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Repurposing of the Open Access Malaria Box for Kinetoplastid Diseases Identifies Novel Active Scaffolds against Trypanosomatids

Marcel Kaiser[1,2], Louis Maes[3], Leela Pavan Tadoori[4], Thomas Spangenberg[5], and Jean-Robert Ioset[4]

Abstract

Phenotypic screening had successfully been used for hit generation, especially in the field of neglected diseases, in which feeding the drug pipeline with new chemotypes remains a constant challenge. Here, we catalyze drug discovery research using a publicly available screening tool to boost drug discovery. The Malaria Box, assembled by the Medicines for Malaria Venture, is a structurally diverse set of 200 druglike and 200 probelike compounds distilled from more than 20,000 antimalarial hits from corporate and academic libraries. Repurposing such compounds has already identified new scaffolds against cryptosporidiosis and schistosomiasis. In addition to initiating new hit-to-lead activities, screening the Malaria Box against a plethora of other parasites would enable the community to better understand the similarities and differences between them. We describe the screening of the Malaria Box and triaging of the identified hits against kinetoplastids responsible for human African trypanosomiasis (*Trypanosoma brucei*), Chagas disease (*Trypanosoma cruzi*), and visceral leishmaniasis (*Leishmania donovani* and *Leishmania infantum*). The in vitro and in vivo profiling of the most promising active compounds with respect to efficacy, toxicity, pharmacokinetics, and complementary druggable properties are presented and a collaborative model used as a way to accelerate the discovery process discussed.

Keywords

ADME/PK, phenotypic drug discovery, neglected diseases, open access, Malaria Box

Introduction

Kinetoplastids are a group of flagellated protozoa responsible for potentially fatal diseases in humans as well as in other mammals. Human African trypanosomiasis (HAT; better known as sleeping sickness), human American trypanosomiasis (Chagas disease), and visceral leishmaniasis (VL; also known as kala-azar) are caused by *Trypanosoma brucei* (with *rhodesiense* and *gambiense* subspecies), *Trypanosoma cruzi*, and *Leishmania donovani*, respectively, and account for the major kinetoplastid diseases. Neglected diseases affect populations mainly located in low-income tropical countries and are closely associated with high burdens of mortality and morbidity as well as social stigmatization.

Current existing drugs used in the treatment of kinetoplastid diseases have several drawbacks and limitations in terms of efficacy, as exemplified by the low drug susceptibility profile of specific *T. cruzi* lineages to benznidazole or nifurtimox for Chagas disease,[1] the resistance of VL to antimonials in India or Nepal,[2] and the lack of efficacy of miltefosine and AmBisome for VL in East Africa.[3,4] The limited safety of some of the drugs used to treat kinetoplastid diseases is also a major concern, leading in some instances to the discontinuation of treatment, as in the case

[1]Parasite Chemotherapy, Swiss Tropical and Public Health Institute, Basel, Switzerland
[2]University of Basel, Basel, Switzerland
[3]Laboratory of Microbiology, Parasitology and Hygiene, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, Antwerp University, Antwerp, Belgium
[4]Drugs for Neglected Diseases *initiative*, Geneva, Switzerland
[5]Medicines for Malaria Venture, Geneva, Switzerland

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Corresponding Author:
Jean-Robert Ioset, Drugs for Neglected Diseases *initiative*, 15 Chemin Louis-Dunant, 1202 Geneva, Switzerland.
Email: jrioset@dndi.org

of benznidazole and nifurtimox for Chagas disease,[5,6] or causing a non-negligible drug-induced mortality in HAT patients when treated with melarsoprol.[7] The lack of pediatric formulations for some of those drugs together with contraindications for pregnant women and those of childbearing age further limit the use of existing treatments.[8] In addition, the various requirements of a cold chain for transport and storage (e.g., AmBisome), parenteral administration by clinical staff, and sometimes lengthy hospitalization (e.g., eflornithine) make them inadequate treatment options for field use and are clear obstacles in terms of drug access for the patients. Finally, some of those drugs have suffered from a lack of availability due to production as well as distribution shortages, as recently documented in the case of benznidazole for the treatment of Chagas disease.[9] The next generation of drugs therefore aim to be effective and safe orally administrated drugs, with a long shelf life in tropical field conditions and simple, short-course drug administration regimens (maximum 10 d, ideally 1–3 d).

Several discovery approaches have been investigated to identify new active starting points for drug development against kinetoplastid pathogens, based on the selection of compound collections and evaluation in either target-based or whole-cell assays. These include, on one hand, the screening of drugs and preclinical candidates developed for other indications against the parasite in question, which may lead to drug repurposing, and, on the other hand, the screening of libraries made up of compounds or chemical classes that are known to affect mammalian targets/biochemical pathways that may also be present in parasites. These compounds/drugs can originate from data mining the literature. More recently, the development of technological platforms for high-throughput screening (HTS) has opened new areas of investigation, allowing the evaluation of significantly larger compound collections (10,000 to several million) representative of a much broader chemical diversity against *T. b. brucei, T. cruzi*, and *L. donovani* whole-cell assays. The merits and drawbacks of these various discovery strategies with respect to compound selection and in vitro activity have been detailed and critically discussed elsewhere.[10]

The Medicines for Malaria Venture (MMV) has compiled the "Open Access Malaria Box," a collection of 400 compounds selected from more than 19,000 structurally unique molecules with confirmed activity against the blood stage of *Plasmodium falciparum* in three large phenotypic (HTS) campaigns undertaken by GlaxoSmithKline (GSK),[11] Novartis-GNF,[12] and St. Jude Children's Research Hospital.[13,14] The Malaria Box, launched at the end of 2009, aims to provide the research community with a collection of starting points for hit-to-lead drug discovery campaigns and a tool to facilitate the understanding of the underlying parasite and host biology. All compounds are commercially available, allowing sourcing of compounds and their near neighbors for efficient confirmation of activity and exploration of structure-activity relationships. Repurposing of the Malaria Box has successfully identified new scaffolds in the field of cryptosporidiosis[15] and schistosomiasis.[16] In addition to initiating new hit-to-lead activities, screening the Malaria Box against other parasites should enable the community to better understand the similarities and differences between them.

