniasis.

In total, 10,000 compounds from the ChemBridge DIVERset™ library cassette #5 were screened. Each compound was screened at 50 μM and miltefosine was screened at 50 μM as the control in the presence of 1×10^6 transgenic axenic amastigotes in a single well. The assay was repeated and the average percent parasite killing was determined. Two hundred and ten compounds killed 80% of parasites, resulting in a hit rate of 2.1%; 116 of those compounds killed 90% of parasites and 61 compounds were as effective as 50 μM miltefosine (Table 1). The 210 positive hits were grouped into 109 different scaffolds. Thirty-three scaffolds were represented by two or more compounds resulting in 134 compounds; 76 compounds represented unique scaffolds (Table 1).

3.2. Compound activity and host cell toxicity screening

Of the 33 scaffolds represented by two or more compounds, the most well represented was 2,4-diaminoquinazoline (2,4-DAQ). In total, 188 2,4-DAQ compounds were present in the library and 30 of these killed 80% of axenic amastigotes (Table 1); 96 of the compounds killed ≥50% of axenic amastigotes and 163 killed ≥30% of axenic amastigotes (Table 1).

Twenty-six of the hit 2,4-DAQ compounds were screened against axenic amastigotes and THP-1 macrophages to determine compound activity and host cell toxicity, respectively. All 26 compounds had IC_{50} values <10 μM when screened against axenic amastigotes with SI values between 0.06 and 183, and four compounds had CC_{50} values >10 μM (Table 2). Nine of these 26 compounds were then screened against *bone fide* intracellular amastigotes; eight compounds had IC_{50} values <10 μM with SI values between 0.09 and 18.37 (Table 2).

3.3. ADME/in-vitro DMPK

A representative 2,4-DAQ, compound 9251265, was evaluated for its in-vitro pharmacokinetic properties in standard in-vitro ADME assays (run at Q^2 Solutions, Quintiles Quest Join Venture). 2,4-DAQ 9251265 displayed high intrinsic clearance (>200 mL/min/kg in rats) and >100 μM solubility in both phosphate buffer and HBSS. Human plasma protein binding was moderately high, displaying <2.6% fraction unbound. 2,4-DAQ 9251265 exhibited moderate inhibition of cytochrome P450 isoforms 2C9, 2D6 and 3A4 (322.2%, 62.6% and 23.6%, respectively).

3.4. Cutaneous leishmaniasis BALB/c murine model

Six of the 26 hit compounds were screened in an in-vivo model: 6666551, 9213357, 9222605, 9243327, 9251265 and 9270128. Cohorts of female BALB/c mice were inoculated with transgenic *L. major* promastigotes in the footpad, and lesions developed over 14 days. The mice were then treated with 30-μL intralesional injections of antimony tartrate or compound (0.110 μg/kg) over a 12-day period. Three of the compounds (6666551, 9243327 and 9222605) were less effective at killing parasites, while the other three compounds (9213357, 9270128 and 9251265) were substantially more effective (Table 1 and Fig. 1). These compounds were as effective at parasite killing as the antimony control (Fig. 1),

