MedChemComm

CONCISE ARTICLE

View Article Online
View Journal | View Issue

Cite this: *Med. Chem. Commun.*, 2014, **5**, 142

Synthesis and biological evaluation of 2-acetamidothiophene-3-carboxamide derivatives against *Leishmania donovani*†

Sangmi Oh,[a] Bosung Kwon,[a] Sunju Kong,[a] Gyongseon Yang,[b] Nakyung Lee,[b] Dawoon Han,[b] Junghyun Goo,[b] Jair L. Siqueira-Neto,‡[b] Lucio H. Freitas-Junior,§[b] Michel Liuzzi,[c] Jinhwa Lee[d] and Rita Song*[a]

Received 8th October 2013
Accepted 15th November 2013

DOI: 10.1039/c3md00299c

www.rsc.org/medchemcomm

A high-throughput (HTS) and high-content screening (HCS) campaign of a commercial library identified 2-acetamidothophen-3-carboxamide as a novel scaffold for developing new anti-leishmanial agents. A series of chemical modifications were performed to study the structure–activity relationship (SAR) and *in vitro* anti-leishmanial activities were evaluated using biological assays of not only extracellular promastigotes but also intracellular amastigotes. Compound **6a** showed promising anti-amastigote activity (EC_{50} = 6.41 μM) against *L. donovani* without any cytotoxicity (CC_{50} > 50 μM) towards human macrophages.

Introduction

Leishmaniasis is one of the most neglected tropical diseases caused by *Leishmania*, a protozoan parasite transmitted by the bite of female phlebotomine sandflies.[1] More than 1.2 million people worldwide are estimated to be infected by *Leishmania* and more than 50 000 deaths are reported every year.[2] Three different types of leishmaniasis are characterized: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL). VL caused by *Leishmania donovani* (*L. donovani*) is the most severe form of the disease, being fatal if left untreated.

Most of the current drugs to treat VL, such as pentavalent antimonials, amphotericin B, and miltefosine, are decades-old and have many limitations. Both pentavalent antimonials and amphotericin B are administered parenterally and their high toxicity along with the emergence of drug resistance has posed serious problems. Miltefosine is the only oral drug for the treatment of VL; however, this drug is teratogenic and causes extremely severe side effects.[3,4] In addition, the drugs currently in clinical development are mostly either reformulations or combination therapies based on old drugs.[5] Therefore, there is an urgent unmet medical need for the development of novel anti-leishmanial drugs with improved efficacy and safety. Drug discovery for leishmaniasis has been complicated by the lack of validated drug targets and relevant drug assays. Target free cell-based assays have been widely used, as they bypass the need for a known target. In this context, we previously reported a high-content screening (HCS) assay for finding novel chemical scaffolds targeting intracellular *L. donovani* amastigotes in human macrophages.[6] Most of the compounds reported as potential anti-leishmanial agents such as pentamidine-like molecules,[7,8] natural product-inspired hybrid compounds,[9,10] and a novel series of *N*-benzoyl-2-hydroxybenzamide have been validated from target-free cell-based assays.[11] Here we report the synthesis and anti-leishmanial activity of 2-acetamidothiophene-3-carboxamide derivatives which were tested against both *L. donovani* promastigotes and intracellular amastigotes.