Here we present and critically comment on the results of a novel approach of addressing drug discovery in a collaborative and open research framework in relation to kinetoplastid diseases through the Open Access Malaria Box initiative. This exemplifies the collaborative work between Product Development Partnerships organizations (i.e. MMV and Drugs for Neglected Diseases *initiative* [DND*i*]) and shows coordination efforts in drug discovery activities with international expert centers in parasitology.

Materials and Methods

Open Access Malaria Box

The Malaria Box was supplied in V-shaped 96-well plates in 20 µL of a 10 mM DMSO solution and shipped frozen. The chemical purity based on liquid chromatography–mass spectrometry (LC/MS) was ≥90%. Plate mapping and full data on the Malaria Box with the original GSK/St Jude/Novartis compound number, structure, canonical SMILES, biological data, and select in silico physicochemical parameters is available (in pone.0062906.s001) as well as on the MMV Web site (http://www.mmv.org/research-development/malaria-box-supporting-information), together with a list of vendors and their contact details (in pone.0062906.s002). For those compounds of most interest after a first round of screening, a fresh solid sample was repurchased from vendors (MMV665961 from Otava Ltd with 95% purity, MMV019017 from Pharmeks Ltd with ≥90% purity, MMV000498 and MMV019746 from ChemDiv Inc. with ≥95% purity, MMV000248 as AG-690/13706332 and MMV000963 as AS-724/STK154807, MMV-006319 as AS-724/STK839477, and MMV020505 as AN-465/43369387 from SPECS, all with >90% purity) prior to retesting for biological activity.

Primary Screening Campaign

Primary screening of the library, consisting of 400 compounds, was undertaken in a dose-response scheme using 96-well plates against *T. b. brucei, T. b. rhodesiense, T. cruzi, and L. infantum*. Standard screening methodologies were adopted as previously described with slight modifications.[17] All assays were performed in duplicate at the Laboratory of Microbiology, Parasitology and Hygiene at the University of Antwerp (LMPH, Belgium). Compounds were tested at 5 concentrations (32, 8, 2, 0.5, and 0.125 µM) to establish a full dose titration and determination of the

IC_{50} (inhibitory concentration 50%) using Graph Pad Prism software (GraphPad Software, Inc., La Jolla, CA). The IC_{50} values were calculated using blank noninfected controls as 100% and infected nontreated controls as 0% inhibition. The final test volume was 200 µL, and the in-test concentration of DMSO was below 0.5%, a level that is known not to interfere with the different assays. Selectivity of activity was assessed by simultaneous evaluation of cytotoxicity on a human fibroblast (MRC-5) cell line.

Antileishmanial Activity

L. infantum MHOM/MA(BE)/67 amastigotes were collected from the spleen of an infected donor hamster and used to infect primary peritoneal mouse macrophages. To determine in vitro antileishmanial activity, 3×10^4 macrophages were seeded in each well of a 96-well plate. Culture medium was RPMI-1640 supplemented with L-glutamine (20 mM), 16.5 mM sodium hydrogen carbonate, and 10% fetal calf serum (FCS). After 2 d of outgrowth, 5×10^5 amastigotes/well were added and incubated for 2 h at 37 °C. Prediluted samples to be tested were subsequently added, and the plates were further incubated for 5 d at 37 °C and 5% CO_2. Total parasite burdens in the well were microscopically assessed after Giemsa staining and expressed as a percentage of the total burden in the blank controls without compound. In treated wells with high amastigote burdens, an overall estimate of the total burden per well was made without discrimination between the number of infected macrophages and the number of amastigotes per infected cell. In treated wells with low burdens, exact counting was performed. Miltefosine was used as reference and obtained an IC_{50} value of 7.56 ± 1.25 µM. The data set was deposited in the ChEMBL database under the assay ID CHEMBL2028076.

Antitrypanosomal Activity

For HAT assays, *T. b. brucei* (strain Squib-427) and *T. b. rhodesiense* (strain STIB 900) were cultured at 37 °C and 5% CO_2 in Hirumi-9 medium,[18] supplemented with 10% FCS. Assays were performed in 96-well plates, each well containing 10 µL of the compound dilution and 190 µL of the parasite suspension (1.5×10^4 trypomastigotes/well for *T. b. brucei* and 4×10^3 for *T. b. rhodesiense*). Parasite growth was assessed after 72 h at 37 °C by adding resazurin.[19] After 6 h (*T. b. rhodesiense*) or 24 h (*T. b. brucei*), fluorescence was measured (λ_{ex} 550 nm, λ_{em} 590 nm). Suramin was used as reference and obtained an IC_{50} value of 0.05 ± 0.01 µM. The data sets were deposited in the ChEMBL database under the assay IDs CHEMBL2028074 and CHEMBL2028075, respectively.

For Chagas disease, *T. cruzi* Tulahuen CL2 was maintained on MRC-5 cells in minimal essential medium (MEM) supplemented with 20 mM L-glutamine, 16.5 mM sodium hydrogen carbonate, and 5% FCS. In the assay, 4×10^3 MRC-5 cells and 4×10^4 parasites were added to each well, and after incubation at 37 °C for 7 d, parasite growth was assessed by adding the alpha-galactosidase substrate chlorophenol red alpha-D-galactopyranoside.[20] The color reaction was read at 540 nm after 4 h, and absorbance values were expressed as a percentage of the blank controls. Benznidazole was used as reference and obtained an IC_{50} value of 3.18 ± 0.11 µM. The data set was deposited in the ChEMBL database under the assay ID CHEMBL2028073.

Cytotoxicity against MRC-5 Cells

MRC-5 SV2 cells were cultivated in MEM, supplemented with L-glutamine (20 mM), 16.5 mM sodium hydrogen carbonate, and 5% FCS. For the assay, 10^4 MRC-5 cells/well were seeded onto the test plates containing the prediluted sample and incubated at 37 °C and 5% CO_2 for 72 h. Cell viability was assessed fluorimetrically 4 h after the addition of resazurin. Fluorescence was measured (λ_{ex} 550 nm, λ_{em} 590 nm) and the results expressed as percentage reduction in cell viability compared with control. Tamoxifen was used as reference and obtained an IC_{50} value of 11.17 ± 0.16 µM. The data set was deposited in the ChEMBL database under the assay ID CHEMBL2028077.