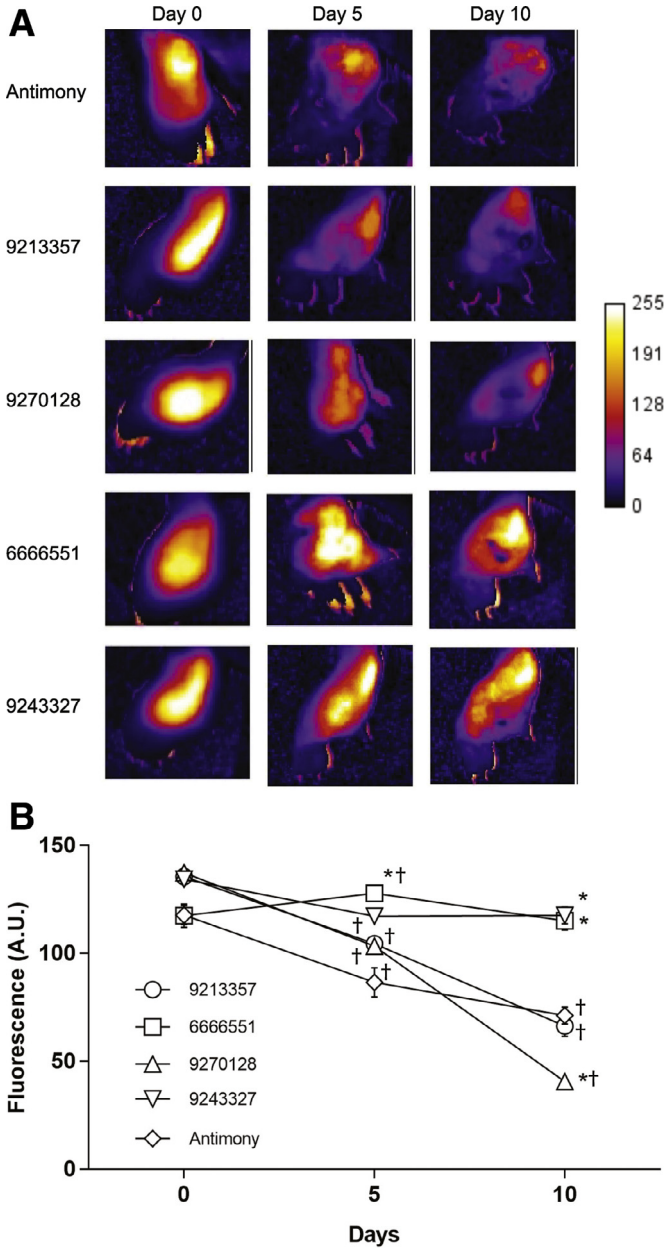
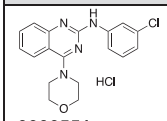
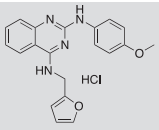
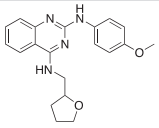
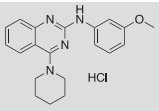
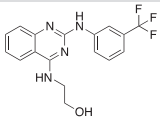
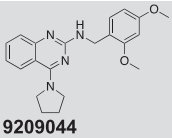
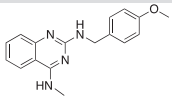
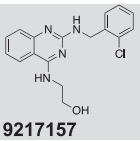
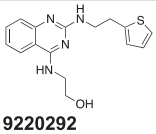
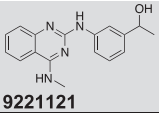
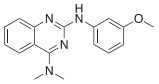
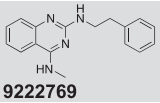
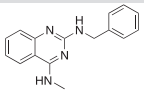


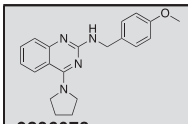
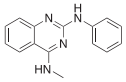
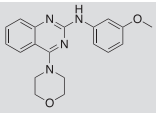
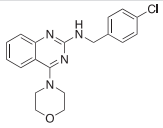
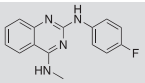
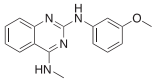
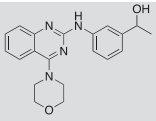
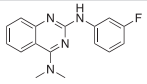
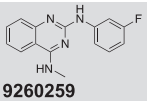
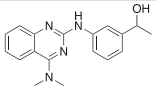
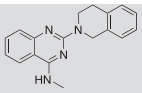
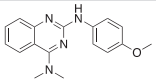
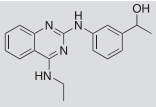
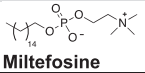
Fig. 1. In-vivo cutaneous leishmaniasis murine model. BALB/c mice were inoculated with *Leishmania major* mCherry promastigotes (10^6) in a footpad. After 14 days, fluorescence values as a proxy for parasite load were obtained using an IVIS Lumina system (Day 0). The mice were then treated with antimony (III) tartrate (five doses per week), #9213357 (five doses per week), #6666551 (five doses per week), #92791028 (five doses per week) and #9243327 (five doses per week) over a 12-day period. All doses were 30-μL intralesional injections of aqueous solutions (0.015 μg/kg). (A) Representative images shown. The fluorescence scale bar applies to all images and is given in arbitrary units. (B) Quantification of IVIS Lumina images. Two-way analysis of variance with Bonferroni's post-hoc analysis was conducted. Differences were regarded as extremely significant when $P<0.0001$ (*) compared with antimony treatment and $P<0.0001$ (†) compared with the Day 0 control.

