Identification of Leucinostatins from *Ophiocordyceps* sp. as Antiparasitic Agents against *Trypanosoma cruzi*

Jean A. Bernatchez, Yun-Seo Kil, Elany Barbosa da Silva, Diane Thomas, Laura-Isobel McCall, Karen L. Wendt, Julia M. Souza, Jasmin Ackermann, James H. McKerrow, Robert H. Cichewicz,* and Jair L. Siqueira-Neto*

Cite This: ACS Omega 2022, 7, 7675–7682

**INTRODUCTION**

Chagas disease, caused by the unicellular parasite *Trypanosoma cruzi*, remains a serious global health burden, with 6–7 million individuals infected worldwide; an estimated 300,000–1 million cases are found in the United States alone. *T. cruzi* is usually transmitted via a blood meal by triatomine insects (commonly known as kissing bugs) but can also be acquired congenitally, through blood transfusions, or orally from the ingestion of *T. cruzi*-contaminated food. Within the mammalian host, *T. cruzi* can be found in two lifecycle stages: the circulating trypomastigote stage and the intracellular amastigote stage. Parasites can persist at low levels in multiple organs including the heart and gastrointestinal tract, leading to cardiac and gastrointestinal pathology in chronically infected patients, which can be fatal. Benzimidazole and nifurtimox, the sole available drugs for *T. cruzi* treatment, are known to cause severe adverse effects that complicate their clinical use, and their utility in the chronic phase of infection is debated within the scientific community. Chagas disease is classified as a neglected tropical disease, and its historical prevalence in resource-limited countries in Latin America has restricted industrial interest in the development of a therapeutic. Given the paucity of safe and effective pharmaceutical options for *T. cruzi*, there is an urgent need for the discovery and advancement of new chemical entities for the treatment of this disease.

Natural products represent a rich source of chemical space which has yielded many successful drugs. Recent work has utilized natural product scaffolds to develop drug leads for trypanosomes. Plant- and marine-derived molecules against *T. cruzi* and *Trypanosoma brucei* have been successfully isolated and characterized in ongoing drug discovery ventures including derivatives of flavanones, gallinamides, and polynye macro-lactams. Furthermore, amphotericin B and paromomycin are compounds derived from bacterial sources that are used in the treatment of *Leishmania* infections.

Fungi are also an important source of antiparasitic drug scaffolds: our recent work screening for active compounds against *Trichomonas vaginalis* revealed anthraquinones, xan-thone–anthraquinone heterodimers, and decalin-linked tetra-mic-acid-containing metabolites as moderate to potent inhibitors of this pathogen. More specifically against *T. cruzi*, a sesquiterpene hypnophilin derived from *Lentinus* species of fungi is a potent inhibitor of the trypanothione reductase and...
showed activity against the intracellular parasite in vitro. Other fungi-derived secondary metabolites with antiparasitic activity have been reported. In light of this, we screened a library of fungal natural products against *T. cruzi* to identify new chemical entities with antitrypanosomal activity. The fungal natural products were obtained via a citizen-science soil-sampling initiative (https://whatsinyourbackyard.org/), as well as a survey of Great Lakes sediments. Here, we report leucinostatins A, B, and F, as well as novel leucinostatins NPDG C and NPDG D (recently characterized), as having potent activity against the replicative, intracellular amastigote form of the parasite and no host cell toxicity up to 1.5 μM using a phenotypic, high-content imaging assay. We further demonstrated that leucinostatin B reduced parasite growth in an in vivo bioluminescent model of *T. cruzi* infection. These results represent a framework for further development of leucinostatins and derivatives for Chagas disease treatment.