Medicinal Chemistry Analysis of Hits Identified from Primary Screening

Cluster analysis of the entire Open Access Malaria Box (n = 400) was completed using a predefined fingerprinting set (DotFPCF_1024, number of clusters = 50, Tanimoto similarity). A priority hit list was defined based on the following criteria: (1) number of hits within the cluster based on predefined activity and selectivity criteria; (2) absence of toxicophores based on filters developed in house, which include a list of 110 undesirable chemical moieties, permanently charged molecules, and frequent hitters based on PAINS filters[21]; (3) druglike structural features (based on Lipinski's rule of 5 scoring)[22]; (4) scaffold novelty with respect to kinetoplastid activity; and (5) potential for central nervous system (CNS) penetration, in the case of *T. b. rhodesiense* hits only (TPSA <70 $Å^2$).

Pharmacokinetic Studies for Selected Hits in the Malaria Box

Fasted animals (male CD-1 mouse, 7–9 wk of age, 30 ± 10 g, n = 3) obtained from an approved vendor (SLAC Laboratory Animal Co. Ltd., Shanghai, China; or Sino-British SIPPR/BK Laboratory Animal Co. Ltd, Shanghai, China) were administered with a single oral dose (~50 mg/kg) using DMSO:1% HPMC (5:95) as vehicle. Blood sampling

was performed via the submandibular or saphenous vein (t = 0.083, 0.25, 1, 2, 4, 6, and 9 h) and transferred into microcentrifuge tubes containing sodium heparin (1000 IU/μΛ) as anticoagulant and placed on wet ice until processed for plasma by centrifugation at 3000×g for 15 min at 4 °C. Plasma samples were stored in polypropylene tubes, quick frozen over dry ice, and kept at −70 ± 10 °C until liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis. LC/MS/MS methods for the quantitative determination of compound plasma levels were developed with an internal standard. Finally, concentration in plasma versus time data were analyzed by noncompartmental approaches using the WinNonlin software program (Phoenix WinNonlin, version 6.2.1; Pharsight, Mountain View, CA) and expressed as Cmax, Tmax, T½, and AUC(0-t). Graphs of plasma concentration versus time profile are provided in the supplementary material (**Suppl. Fig. S2**). The data set is freely available upon request to MMV and will be deposited in the ChEMBL database in due course.

Hit Confirmation against T. b. rhodesiense *STIB 900*

Compounds prioritized from primary screening were retested at Swiss Tropical and Public Health Institute (Swiss TPH), Basel, Switzerland, against *T. b. rhodesiense* in duplicate and at varying concentrations to obtain a dose-response curve. The STIB 900 strain was isolated in 1982 from a human patient in Tanzania and after several mouse passages cloned and adapted to axenic culture conditions.[23] MEM (50 μL) supplemented with 25 mM HEPES, 1 g/L glucose, 1% MEM nonessential amino acids (100×), 0.2 mM 2-mercaptoethanol, 1 mM Na-pyruvate, and 15% heat-inactivated horse serum was added to each well of a 96-well plate. Serial drug dilutions of 11 threefold dilution steps covering a range from 100 to 0.002 μM were prepared. Then, 4×10^3 bloodstream forms of *T. b. rhodesiense* STIB 900 in 50 μL were added to each well, and the plate was incubated at 37 °C under a 5% CO_2 atmosphere for 70 h. Ten microliters of Alamar Blue (resazurin, 12.5 mg in 100 mL double-distilled water) was then added to each well and incubation continued for a further 2 to 4 h.[19] The plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Corporation, Sunnyvale, CA) using an excitation wave length of 536 nm and an emission wave length of 588 nm. The IC_{50} values were calculated by linear regression[24] from the sigmoidal dose inhibition curves using SoftmaxPro software (Molecular Devices Corporation). For the retests, newly delivered solid compound stocks were used. Melarsoprol (Arsobal Sanofi-Aventis, received from the World Health Organization) was used as control (IC_{50}: 0.008 μM).

To investigate the effect of serum on compound activity, the serum concentration in vitro was varied at 5%, 15%, and 30%. IC_{50} and IC_{90} values were determined as described above.

Examination of Drug Action

Microcalorimetry Studies Using T. b. rhodesiense. In vitro time of drug action was monitored using isothermal microcalorimetry. Previous experiments have shown that heat flow data can be used as a proxy for the number of viable cells,[25] defined as trypanosomes still moving under the microscope. With this method, the time to the onset of drug action and the time to kill can be determined on a real-time basis.[26] For experiments with permanent drug exposure, bloodstream trypanosomes (2 mL at 1×10^5 organisms/mL per ampoule) were placed in 4 mL ampoules and spiked with the appropriate test compound at concentrations of 1×, 3×, 10×, and 30× IC_{50} with a final DMSO concentration of 0.1% (vol/vol). The same culture medium with 15% horse serum was used as described above. Trypanosome-free negative controls contained only culture medium. After the ampoules had been inserted into the isothermal microcalorimetry instrument (Thermal Activity Monitor, model 249 TAM III; TA Instruments, New Castle, DE), heat flow was continuously measured (1 reading/s) at 37 °C. Each experiment with permanent compound exposure was set up in duplicate and repeated two times. The IC_{50} values against *T. b. rhodesiense* were first determined at Swiss TPH as described above.

Reversibility of Trypanocidal Effects

Trypanosomes were seeded in clear 96-well V-bottom plates at a density of 1×10^5 parasites per well and incubated with the serially diluted test compound. One plate was prepared for each time point. At the designated time, 1, 6, and 48 h, a plate was removed from the incubator and spun at 4.4×10^3 rpm for 5 min to collect the parasites. The supernatant was aspirated, and 100 μL of warmed medium (37 °C) was added to each well. The wash was repeated twice more. The parasites were resuspended in 100 μL of warmed medium, and 20 μL of this suspension was added to 80 μL of medium in triplicate plates. Following 72 h of incubation, resazurin was added and trypanocidal activity determined.