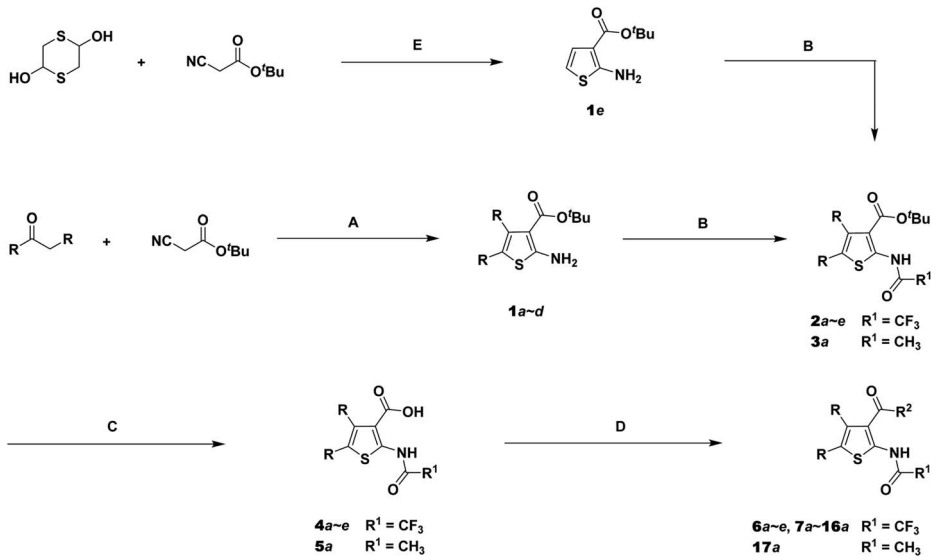
Results and discussion

A high-throughput (HTS) and high-content screening (HCS) campaign of a 200 000 chemical library utilizing amastigotes infected cell-based assay identified the 2-acetamidothophen-3-carboxamide scaffold. In search of compounds with improved activity, a series of 2-acetamidothiophene-3-carboxamide derivatives were prepared by introducing diverse substitutions in three parts: change of the aliphatic ring fused with thiophene, modification of both the 2-amino functional group and 3-carboxamide part with different substituents. A general synthesis scheme for 2-acetamidothiophene-3-carboxamide derivatives is depicted in Scheme 1. 2-Aminothiophenecarboxylates **1a–e** were obtained using Gewald cyclization as previously reported, and the reaction

[a]*Medicinal Chemistry Group, Institut Pasteur Korea, 696 Sampyeong-dong, Bundang-gu, Seongnam-si, Gyeonggi-do 463-400, Korea. E-mail: rsong@ip-korea.org*
[b]*Center for Neglected Diseases Drug Discovery (CND3), Institut Pasteur Korea, 696 Sampyeong-dong, Bundang-gu, Seongnam-si, Gyeonggi-do 463-400, Korea*
[c]*Early Discovery Program, Institut Pasteur Korea, 696 Sampyeong-dong, Bundang-gu, Seongnam-si, Gyeonggi-do 463-400, Korea*
[d]*Late Discovery Program, Institut Pasteur Korea, 696 Sampyeong-dong, Bundang-gu, Seongnam-si, Gyeonggi-do 463-400, Korea*

† Electronic supplementary information (ESI) available: Experimental details in biology and chemistry, melting point and NMR spectra of all new compounds. See DOI: 10.1039/c3md00299c

‡ Present address: University of California, San Francisco, USA.
§ Present address: Laboratório Nacional de Biociências (LNBio), Brazil.



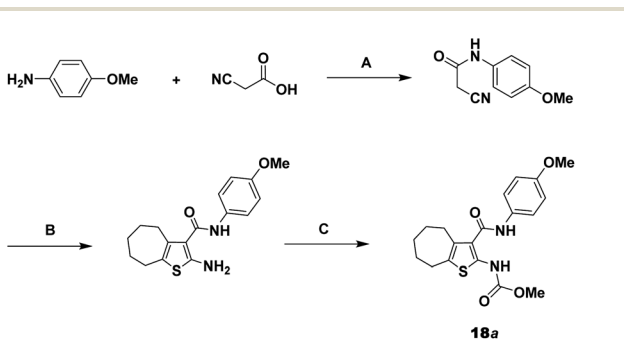
Scheme 1 *Reagents and conditions*: (A) sulfur 1.2 eq., morpholine 1.5 eq., EtOH, 45 °C, 24 h; (B) TFAA or Ac_2O 2.0 eq., DIPEA 2.0 eq., MC, 0 °C, 24 h; (C) TFA 5.0 eq., MC, RT, 4 h; (D) R^2H 1.2 eq., EDC 2.0 eq., HOBt 2.0 eq., TEA 3.0 eq., DMF, 50 °C, 24 h; (E) TEA 4.0 eq., DMF, 45 °C, 3 h.

of acylation of the 2-amino group followed by de-esterification and EDC coupling reaction yielded the desired final compounds **6a–e** and **7a–17a.**[12,13]

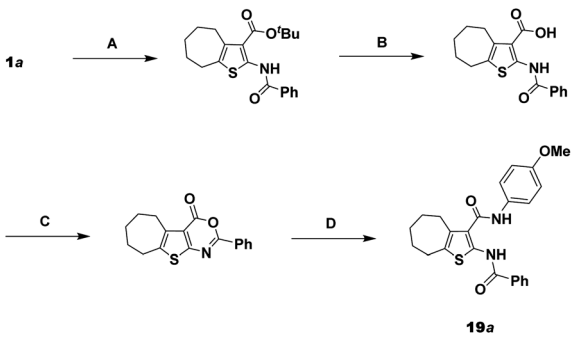
Due to the fact that the final coupling reaction did not yield compound **18a** as shown in Scheme 1, the synthesis of **18a** was first started from an amide coupling reaction (Scheme 2). In the case of compound **19a** with a phenyl moiety at the R^1 position, an unexpected cyclocondensation reaction occurred during EDC coupling resulting in thienopyrimidinone ring formation. Opening the ring by the reaction of LiHMDS in the presence of *p*-anisidine finally yielded compound **19a** (Scheme 3).