Table 2
Secondary screening of 2,4-diaminoquinazoline compounds

Structure	THP-1 MP	Axenic amastigote		Intramacrophage		*In vivo*
	CC_{50}	IC_{50}	SI	IC_{50}	SI	CL
 **6666551**	73.60	1.97	37.36	48.00	1.53	+
 **6674271**	1.96	0.69	2.84			
 **7162450**	4.62	9.70	0.48			
 **7749545**	9.64	2.73	3.53			
 **9201953**	6.01	1.38	4.36	2.85	2.11	N/A
 **9209044**	3.63	3.44	1.06			
 **9213357**	9.92	2.32	4.28	0.54	18.37	+++
 **9217157**	8.48	3.27	2.59			
 **9220292**	1.93	1.43	1.35	1.50	1.29	N/A
 **9221121**	0.42	6.85	0.06			
 **9222605**	0.61	1.41	0.43	6.62	0.09	+
 **9222769**	3.08	0.37	8.32	0.68	4.53	N/A
 **9228504**	8.49	1.45	5.86	0.57	14.89	N/A

(*continued on next page*)

Table 2 (*continued*)

 **9236078**	1.92	2.40	0.80			
 **9238700**	0.93	2.94	0.32			
 **9243255**	7.18	1.85	3.88			
 **9243327**	15.06	0.86	17.51	1.80	8.37	+
 **9244914**	1.24	2.68	0.46			
 **9251265**	1.00	1.73	0.58	N/A	N/A	+++
 **9257644**	28.73	7.76	3.70			
 **9258839**	2.83	1.36	2.08			
 **9260259**	1.34	2.92	0.46			
 **9263467**	2.26	6.36	0.36			
 **9270128**	73.20	0.40	183.00	9.70	7.55	+++
 **9271294**	1.00	3.21	0.31			
 **9284699**	0.60	6.14	0.10			
 **Miltefosine**	36.16	3.25	11.13	2.90	12.47	N/A

IC_{50}, half maximal inhibitory concentration; SI, selectivity indices; CL, cutaneous leishmaniasis.

demonstrating that 2,4-DAQ compounds are effective treatment options for cutaneous leishmaniasis.

4. Discussion

A simple fluorometric method for primary drug screening was developed that is a target-free, unbiased approach allowing differentiation between cytostatic and cytotoxic compounds *in vitro* and imaging for in-vivo efficacy testing against *L. donovani* parasites.

For the assay, a relatively stringent cut-off value of 80% parasite killing was considered; similar studies considered as little as 30% parasite killing as effective [15]. The compound library from ChemBridge was a broad, non-target-based library with small molecules with drug-like properties. By using a diverse library with a stringent cut-off value, it was possible to probe the chemical space while also identifying the most potent antileishmanial compounds. The 2,4-DAQ scaffold has previously been identified as having antileishmanial activity [16–20], thus validating the screening methods used in this study.

5. Conclusion

In summary, a simple in-vitro fluorometric method for primary drug screening was developed that is target-free and allows for imaging of in-vivo efficacy testing against leishmania parasites. Validating the screening platform, 2,4-DAQ compounds that were previously shown to have good efficacy as antileishmanial agents were identified, and the efficacy and selectivity of a new series of 2,4-DAQ compounds not previously tested for antileishmanial activity were reported. As reported previously, this series suffers from poor pharmoacokinetic properties and possibly dose-limiting toxicity in murine models; however, these limitations do not disregard their potential in combination therapies at lower overall doses. Further studies, including pharmacokinetic, combination therapy and mechanism-of-action studies, are needed to fully understand the therapeutic feasibility of 2,4-DAQ compounds.

Acknowledgements

The authors wish to thank the staff of the Freimann Life Science Center and the University of Notre Dame for their expertise and care.

Funding

This project was funded, in part, with support from the Indiana Clinical and Translational Sciences Institute by Award Number UL1TR002529 from the National Institutes of Health, National Center for Advancing Translational Sciences, Clinical and Translational Sciences Award. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This project was also funded, in part, with support from the University of Notre Dame Eck Institute for Global Health.

Competing interest

None declared.

Ethical approval

University of Notre Dame IACUC 15-10-2708.

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