In Vivo T. b. rhodesiense *(STIB 900) Acute Mouse Model*

The STIB 900 acute mouse model mimics the first stage of the disease. Four female NMRI mice were used per experimental group. Each mouse was inoculated intraperitoneally

with 10^4 bloodstream forms. Heparinized blood from a donor mouse with approximately 5×10^6/mL parasitaemia was suspended in PSG buffer to obtain a trypanosome suspension of 1×10^5/mL. Each mouse was injected with 0.25 mL. Compounds were formulated in 100% DMSO, diluted 10-fold in 1% HPMC. Compound treatment was on 4 consecutive days, day 3 to 6 postinfection. The compounds were administered intraperitoneally or orally by gavage in a volume of 0.1 mL/10 g. Four mice served as infected-untreated controls. Parasitaemia was monitored using smears of tail-snip blood twice a week after treatment. Mice are considered cured when no parasitaemia relapse was detected in the tail blood over the 60-day observation period. Mean relapse days are determined as day of relapse postinfection of mice. Mice were euthanized when high levels of parasitemia were evident (10^7 trypanosomes/mL blood) on peripheral blood slides. In vivo efficacy studies in mice were conducted at the Swiss Tropical and Public Health Institute (Basel, Switzerland) according to the rules and regulations for the protection of animal rights ("Tierschutzverordnung") of the Swiss "Bundesamt für Veterinärwesen." They were approved by the veterinary office of Canton Basel-Stadt, Switzerland.

Organization and Responsibilities of the Collaborative Framework

The collaborative frame included the following contributing partners: MMV provided the physical supply of 400 compounds of the Open Access Malaria Box, including chemical structures, physiochemical properties, and their associated in vivo pharmacokinetic (PK) profiles. The Laboratory of Microbiology, Parasitology and Hygiene from University of Antwerp undertook the primary screening of the Open Access Malaria Box against the kinetoplastid assay panel as well as the cytotoxicity counterscreening assay. The Parasite Chemotherapy Unit of the Swiss Tropical and Public Health Institute was responsible for hit reconfirmation against *T. b. rhodesiense*, secondary assays against *T. b. rhodesiense* (as listed above), as well as the execution of the in vivo assays in the acutely infected *T. b. rhodesiense* mouse model. The DND*i* was responsible for the coordination of the research collaboration, guidance with respect to hit and lead selection criteria, and management of data from the collaboration.

Results

The 400 compounds of the Open Access Malaria Box were evaluated in duplicate in an in vitro dose-response screening scheme against the bloodstream forms of *T. b. rhodesiense* and *T. b. brucei* as well as against the intracellular amastigote forms of *T. cruzi and L. infantum.* A counterscreen using MRC-5 cells was used to determine a selectivity index (ratio of IC_{50}s). All data generated in the primary screening campaign against the *T. b. brucei* (CHEMBL2028074), *T. b. rhodesiense* (CHEMBL2028075), *L. infantum* (CHEMBL2028076), *T. cruzi* (CHEMBL2028073), and the MRC-5 cell line (CHEMBL2095143) are available from the CHEMBL Web site (https://www.ebi.ac.uk/chembl) together with the 400 chemical structures of the Open Access Malaria Box. The very good correlation (r^2 of 0.80, **Suppl. Fig. S3**) observed between *T. b. rhodesiense* 900 and *T. b. brucei* S427 assays indicated the nonhuman pathogenic and safer-to-handle *T. b. brucei* subspecies can be used as a predictive assay for screening purposes. The hit selection of the Malaria Box primary screening was based on the *T. b. rhodesiense* data set, which is ultimately the relevant human subspecies. Based on predefined activity and selectivity criteria, the screening led to the identification of 55 hits against *T. b. rhodesiense* (IC_{50} <5 μM and SI >10, 13.7%), 21 against *T. cruzi* (IC_{50} <5 μM and SI >10, 5.2%), and 8 against *L. infantum* (IC_{50} <10 μM and SI >10, 2.0%). The number of hits identified in each of the three kinetoplastid screening assays as well as the respective overlapping hit rate between the assays are presented in **Figure 1a**. All hits were finally examined using a cluster analysis of the entire Malaria Box compound collection (see the Materials and Methods section: medicinal chemistry analysis of hits identified from primary screening) and prioritized according to the following set of criteria: number of active analogues in the cluster (clusters with $n > 1$ were preferred to singletons), removal of any toxicophores ($n = 6$), permanently charged molecules ($n = 4$), and frequent hitters (filter A: $n = 52$, filter B: $n = 12$ as filter B, and filter C: $n = 5$), druglike structural features based on Lipinski's rule of 5 scoring including low molecular weight, low clogP, and low number of H-bond donor/acceptors. In addition, any hits associated with *T. b. rhodesiense* were further reviewed for their potential for CNS penetration based on a TPSA surrogate filter (<70 Å^2).

From an initial list of active clusters (4 for *L. infantum*, 5 for *T. cruzi*, and 15 for *T. b. rhodesiense*) based on the distribution of the active compound scaffolds identified from cluster analysis, the application of the aforementioned set of criteria led to the selection of two priority hits (**1** and **2**) for *L. infantum* based on 1,3-dihydrobenzimidazol-2-imine (cluster A) and a 2,4-diaminopyrimidine (cluster B) moieties, respectively. Two priority hits (**2a** and **3**) were identified for *T. cruzi* that belong to the 2,4-diaminopyrimidine scaffold mentioned above (cluster B) and to a 2-amidothiazole/2-aminothiazole chemical core (cluster C), respectively. Finally, an additional 6 priority hits (**4–9**) were reported against *T. b. rhodesiense*, including 9-substituted-carbazole (cluster D), 2-aminobenzothiazole/2-aminoimidazole (cluster E and F), 2-benzyl, 4-aminoquinazoline (cluster G), 6-amidoquinoline (cluster H), and phenylfuran (cluster I) moieties, respectively. Chemical structures of priority hits **1–9** are shown in **Figure 1b** along with core scaffolds defined by the cluster analysis cluster shown in bold. More details