For assessing the anti-leishmanial activity of the compounds, two different stages in the *Leishmania* life cycle were tested. One, the amastigote form, is intracellular and specifically found in the host macrophage. The other, the promastigote form, is predominately found in the insect vector. Even though amastigotes and promastigotes are substantially different from a molecular perspective, drug discovery for leishmaniasis has traditionally been performed using either promastigotes or axenic amastigotes; the latter usually *in vitro* cultured under conditions of acidic pH and elevated temperature.[14] Ease of mass production of the parasites facilitated the adaption of both species to a high-throughput assay format.

To evaluate the anti-leishmanial activity of the compounds under more physiologically relevant conditions, we measured the *in vitro* activity of the compounds against amastigotes in human macrophages and extracellular promastigotes.[6] The intracellular amastigote cell-based assay was developed by infecting differentiated THP-1 macrophages with *L. donovani* metacyclic promastigotes to lead to amastigote transformation.[6] Each compound was added to the plate 24 hours after infection and the quantification of the infection ratio was done 3 days after compound treatment. Fluorescent image analysis using automated confocal microscopy and customized software allowed for the quantitative assessment of diverse information such as number of cells per well, number of parasites per cell, infection ratio, and cell cytoplasmic area.[6] Cytotoxicity against host cells was also quantified



Scheme 2 *Reagents and conditions*: (A) EDC 2.0 eq., HOBt 1.5 eq., TEA 2.0 eq., DMF, 55 °C, 22 h, 62%; (B) cycloheptanone 1.0 eq., sulfur 1.2 eq., morpholine 3.0 eq., EtOH, 55 °C, 30 h, 5%; (C) MeOCOCl 1.1 eq., In 0.7 eq., ACN, RT, 12 h, 18%.



Scheme 3 *Reagents and conditions*: (A) BzOH 1.2 eq., EDC 2.0 eq., HOBt 1.5 eq., TEA 1.5 eq., DMF, 50 °C, 6 h, 50%; (B) TFA, RT, 1 h, 45%; (C) *p*-anisidine 1.2 eq., EDC 2.0 eq., HOBt 1.5 eq., TEA 1.5 eq., DMF, 50 °C, 6 h, 80%; (D) *p*-anisidine 1.0 eq., LiHMDS 1.0 eq., THF, RT, 15 min, 15%.

by ascertaining the cell number. The viability of promastigotes, on the other hand, was analysed by resazurin assay after compound treatment.[15,16] The *in vitro* anti-leishmanial activity of the compounds is summarized in Table 1.

Compound **6a** which contained cycloheptane fused with a thiophene moiety exhibited comparable anti-amastigote activity (6.41 μM) to that of the reference drug, miltefosine (7.10 μM). In the case of cytotoxicity, compound **6a** did not show any toxicity against host macrophages (CC_{50} > 50 μM), but miltefosine was found to be toxic at lower concentrations (CC_{50} = 22.7 μM), resulting in a lower selectivity index, 3.19. Considering that miltefosine was originally developed as an antineoplastic agent for topical use, a lower selectivity index was expected.