### RESULTS

**Fungal Natural Product Library.** The natural products used for bioactivity screening were derived from fungi collected using two distinct sampling strategies. The University of Oklahoma Citizen Science Soil Collection is a program aimed at offering citizen scientists throughout the United States the opportunity to share soil samples from which fungal isolates are obtained. Today, the collection contains fungi collected from across the country, representing every major ecological biome throughout the region. A map of sample sites and descriptions of the samples have been archived and can be viewed at the

---

**Figure 1.** Primary screen of a fungal natural product library against *T. cruzi*. A) collection of 5631 samples from the University of Oklahoma natural product collection was screened at a final assay concentration of 2 μg/mL against *T. cruzi* CA-1/72 amastigotes using a phenotypic high-content imaging assay. Benznidazole-treated (50 μM), DMSO vehicle-treated (0.1% final concentration), and natural-product-treated samples are shown in red, blue, and yellow, respectively. DMSO control-normalized percent cell viability of host cells and percent antiparasitic activity are represented on the y-axis and x-axis of the graph, respectively. Vertical and horizontal lines on the graph represent the hit threshold cutoffs for antiparasitic activity (75%) and host cell viability (50%).

---

**Figure 2.** Chemical structures of the five leucinostatins active against *T. cruzi*.
SHAREOK data sharing portal (https://shareok.org/handle/11244/28096). The University of Oklahoma natural product screening collection contains additional sets of fungi and their secondary metabolites obtained through special focused collection initiatives. One such subset was prepared from fungi derived from sediment samples collected throughout Lake Michigan and Lake Superior, as well as a scattering of samples collected in northern Lake Huron. The natural product collection contains >78,000 samples consisting of mixtures of fungal biosynthetic products that had been prepared by subjecting cultures of the isolates to organic solvent extraction, followed by partitioning using ethyl acetate-water.

**Primary Screening of Fungal Extract Samples.** A subset of 5,631 samples dissolved in absolute dimethyl sulfoxide (DMSO) from the University of Oklahoma fungal natural product library were tested at 2 μg/mL against *T. cruzi* strain CA-1/72 in a phenotypic high-content imaging assay we had previously developed and implemented in other drug discovery initiatives.19–22 DMSO vehicle-treated infected mouse myocytes were included as positive infection controls, while infected mouse myocytes treated with 50 μM benznidazole were included as the negative infection control. The results from this screen are presented in Figure 1; hit cutoff conditions were set at >75% antiparasitic activity and >50% host mouse myoblast cell viability. The antiparasitic hit cutoff criteria were determined based on previous screening campaigns conducted by the authors using other chemical libraries giving the optimum hit rate, minimizing the probability of false negatives versus the number of false positives that can be later eliminated by follow-up assays.19,24 The cytotoxicity criteria were based on the average number of cells from untreated controls minus three standard deviations of the mean, which was rounded to 50% cell viability.24 From this initial screen, a subset of 259 bioactive samples (4.6% hit rate) were retained for follow-up.

**Identification of Five Leucinostatins from an Ophiocordyceps sp. as the Active, Pure Compounds against *T. cruzi*.** An iterative bioactivity-guided fraction process led to the identification of an active fraction enriched in putative peptidic natural products (as previously described18). Subjecting this sample to further chromatographic steps led to the purification of five natural products, which included three previously reported metabolites (leucinostatins A, B, and F) and two leucinostatin analogues (leucinostatins NPDG C and NPDG D) that were recently reported as new natural products18 (Figure 2).

The structures of the metabolites were determined using a combination of spectrometric, spectroscopic, and chemical methods.18 The leucinostatins and the positive control benznidazole were tested to assess their concentration-dependent antiparasitic activities (Figures 3 and 4) and cytotoxicity (Figure 5).

All five leucinostatins exhibited EC50 values in the low nanomolar range, with no host cell toxicity detected at concentrations up to 1.5 μM, which translated into a good selectivity index of >120 for all hits (Table 1).