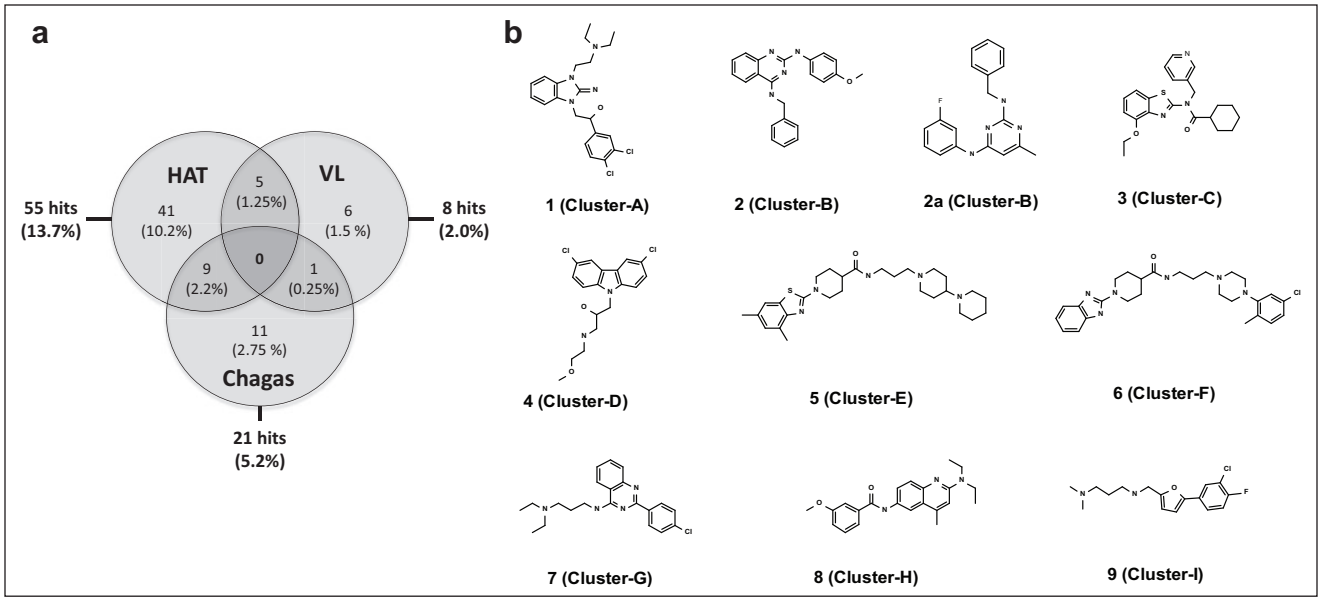

Figure 1. (**a**) Hit overlap identified from the 3 kinetoplastid screening assays including numbers and respective percentages of hits exclusively/commonly identified by each assay: Human African trypanosomiasis (HAT), *Trypanosoma brucei rhodesiense* (STIB 900); visceral leishmaniasis (VL), *Leishmania infantum* MHOM/MA(BE)/67); and Chagas disease (*Trypanosoma cruzi*, Tulahuen CL2). Hit criteria: IC_{50} <5 μM and SI >10 for HAT and Chagas disease, IC_{50} <10 μM and SI >10 for VL. Hit percentages: number of hits divided by 400 (size of compound library). (**b**) Chemical structures of the prioritized hits **1–9** (core scaffolds as defined by the clustering analysis are shown in bold).

regarding those clusters including chemical structures of hits as well as number of hits and inactives within a cluster are available in **Supplemental Table S7**.

Compound **3** was discarded at this stage because of its relatively high IC_{90} value (20.53 μM) against *T. cruzi.* It showed a 128-fold difference in comparison to IC_{50} as well as a clearly suboptimal drug plasma concentration observed from the in vivo PK murine study following oral administration (see **Suppl. Fig. S2**). In the absence of any PK data for compound **2** (not available at the time the decision was made to follow up on priority hits but now included in **Suppl. Fig. S2**), its chemical analogue **2a**, active against both *L.infantum* and *T. cruzi*, was instead considered for progression into in vivo efficacy models for visceral leishmaniasis and Chagas disease. This priority hit was, however, discarded because of its relatively high IC_{90} activity value against *L. donovani* (7.05 μM) in comparison with a lower plasma exposure measured in the in vivo PK study following oral administration (see **Suppl. Fig. S2**). It was similarly disregarded for Chagas disease as its IC_{90} value against *T. cruzi* could not be determined (>64 μM; **Suppl. Table S1b**) upon reconfirmation screening. The recent availability of the in vivo PK profile of compound **2** showed a poor plasmatic exposure (see **Suppl. Fig. S2**) that did not trigger a revival in this molecule despite confirmation of its in vitro activity against *L. infantum* (IC_{90} = 1.81 μM; **Suppl. Table S1b**). All other prioritized hits **1–9** were reassayed and consistently reconfirmed for their in vitro antiprotozoal and cytotoxicity activities (**Table 1**; **Suppl. Table S1b**) with respect to data obtained from the primary screening campaign (**Suppl. Table S1a**). Previously generated and freely accessible in vivo drug exposure data obtained from a single-dosing of mice following oral drug administration were then used to make an informed decision whether the prioritized hits had the potential to reach a sufficient drug plasma concentration to have a therapeutic effect in the mouse challenge model (**Table 1**; **Suppl. Figure S2**). Based on the in vitro activity and in vivo PK data, four hits against *T. b. rhodesiense* (**4**, **6**, **7**, and **8**) were prioritized as potential candidates for in vivo testing in the acute *T. b. rhodesiense* murine model. The screening of a small set of 20 structurally related commercially available analogues to **1** and **9** (data not presented) did not show any significant improvement in in vitro activities against *L. infantum* (**1**) or *T. b. brucei* (**1** and **9**), leading to the discontinuation of these scaffolds.

Before proceeding with in vivo efficacy assays, the in vitro activity of **4**, **6**, **7**, and **8** was reconfirmed against *T. b. rhodesiense* (STIB 900) using a new batch of compound. The compounds confirmed similar in vitro activity as in the previous assay (**Table 1**). Surprisingly, none of the tested compounds showed efficacy in the *T. b. rhodesiense* (STIB 900) acute mouse model (**Suppl. Table S3**). Compound **7**, having a very favorable PK profile (C_{max} 2.3 μM, $T_{1/2}$ >9 h) with respect to its in vitro activity, was also tested orally at 50 mg/kg twice daily and intraperitoneally at 50 mg/kg,

Table 1. In Vitro Activity against *T. b. rhodesiense* (STIB 900) and Pharmacokinetic Profile of Four Selected Compounds.