Compounds incorporating sterically hindered aliphatic cycles such as cycloheptyl (**6a**) exhibited the best activity within the series, while compounds with cyclohexyl (**6b**) or cyclopentyl (**6c**) showed gradually decreasing activity (14.6 and >50 μM, respectively). Likewise, substitution with two methyl groups (**6d**) enhanced anti-amastigote activity compared to compounds with a less hindered cyclopentyl (**6c**) or without a substituent (**6e**) (10.1 and >50 μM, respectively). Given that the sterically hindered aliphatic substitution improved the activity, lipophilicity of the compound seems to be an important factor for exhibiting anti-leishmanial activity. A significant decrease of anti-amastigote potency was observed when the *para*-methoxy group in R^2 was changed to hydrogen (**9a**) or *ortho*-methoxy (**8a**). The methoxy group at the *para*-position plays a critical role in retaining anti-amastigote activity and in reducing cytotoxicity. It is believed that this is an electronic effect because other compounds containing an electron withdrawing group at the *para*-position were totally inactive (**11a**) or showed high cytotoxicity against host macrophages (**12a–14a**). And also, it can be inferred that a hydrogen bond acceptor is essential at this position (**6a**), because methyl-substituted compound (**10a**) lost their activity. The aminophenyl moiety at the R^2 position is also important for anti-amastigote activity, as one carbon elongated-aminobenzyl compound (**15a**) was found to be inactive. Other substituents at the R^1 position such as methyl (**17a**), methoxy (**18a**), or phenyl (**19a**) instead of trifluoromethyl groups were not effective in improving the anti-amastigote activity. It is interesting to note that most of the compounds described here were inactive in the *L. donovani* promastigote assay although compounds **6a** and **6d** showed considerable activity in the amastigote cell-based assay. As described above, traditional drug screening has been carried out using promastigotes or axenic amastigotes. However, it has been known that promastigotes and axenic amastigotes are not fully representative of human leishmaniasis because amastigotes are exclusively intracellular *in vivo*.[6] The finding that 2-acetamidothiophene-3-carboxamide derivatives show anti-leishmanial activity only against intracellular amastigotes may imply that the target of the compound in this series is amastigote-specific or that the compound acts through a novel mechanism of action; for example, by interacting with one or more cellular factors.

Table 1 *In vitro* activity of 2-acetamidothiophene-3-carboxamide derivatives against the amastigote and promastigote forms of *L. donovani*

	Chemical structure			Activity against *L. donovani*			
				Amastigote form			Promastigote form
Compounds[a]	R	R^1	R^2	EC_{50} (μM)[b]	CC_{50} (μM)[c]	SI[d]	EC_{50} (μM)[e]
1a	$-(CH_2)_5-$	—	^{tert}Butoxy	>50	>50	N/A[f]	N/T[g]
3a	$-(CH_2)_5-$	Methyl	^{tert}Butoxy	>50	>50	N/A	N/T
6a	$-(CH_2)_5-$	Trifluoromethyl	4-Methoxyphenylamino	6.41	>50	>7.80	>50
6b	$-(CH_2)_4-$	Trifluoromethyl	4-Methoxyphenylamino	14.6	>50	>3.42	>50
6c	$-(CH_2)_3-$	Trifluoromethyl	4-Methoxyphenylamino	>50	>50	N/A	N/T
6d	Me	Trifluoromethyl	4-Methoxyphenylamino	10.1	>50	>4.95	>50
6e	H	Trifluoromethyl	4-Methoxyphenylamino	>50	>50	N/A	>50
7a	$-(CH_2)_5-$	Trifluoromethyl	3-Methoxyphenylamino	10.1	37.6	3.71	21.5
8a	$-(CH_2)_5-$	Trifluoromethyl	2-Methoxyphenylamino	>50	>50	N/A	42.0
9a	$-(CH_2)_5-$	Trifluoromethyl	Phenylamino	>50	>50	N/A	N/T
10a	$-(CH_2)_5-$	Trifluoromethyl	4-Methylphenylamino	>50	>50	N/A	N/T
11a	$-(CH_2)_5-$	Trifluoromethyl	4-Chlorophenylamino	>50	>50	N/A	N/T
12a	$-(CH_2)_5-$	Trifluoromethyl	4-Trifluoromethylphenylamino	17.6	17.2	0.90	>50
13a	$-(CH_2)_5-$	Trifluoromethyl	4-Trifluoromethoxyphenylamino	9.85	11.9	1.20	>50
14a	$-(CH_2)_5-$	Trifluoromethyl	4-Cyanophenylamino	8.53	9.11	1.06	>50
15a	$-(CH_2)_5-$	Trifluoromethyl	4-Methoxybenzylamino	>50	>50	N/A	>50
16a	$-(CH_2)_5-$	Trifluoromethyl	^{tert}Butylamino	>50	>50	N/A	N/T
17a	$-(CH_2)_5-$	Methyl	4-Methoxyphenylamino	>50	>50	N/A	>50
18a	$-(CH_2)_5-$	Methoxy	4-Methoxyphenylamino	>50	>50	N/A	N/T
19a	$-(CH_2)_5-$	Phenyl	4-Methoxyphenylamino	>50	>50	N/A	N/T
Amphotericin B	—	—	—	0.25	7.57	30.2	0.18
Miltefosine	—	—	—	7.10	22.7	3.19	7.40

[a] Compounds **1a, 6a–6c, 8a–12a, 17a, 19a** are already known molecules. [b] EC_{50} indicates half maximal effective concentration against intracellular *L. donovani* (2-fold dilution with 10 point dose response). The average of duplicate determinations. [c] CC_{50} indicates cytotoxicity against THP-1 cells, which was determined by THP-1 cell number. The average of duplicate determinations. [d] SI is the selectivity index (EC_{50}/CC_{50}). [e] EC_{50} indicates half maximal effective concentration against extracellular *L. donovani*. The average of duplicate determinations. [f] N/A: not available. [g] N/T: not tested.