A representative compound from the class which was retained for animal studies, leucinostatin B, demonstrated activity against *T. cruzi* in vitro, whereby the majority of parasites appeared to be cleared from host cells using our in vitro assay in two additional luminescent parasite strains that can be used for in vivo mouse experiments, Brazil-luc25 and CL-luc26 (Figure 3). In Vivo Efficacy Proof-Of-Concept Study. To test if the in vitro antiparasitic activity of the leucinostatins would translate to in vivo efficacy, we used a mouse model of acute Chagas disease.27 Mice were infected with luciferase-expressing *T. cruzi* trypomastigotes (CL-luc), sorted into groups to equalize the parasite load 3 days post infection, and dosed by i.p. injection with either vehicle, benznidazole, or leucinostatin B from days 3–6 post infection. The doses of leucinostatin B chosen for the in vivo efficacy study were based on previously published experiments that tested the efficacy of leucinostatin B in doses ranging from 0.3 up to 2.5 mg/kg by i.p. administration for up to 4 days against *T. brucei* infection in mice.28 Therefore, we employed a dosing regimen with escalating doses starting at 0.25 mg/kg at day 3 post infection, increasing to 0.5 mg/kg at day 4 post infection, and topping out at 1 mg/kg at days 5 and 6 post infection to minimize the risk of toxicity while maximizing the chance of reaching a therapeutic dose. Leucinostatin B and benznidazole successfully reduced parasite growth compared to vehicle-treated mice (Figures 6 and 7).

Variability was observed in the vehicle-treated mouse group in terms of parasite burden, which can be attributed to the intrinsic individual variability of infection progression. A significant difference in the slope of parasite-derived luminescence over time was observed between benznidazole and vehicle groups (*p* = 0.0007) and between leucinostatin B and vehicle groups (*p* = 0.0007).
(0.02), even though benznidazole showed superior efficacy to leucinostatin B \((p = 0.0004\) at day 7).

**DISCUSSION**

Treatment options for Chagas disease remain limited. Efforts to expand the scope of chemical scaffolds for drug discovery programs against *T. cruzi* using natural product, de novo design, and drug repositioning approaches have yielded promising new leads in recent years. Here, we used a high-throughput screening and bioactivity-guided fractionation pipeline to identify five potent leucinostatins from an *Ophiocordyceps* sp. with low nanomolar EC\(_{50}\) values and no host cell toxicity at concentration up to 1.5 \(\mu\)M. These EC\(_{50}\) values are particularly promising since selection criteria for *T. cruzi*-screening campaigns usually consider <10 \(\mu\)M EC\(_{50}\) values as hit molecules. The benznidazole EC\(_{50}\) of 2.2 \(\mu\)M, when used as a point of comparison, demonstrates how much more potent the leucinostatins are as *T. cruzi* inhibitors than the reference drug in vitro. Furthermore, the large selectivity index ranging from >120 to >530 for the leucinostatin hits in our study greatly exceeded...
standard hit selection criteria for emerging infectious diseases (SI of >10). Using a mouse model of Chagas disease, we were also able to demonstrate significant antiparasitic activity in vivo, which is an encouraging proof-of-concept for future development of this chemical scaffold as a therapeutic agent. While benznidazole had better activity against T. cruzi in our in vivo experiment than leucinostatin B, it is unlikely that this is driven by pharmacokinetic parameters as previous work on this compound against T. brucei showed efficacy in the compound dosing range we tested and showed toxicity at higher concentrations of leucinostatin B. DMPK studies would have to be performed to confirm this hypothesis. Both of these findings point to an adequate organisal exposure to the compound in vivo.

Previous work has shown that both leucinostatins A and B have activity against the causative agent of African trypanosomiasis, T. brucei; the authors of the study speculated that the mechanism of action of these compounds may involve the targeting of the ATP synthetase and/or Ca\(^{2+}\) and pH homeostasis in T. brucei. We have confirmed that both these compounds inhibit the related kinetoplastid T. cruzi and discovered three additional leucinostatin scaffolds. Recent work with SAR using a leucinostatin A scaffold against T. brucei and Leishmania donovani has further demonstrated experimentally that the principal mechanism of action of leucinostatins is through destabilization of the inner mitochondrial membrane in these parasites.

Leucinostatins are chemically tractable natural products for an SAR campaign, and the total synthesis of molecules in this class has been demonstrated. Peptidic leads for drug discovery programs for Chagas disease have been explored in a number of other publications from our group and others.

Our findings suggest a potential common mechanism of inhibition across trypanosomes for this class of compounds. Future work will focus on testing these compounds for potential activity against other strains of T. cruzi including clinical isolates and the mechanism of action studies to confirm if they also target the membrane stability in T. cruzi. In addition, SAR studies and pharmacokinetic optimization will be logical next steps in the development of these compounds as potential anti-Chagas drug leads while ensuring adequate drug tolerability and oral bioavailability since the target product profile for Chagas disease requires oral administration for new drugs.