	MW	LogP	IC_{50} (μM)[a]	IC_{90} (μM)[a]	IC_{50} (μM) MRC-5[a]	SI	IC_{50} (μM)[b]	IC_{90} (μM)[b]	C_{max} (μM)[c]	T_{max} (h)[c]	$T_{1/2}$ (h)[c]	$AUC_{0\text{-last}}$ (h*μmol/L)[c]	PPB (%)[d]
4	367.3	3.8	0.36 ± 0.08	1.41 ± 0.25	4.81 ± 1.12	13.4	0.3 ± 0.1	0.5 ± 0.2	3.6 ± 0.5	1.7 ± 0.6	NR	24.1 ± 2.1	99.9
6	495.1	4.1	1.01 ± 0.18	1.76 ± 0.53	14.52 ± 2.75	14.4	1.2 ± 0.2	2.3 ± 0.6	1.7 ± 0.3	4.7 ± 2.3	NR	8.5 ± 01.7	98.6
7	368.9	4.5	0.25 ± 0.05	0.44 ± 0.09	15.77 ± 3.02	63.1	0.4 ± 0.13	0.5 ± 0.2	2.3 ± 0.1	2.0 ± 0.0	NR	17.1 ± 0.8	99.2
8	363.5	4.7	0.68 ± 0.15	1.74 ± 0.45	15.33 ± 2.95	22.5	0.2 ± 0.3	5.1 ± 0.9	13.9 ± 4.0	0.3 ± 0.0	1.98	34.5 ± 5.1	99.75

[a]Primary screening (DMSO stock solutions) at Laboratory of Microbiology, Parasitology and Hygiene at the University of Antwerp, suramin as control for *T. b. rhodesiense*. IC_{50} 0.05 ± 0.01 μM, tamoxifen as control for MRC-5 IC_{50} 11.17 ± 0.16 μM.
[b]Retested from new solid stocks at Swiss Tropical and Public Health Institute, melarsoprol as control for *T. b. rhodesiense*. IC_{50} 0.008 ± 0.002 μM.
[c]Administered dose (orally) for **4**, **6**, **7**, and **8** was 59, 56, 55, and 45 mg/kg, respectively.
[d]CD-1 mouse plasma.
[e]NR, not reported because of lack of terminal phase.

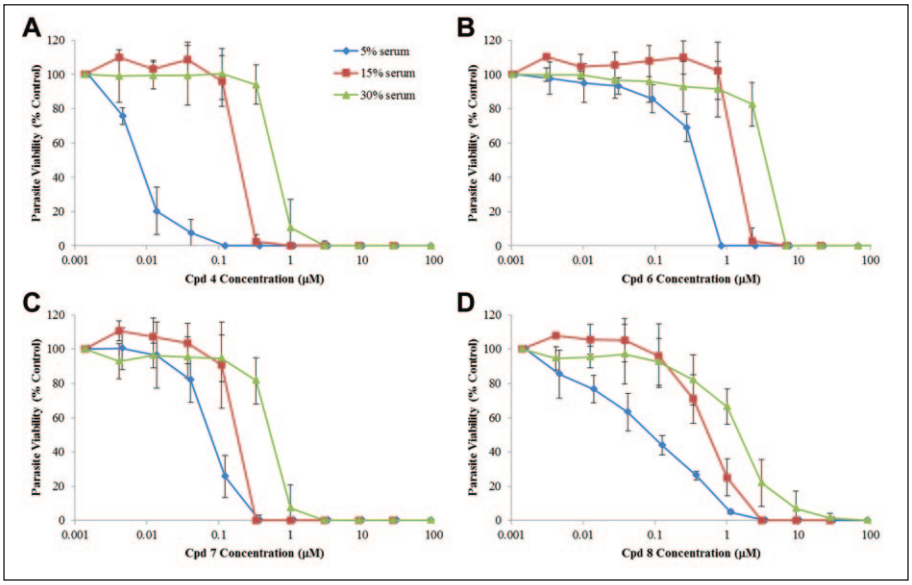

Figure 2. In vitro dose-response curve of compounds **4**, **6**, **7**, and **8** at different serum concentrations.

with the duration of treatments being, respectively, 5 d and 4 d. However, none of these additional experiments were able to demonstrate any sign of in vivo efficacy potential (**Suppl. Table S3**).

Possible causes of failure, including influence of serum protein binding on the in vitro activity, the time course of drug action, and reversibility of the drug effect, were examined to explain these disappointing in vivo results. As all 4 compounds (**4**, **6**, **7**, and **8**) showed high plasma protein binding levels (>98.6% or higher; see **Table 1**), they were progressed into serum shift assays to determine the effect of serum protein binding on the trypanocidal in vitro activity. All compounds exhibited an IC_{50} shift of two to three times to a higher value when the serum content in the test was doubled from 15% to 30%, demonstrating decreased in vitro activity (**Fig. 2**; **Suppl. Table S4**). Similarly, all four compounds showed significantly lower IC_{50} values and therefore increased in vitro activity when the serum content was decreased to 5% (**Fig. 2**; **Suppl. Table S4**).

To obtain a better understanding of the PK/pharmacodynamic parameters of the in vitro activity of compounds **4**, **6**, **7**, and **8**, the time of drug action in vitro against *T. b. rhodesiense* (STIB 900) was recorded and analyzed using isothermal microcalorimetry analysis.[25,26] Onset of drug action, time to peak, and time to kill were evaluated as measures of drug action.[27] The results are presented in **Figure 3**.

Compound **8** exhibited a dose-dependent response for all measured parameters (**Fig. 3**). The onset of drug action ranged from 8 to 12 h, time to peak ranged from 19 to 46 h, and time to kill was between 55 and 122 h (**Suppl. Table S5**). A concentration of 0.9 μM (1× IC_{50}) gave a slight growth inhibition. The parasites were killed within 84 h at a constant drug exposure of 2.8 μM (3× IC_{50}). At 10× and 30× IC_{50} (Cmax is 20× IC_{50} at 45 mg/kg orally), the parasites were killed in about 55 h. The microcalorimetric study showed that a constant drug concentration superior to 2.8 μM is required to kill *T. b. rhodesiense* parasites within 50 to 90 h (**Fig. 3**). The compound had a good C_{max} (13.9 μM) in the mouse but a short $T_{1/2}$ (2 h).