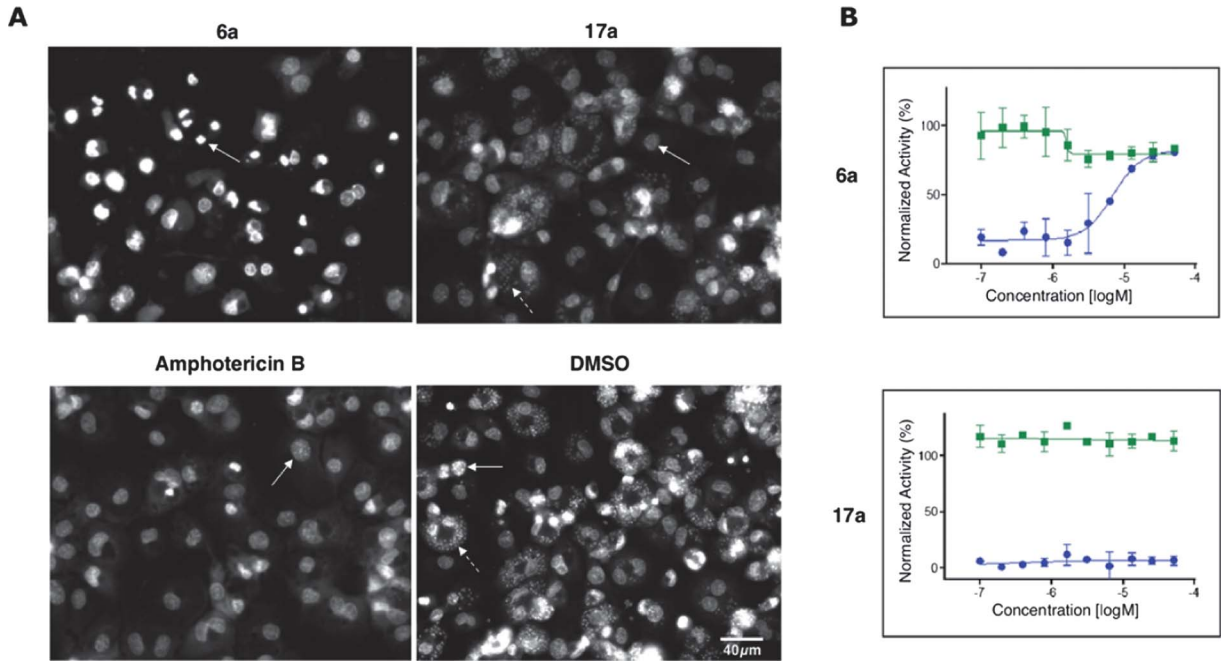


Fig. 1 Confocal microscopy images of compounds **6a** and **17a**. (A) Infected THP-1 cells in the presence of 50 μM of compound **6a** (upper left) showed complete parasite clearance from the host cells while 50 μM of compound **17a** (upper right) showed no effect. Amphotericin B (4 μM) was used as a positive control (lower left), and DMSO (1%) was used as a negative control (lower right). Fluorescent DNA dye Draq5 was used to visualize DNA within host cells and parasites. The solid arrow indicates nucleus and the dotted arrow indicates parasites in cytosol. (B) Dose response curves of compounds **6a** and **17a** plotting the normalized anti-leishmanial activity (●) and the relative percentage of cell number compared to DMSO represented cytotoxicity control (■).

The images from automated confocal microscopy and the corresponding dose response curves of the representative compounds **6a** and **17a** are shown in Fig. 1. In the case of compound **6a**, which showed comparable anti-leishmanial activity to that of the marketed drug miltefosine, no parasite was observed inside of host THP-1 cells at a concentration of 50 μM, while most of the parasites still remained using the same concentration of the inactive compound **17a**. In addition to the promising anti-leishmanial activity, no significant decrease in cell number was observed when host THP-1 cells were treated with compound **6a**. The calculated percentage infection based on the EC_{100} (effective concentration showing 100% activity as a minimum measured infection ratio) of amphotericin B and 1% DMSO (0% activity as a maximum measured infection ratio) was normalized to percentage activity. Cytotoxicity was determined by the percentage of cell number normalized against DMSO control.