In summary, we have determined that naturally occurring leucinostatins are promising natural products with potent in vitro and in vivo activities against T. cruzi and cross-trypanosome activity. These results set the stage for the development of this class of antitrypanosomal molecules as leads for the treatment of Chagas disease.

### EXPERIMENTAL SECTION

**Cells.** CA-I/72 T. cruzi (a gift from J. Dvorak, NIH) and C2C12 mouse myoblasts (ATCC CRL-1772) were passaged in Dulbecco’s modified Eagle medium (Invitrogen, 11095–080) with 5% HyClone iron-supplemented calf serum (Cytivia, SH30073.03) and 1% penicillin–streptomycin (Invitrogen, 15140122) and were maintained at 37 °C and 5% CO\(_2\). CA-I/72 T. cruzi was passaged weekly through coculture with C2C12 mouse myoblasts, essentially as described.

**Fungi and Natural Products.** The methods related to the identification and culture of the Ophiocordyceps ssp., purification of the leucinostatins, and methods used to solve the structures of the natural products have been reported.

**In Vitro Antiparasitic Assay.** Samples and pure compounds from the fungal natural product library, benznidazole (Sigma cat. no. 419656), and DMSO (Sigma cat. no. D2650) were spotted onto black, clear-bottom 384-well plates (Greiner Bio One, 782092) using an Acoustic Transfer System (ATS) instrument (EDC Biosystems). Using a Multidrop Combi liquid handler (Thermo Scientific), 500 cells per well of C2C12 cardiomyoblasts and 7500 cells per well of CA-I/72 T. cruzi
parasites were added to each well plate. This was followed by incubation at 37 °C and 5% CO₂ for 72 h using plate-holding trays to reduce the evaporation of cultures at the edges of the plates. Paraformaldehyde (4% final concentration) in 1× phosphate-buffered saline (Invitrogen, 10010023) was then added to the plates to fix the cells. Subsequent treatment with 5 μg/mL DAPI staining solution (Sigma-Aldrich, D9542) was applied for 1 h to stain host cells and parasite nuclei. Imaging of well plates was conducted with a 10× fluorescence objective using an ImageXpress Micro XLS automated high-content imager (Molecular Devices). A custom image analysis module generated in MetaXpress (Molecular Devices) was then used to count host cells and parasite nuclei to be used as an assay readout, as previously described. Microscopic figure images were prepared using ImageJ.

In Vivo Antiparasitic Proof-Of-Concept Study. Six-week-old BALB/c mice (three males and three females per group) were each infected with 1 × 10⁵ trypomastigotes of P. y. reiquingh luciferase-expressing T. cruzi CL Brener. Three days post infection (dpi), the mice were sorted to equalize parasite load into three groups and received the following treatments for 4 consecutive days (from 3 dpi to 6 dpi): vehicle, 10% DMSO, bis-in-die (b.i.d.), intraperitoneally (i.p.); benznidazole, 50 mg/kg, b.i.d., i.p.; and leucinostatin B in an escalating dose regimen: 3 dpi: 0.25 mg/kg; 4 dpi: 0.5 mg/kg; and 5 and 6 dpi: 1 mg/kg; all b.i.d.; i.p. Mice were each injected (i.p.) with 0.05 μL of 25 mg/mL D-luciferin potassium salt (GoldBio, St. Louis, MO) and applied for 1 h to stain host cells and parasite nuclei. Imaging of well plates was conducted with a 10× fluorescence objective using an ImageXpress Micro XLS automated high-content imager (Molecular Devices). A custom image analysis module generated in MetaXpress (Molecular Devices) was then used to count host cells and parasite nuclei to be used as an assay readout, as previously described. Microscopic figure images were prepared using ImageJ.

Software. GraphPad Prism 8 (GraphPad Software) was used to generate EC₅₀ and CC₅₀ curves for the phenotypic data. CDD Vault (Collaborative Drug Discovery Inc.) was used to curate primary screening and counter-screening data. In vivo data were analyzed using R version 3.6.1 in Jupyter Notebook. The table of contents file was generated using BioRender.com.