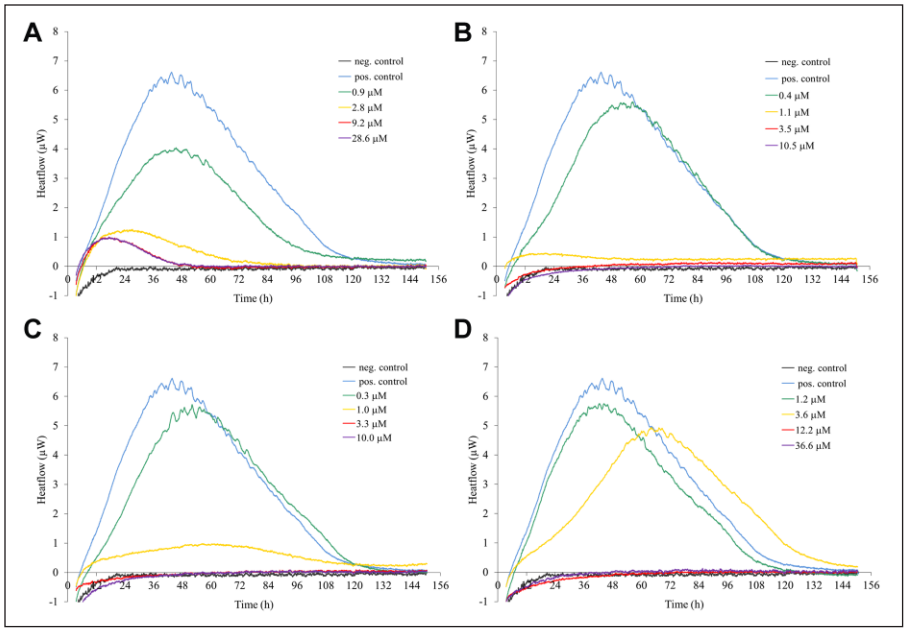

Figure 3. Microcalorimetric growth profiles of *T. b. rhodesiense* strain STIB900 in the presence of various concentrations of **8** (**A**), **7** (**B**), **4** (**C**), and **6** (**D**). The positive control included parasites (1×10^5/mL inoculum) without drug treatment, and the negative control did not include parasites or drug treatment. Each curve represents the mean of two incubations.

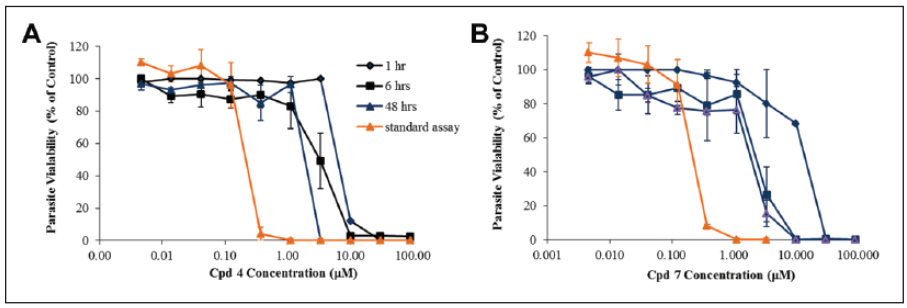

Figure 4. Irreversibility of trypanocidal effect A (**4**), B (**7**): Parasite survival was measured at various times after compound washout at specified times and viability assessment at 72 h. For comparison, the dose-response curve of the standard drug sensitivity assay is added.

Compound **7** killed the parasites in about 56 h at a concentration of 1.1 µM (3× IC_{50}; **Fig. 3**; **Suppl. Table S5**). A constant drug pressure at higher concentrations (10× and 30× IC_{50}) suppressed parasite growth over the whole monitoring period of 150 h. The washout study showed that a fairly long period exposure to the compound at high concentration is required to obtain an irreversible drug effect in vitro (**Fig. 4**). It seems that **7** has rather a static than a cidal effect.

Compound **4** showed a clearly decreased heat flow curve at a concentration of 1.0 µM (3× IC_{50}) compared with the drug-free control curve (**Fig. 3**; **Suppl. Table S5**). But at this concentration, the parasites were not killed, and the time to peak was similar to the control curve. A drug pressure at higher concentrations (10× and 30× IC_{50}) suppressed parasite growth over the whole monitoring period. The washout experiment revealed a potentially static effect. A long period of exposure is required to produce irreversible effects on trypanosome survival (**Fig. 4**). Persistency or irreversibility of the drug effect of compounds **4** and **7** was demonstrated to be both time and concentration dependent. Increasing drug concentrations led to faster killing after washout and incubation in compound-free medium. IC_{50}s and IC_{90}s determined in the washout experiment are displayed in **Suppl. Table S6**.

A concentration of 1.2 µM (1× IC_{50}) of **6** did not affect the growth of the parasites (**Fig. 3**; **Suppl. Table S5**). A concentration of 3.6 µM (3× IC_{50}) did not kill the parasites either, but the heat flow curve was shifted to the right and thus time to peak and time to kill increased. Higher concentrations (10× and 30× IC_{50}) suppressed parasite growth over the whole monitoring period.