Conclusions

A series of compounds based on the 2-acetamidothophen-3-carboxamide scaffold were synthesized and tested using amastigote infected cell-based assay and extracellular promastigote assay. Compound **6a** showed promising anti-amastigote activity against *L. donovani* without any cytotoxicity toward human macrophages. Further studies to improve the anti-leishmanial activity and to explore the mechanism of action are ongoing.

Acknowledgements

This work was supported by the National Research foundation of Korea (NRF) grant funded by the Korea government (MSIP, no. 2007-00559), Gyeonggi-do (no. K204EA000001-09E0100-00110), and KISTI.

Notes and references

1 N. S. Akopyants, N. Kimblin, N. Secundino, R. Patrick, N. Peters, P. Lawyer, D. E. Dobson, S. M. Beverley and D. L. Sacks, *Science*, 2009, **324**, 265.
2 L. Monzote, *Open Antimicrob. Agents J.*, 2009, **1**, 9.
3 S. Sundar and M. Chatterjee, *Indian J. Med. Res.*, 2006, **123**, 345.
4 A. R. Renslo and J. H. McKerrow, *Nat. Chem. Biol.*, 2006, **2**, 701.
5 http://www.dndi.org/diseases-projects/portfolio.
6 J. L. Siqueira-Neto, S. Moon, J. Jang, G. Yang, C. Lee, H. K. Moon, E. Chatelain, A. Genovesio, J. Cechetto and L. H. Freitas-Junior, *PLoS Neglected Trop. Dis.*, 2012, **6**, e1671.
7 X. Zhu, Q. Liu, S. Yang, T. Parman, C. E. Green, J. C. Mirsalis, M. de Nazaré Correia Soeiro, E. M. de Souza, C. F. da Silva, D. da Gama Jaen Batista, C. E. Stephens, M. Banerjee, A. A. Farahat, M. Munde, W. D. Wilson, D. W. Boykin, M. Z. Wang and K. A. Werbovetz, *Antimicrob. Agents Chemother.*, 2012, **56**, 3690.

8 S. Porwal, S. S. Chauhan, P. M. S. Chauhan, N. Shakya, A. Verma and S. Gupta, *J. Med. Chem.*, 2009, **52**, 5793.
9 M. Sharma, K. Chauhan, R. Shivahare, P. Vishwakarma, M. K. Suthar, A. Sharma, S. Gupta, J. K. Saxena, J. Lal, P. Chandra, B. Kumar and P. M. S. Chauhan, *J. Med. Chem.*, 2013, **56**, 4374.
10 V. Tyagi, S. Khan, R. Shivahare, K. Srivastava, S. Gupta, S. Kidwai, K. Srivastava, S. K. Puri and P. M. S. Chauhan, *Bioorg. Med. Chem. Lett.*, 2013, **23**, 291.
11 J. Stec, Q. Huang, M. Pieroni, M. Kaiser, A. Fomovska, E. Mui, W. H. Witola, S. Bettis, R. McLeod, R. Brun and A. P. Kozikowski, *J. Med. Chem.*, 2012, **55**, 3088.
12 M. Gütschow and U. Neumann, *J. Med. Chem.*, 1998, **41**, 1729.
13 C. Jamieson, S. Basten, R. A. Campbell, I. A. Cumming, K. J. Gillen, J. Gillespie, B. Kazemier, M. Kiczun, Y. Lamont, A. J. Lyons, J. K. F. Maclean, E. M. Moir, J. A. Morrow, M. Papakosta, Z. Rankovic and L. Smith, *Bioorg. Med. Chem. Lett.*, 2010, **20**, 5753.
14 P. A. Bates, C. D. Robertson, L. Tetley and G. H. Coombs, *Parasitology*, 1992, **105**, 193.
15 J. Mikus and D. Steverding, *Parasitol Int.*, 2000, **48**, 265.
16 J. L. Siqueira-Neto, O.-R. Song, H. Oh, J.-H. Sohn, G. Yang, J. Nam, J. Jang, J. Cechetto, C. B. Lee, S. Moon, A. Genovesio, E. Chatelain, T. Christophe and L. H. Freitas-Junior, *PLoS NeglectedTrop. Dis.*, 2010, **4**, e675.