Author Information

Corresponding Authors
Robert H. Cichewicz — Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma 73019, United States; Natural Products Discovery Group and Institute for Natural Products Applications and Research Technologies, University of Oklahoma, Norman, Oklahoma 73019, United States; orcid.org/0000-0003-0744-4117; Phone: 405-325-6969; Email: rhcichewicz@ou.edu
Jair L. Siqueira-Neto — Skaggs School of Pharmacy and Pharmaceutical Sciences and Center for Discovery and Innovation in Parasitic Diseases, University of California, San Diego, La Jolla, California 92093, United States; orcid.org/0000-0001-9574-8174; Phone: 858-822-5595; Email: jairlage@ucsd.edu

Authors
Jean A. Bernatchez — Skaggs School of Pharmacy and Pharmaceutical Sciences and Center for Discovery and Innovation in Parasitic Diseases, University of California, San Diego, La Jolla, California 92093, United States;
Yun-Seo Kil — Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma 73019, United States; Natural Products Discovery Group and Institute for Natural Products Applications and Research Technologies, University of Oklahoma, Norman, Oklahoma 73019, United States
Elany Barbosa da Silva — Skaggs School of Pharmacy and Pharmaceutical Sciences and Center for Discovery and Innovation in Parasitic Diseases, University of California, San Diego, La Jolla, California 92093, United States
Laura-Isobel McCall — Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma 73019, United States; Department of Microbiology and Plant Biology and Laboratories of Molecular Anthropology and Microbiome Research, University of Oklahoma, Norman, Oklahoma 73019, United States
Karen L. Wendt — Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma 73019, United States; Natural Products Discovery Group and Institute for Natural Products Applications and Research Technologies, University of Oklahoma, Norman, Oklahoma 73019, United States
Julia M. Souza — Skaggs School of Pharmacy and Pharmaceutical Sciences and Center for Discovery and Innovation in Parasitic Diseases, University of California, San Diego, La Jolla, California 92093, United States; Research Group on Natural Products, Center for Research in Sciences and Technology, University of Franca, Franca, São Paulo CEP 14404-600, Brazil
Jasmin Ackermann — Skaggs School of Pharmacy and Pharmaceutical Sciences and Center for Discovery and Innovation in Parasitic Diseases, University of California, San Diego, La Jolla, California 92093, United States; Athena Institute, VU University Amsterdam, HV Amsterdam 1081, The Netherlands
James H. McKerrow — Skaggs School of Pharmacy and Pharmaceutical Sciences and Center for Discovery and Innovation in Parasitic Diseases, University of California, San Diego, La Jolla, California 92093, United States;

Notes
The authors declare no competing financial interest.
All animal studies were performed under approved protocol S14187 from the Institutional Animal Care and Use Committee, University of California, San Diego (AAALAC accreditation number 000503) and in compliance with the Animal Welfare Act and adhere to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

Author Contributions
J.A.B. and Y.-S.K. contributed equally. All authors wrote and revised the manuscript.

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c06347
ACKNOWLEDGMENTS

We thank Dr. Lars Eckmann from the University of California, San Diego, CA, USA, for the gift of the BALB/c mice used in the in vivo studies. We thank Dr. John Kelly from the London School of Hygiene and Tropical Medicine, London, UK, for the gift of the PpyRE9h luciferase-expressing T. cruzi CL Brener. This project was funded by NIH grant CA182740 to R.H.C. The LC−MS instrument used for this project was provided in part by a Challenge Grant from the Office of the Vice President for Research, University of Oklahoma, Norman Campus and an award through the Shimadzu Equipment Grant Program (R.H.C.). We are grateful for the gift of soil samples provided by citizen scientists P. Clark, K. Tobey, and K. Weeks that were used to obtain fungi used to produce the natural products described in this report. The antiparasitic activity studies were all performed at the UCSD Screening Core with support in part by the Chancellor’s Fund of the University of California, San Diego.

REFERENCES

(1) Rassi, A.; Rassi, A.; Marin-Neto, J. A. Chagas disease. Lancet 2010, 375, 1388−1402.
(2) McCall, L.-I.; McKerrow, J. H. Determinants of disease phenotype in trypanosomatid parasites. Trends Parasitol. 2014, 30, 342−349.
(3) McCall, L.-I.; Morton, J. T.; Bernatchez, J. A.; de Siqueira-Neto, J. L.; Knight, R.; Dorrestein, P. C.; McKerrow, J. H. Mass Spectrometry-Based Chemical Cartography of a Cardiac Parasitic Infection. Anal. Chem. 2017, 89, 10414−10421.
(4) Hossain, E.; Khanam, S.; Dean, D. A.; Wu, C.; Lostracco-Johnson, S.; Thomas, D.; Kane, S. S.; Parab, A. R.; Flores, K.; Katemauswa, M.; et al. Mapping of host-parasite-microbiome interactions reveals metabolic determinants of tropism and tolerance in Chagas disease. Sci. Adv. 2020, 6, eaaa0215.
(5) Morillo, C. A.; Marin-Neto, J. A.; Aveuzum, A.; Sosa-Estani, S.; Rassi, A.; Rosas, F.; Villena, E.; Quiriz, R.; Bonilla, R.; Britto, C.; et al. Randomized Trial of Benznidazole for Chronic Chagas' Cardiomyopathy. N. Engl. J. Med. 2015, 373, 1295−1306.
(6) Urbina, J. A.; Gascon, J.; Ribeiro, I. Benznidazole for Chronic Chagas' Cardiomyopathy. N. Engl. J. Med. 2016, 374, 188.
(7) Newman, D. J.; Cragg, G. M. Natural Products as Sources of New Drugs from 1981 to 2014. J. Nat. Prod. 2016, 79, 629−661.
(8) Greco, S. d. s.; Reimão, J. Q.; Tempone, A. G.; Sartorelli, P.; Cunha, R. L. O. R.; Romoff, P.; Ferreira, R. de R. P.; Fávero, O. A.; Lago, J. H. G. In vitro antileishmanial and antitypanosomal activities of flavonones from Baccharis retusa DC. (Asteraceae). Exp. Parasitol. 2012, 130, 141−145.
(9) Boudreau, P. D.; Miller, B. W.; McCall, L.-I.; Almaliti, J.; Reher, R.; Hirata, K.; Le, T.; Siqueira-Neto, J. L.; Hook, V.; Gerwick, W. H. Design of Gallinamide A Analogs as Potent Inhibitors of the Cysteine Proteases Human Cathespin L and Trypanosoma cruzi Cruzipain. J. Med. Chem. 2016, 59, 9026−9044.
(10) Schulze, C. J.; Donia, M. S.; Siqueira-Neto, J. L.; Ray, D.; Raskatov, J. A.; Green, R. E.; McKerrow, J. H.; Fischbach, M. A.; Linnington, R. G. Genome-Directed Lead Discovery: Biosynthesis, Structure Elucidation, and Biological Evaluation of Two Families of Polyene Macrolactams against Trypanosoma brucei. ACS Chem. Biol. 2015, 10, 2373−2381.
(11) den Boer, M.; Davidson, R. N. Treatment options for visceral leishmaniasis. Expert Rev. Anti-Infect. Ther. 2006, 4, 187−197.
(12) King, J. B.; Carter, A. C.; Dai, W.; Lee, J. W.; Kil, Y.-S.; Du, L.; Helf, S. K.; Cai, S.; Huddle, B. C.; Cichewicz, R. H. Design and Application of a High-Throughput, High-Content Screening System for Natural Product Inhibitors of the Human Parasite Trichomonas vaginalis. ACS Infect. Dis. 2019, 5, 1456−1470.
(13) da Silva Santos-Júnior, P. F.; Schmitt, M.; de Araújo-Júnior, J. X.; da Silva-Júnior, E. F. Sterol 14α-Demethylase from Trypanosomatidae Parasites as a Promising Target for Designing New Antiparasitic Agents. Curr. Top. Med. Chem. 2021, 21, 1900−1921.
(29) Varghese, S.; Rahmani, R.; Russell, S.; Deora, G. S.; Ferrins, L.; Toynton, A.; Jones, A.; Sykes, M.; Kessler, A.; Eufração, A.; et al. Discovery of Potent N-Ethyleurea Pyrazole Derivatives as Dual Inhibitors of Trypanosoma brucei and Trypanosoma cruzi. ACS Med. Chem. Lett. 2020, 11, 278–285.

(30) Harrison, J. R.; Sarkar, S.; Hampton, S.; Riley, J.; Stojanovski, L.; Sahlberg, C.; Appelqvist, P.; Erath, J.; Mathan, V.; Rodriguez, A.; et al. Discovery and Optimization of a Compound Series Active against Trypanosoma cruzi, the Causative Agent of Chagas Disease. J. Med. Chem. 2020, 63, 3066–3089.

(31) Zmuda, F.; Sastry, L.; Shepherd, S. M.; Jones, D.; Scott, A.; Craggs, P. D.; Cortes, A.; Gray, D. W.; Torrie, L. S.; De Rycker, M. Identification of Novel Trypanosoma cruzi Proteasome Inhibitors Using a Luminescence-Based High-Throughput Screening Assay. Antimicrob. Agents Chemother. 2019, 63, e00309-19.

(32) Thomas, M. G.; De Rycker, M.; Cotillo Torrejon, I.; Thomas, J.; Riley, J.; Spinks, D.; Read, K. D.; Miles, T. J.; Gilbert, I. H.; Wyatt, P. G. 2,4-Diamino-6-methylpyrimidines for the potential treatment of Chagas’ disease. Bioorg. Med. Chem. Lett. 2018, 28, 3025–3030.

(33) Katsuno, K.; Burrows, J. N.; Duncan, K.; van Huijsduijnen, R. H.; Kaneko, T.; Kita, K.; Mowbray, C. E.; Schmatz, D.; Warner, P.; Slingsby, B. T. Hit and lead criteria in drug discovery for infectious diseases of the developing world. Nat. Rev. Drug Discovery 2015, 14, 751–758.

(34) Brand, M.; Wang, L.; Agnello, S.; Gazzola, S.; Gall, F. M.; Ragué, L.; Kaiser, M.; Schmidt, R. S.; Ritschl, A.; Jelk, J.; et al. Antiprotozoal Structure-Activity Relationships of Synthetic Leucinostatin Derivatives and Elucidation of their Mode of Action. Angew. Chem., Int. Ed. Engl. 2021, 60, 15613–15621.

(35) Abe, H.; Ouchi, H.; Sakashita, C.; Kawada, M.; Watanabe, T.; Shibasaki, M. Catalytic Asymmetric Total Synthesis and Stereochemical Revision of Leucinostatin A: A Modulator of Tumor-Stroma Interaction. Chem. Eur J. 2017, 23, 11792–11796.

(36) Li, L.; Chenha, B. C.; Yang, K. S.; Cole, T. R.; Goodall, Z. T.; Giardini, M.; Moghadamchargari, Z.; Hernandez, E. A.; Gomez, J.; Calvet, C. M.; et al. Self-Masked Aldehyde Inhibitors: A Novel Strategy for Inhibiting Cysteine Proteases. J. Med. Chem. 2021, 64, 11267–11287.

(37) Alves, L.; Santos, D. A.; Cendron, R.; Rocho, F. R.; Matos, T. K. B.; Leitão, A.; Montanari, C. A. Nitrile-based peptoids as cysteine protease inhibitors. Bioorg. Med. Chem. 2021, 41, 116211.

(38) Alonso-Padilla, J.; Abril, M.; Alarcón de Noya, B.; Almeida, I. C.; Andheben, A.; Araújo Jorge, T.; Chatelain, E.; Esteva, M.; Gascón, J.; Grijalva, M. J.; et al. Target product profile for a test for the early assessment of treatment efficacy in Chagas disease patients: An expert consensus. PLoS Neglected Trop. Dis. 2020, 14, No. e0008035.

(39) Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 2012, 9, 671–675.