Discussion

Figure 5 provides an overview of the sequence of in vitro and in vivo experiments run in the frame of this collaborative work in association with the criteria used to assist the decision-making process. The respective cutoff values used for hit qualification (IC_{50} <5 µM and SI >10 for *T. b. rhodesiense* and *T. cruzi* and IC_{50} <10 µM and SI >10 for *L. infantum*) have

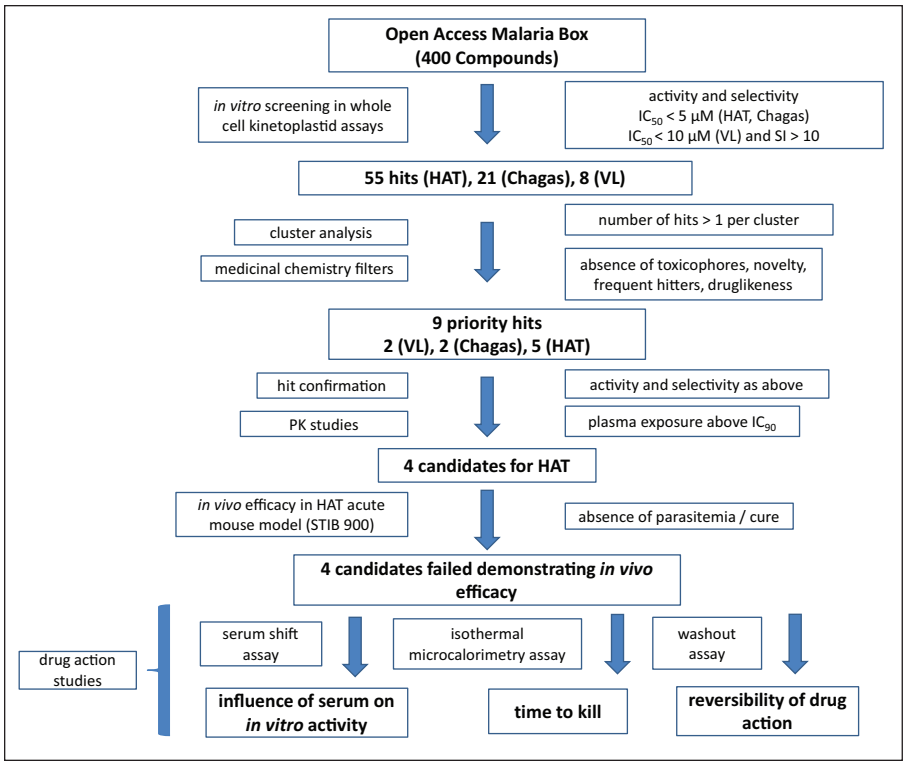

Figure 5. Workflow used to identify and progress hits of the Open Access Malaria Box including key criteria considered in the decision-making process. Human African trypanosomiasis (HAT), *T. b. rhodesiense* (STIB 900); visceral Leishmaniasis (VL), *L. infantum* MHOM/MA(BE)/67); and Chagas disease (*T. cruzi*, Tulahuen CL2).

been arbitrarily set on the basis of the DND*i* empirical experience to progress active starting points identified from in vitro phenotypic screening into drug development programs. The resulting hit rates of 13.7% for *T. b. rhodesiense*, 5.2% for intracellular amastigote form of *T. cruzi*, and 2.0% for the intracellular amastigote form of *L. infantum* were significantly higher than the ones associated with the screening of unbiased selected compound collections, also frequently referred to as "diversity collections." Indeed, recent figures based on previous screening campaigns of diversity collections against the bloodstream form of *T. brucei* subspecies (average 0.45%, ranging from 0.1%–1.2%, approximately 1.3 million compounds assayed), *T. cruzi* (average 0.55%, ranging from 0.03%–0.85%, approximately 500,000 compounds assayed) as well *as L. donovani* 0.06% (ranging from 0.02% to 0.09%, 500,000 compounds assayed) yielded much lower hit rates.[10] Although based on a rather limited number of compounds, this observed enrichment of the hit rate (about 30 times *for T. brucei* and *L. donovani*, 10 times in the case of *T. cruzi*) can be explained by the biological selection bias as well as the druggability properties of the compounds included in the Malaria Box collection. All compounds have indeed previously been demonstrated as being active against the blood-stage form of *P. falciparum* in vitro,[14] suggesting that the hits have favorable properties to reach their biological target in a whole-cell assay. A potential commonality of targets shared between *P. falciparum* and kinetoplastids might additionally contribute to the improved hit rates, as seen for the *T. cruzi* and *L. infantum* hits related to 2,4-diaminopyrimidine and 2, 4-diaminoquinazoline scaffolds (cluster 2; **Suppl. Table S7**). These may be associated with inhibition of the folate synthase pathway, which is well referenced in the literature for *Leishmania, Trypanosoma*, and *Plasmodium*.[28–30] Another example is the 9-substituted carbazole (cluster 4; **Suppl. Table S7**), a scaffold that appears to be selectively active against *T. brucei* subspecies and *P. falciparum*,[31] although the antiplasmodial mechanism of action is still unknown to date. This 9-substituted carbazole, however, also appears to be structurally related to tricyclic antidepressants (e.g., clomipramine and trifluoperazine), previously characterized as trypanothione reductase inhibitors,[32] a well-known target of the trypanothione metabolism pathway unique to *Trypanosomes* and *Leishmania*.[33] Trypanothione reductase also appears to be a putative target for closely related derivatives of the 1,3-dihydrobenzimidazol-2-imine scaffold (**1**), as reported from a recent target-based screening campaign dedicated to this target.[34] In addition, a common 2-aminothiazole/imidazole pharmacophore is associated with hits **3**, **5**, and **6**, which appear to act specifically against *Trypanosom*es, indicating a potential common target. However, the *T. cruzi* activity of **3** and its derivatives (cluster 3; **Suppl. Table S7**) could also be explained by the presence of a commonly shared unencumbered pyridine moiety, a substituent well known for its potential for CYP51 inhibition.[35]

Taken overall, the hit rates recorded in the screening of the Malaria Box advocate for the larger-scale screening of antiplasmodial activity-biased compound collections against kinetoplastids. Finally, the fact that compounds qualify as *P. falciparum* hits with activity ranging from the low-nanomolar to micromolar range in respect to IC_{50} in whole-cell screening assays but have not yet been engineered out to act against any specific *Plasmodium falciparum* target may also account for the increased hit rate.

Free access to chemical structures made public domain before undertaking any screening activities enabled a fast initial triage of the identified hits from primary screening based on activity, selectivity, structural similarity, and drug-likeness. Furthermore, upon request, access to key information generated as part of the Malaria Box Open Access collaboration, such as the in vivo levels of drug exposure in murine noninfected models, greatly supported a quick and scientifically based rationale to move forward a short list of 7 hits (**4, 6**, **7**, and **8**) into in vivo efficacy testing. Conversely, the suboptimal drug exposure profile of other selected hits (in vitro activities of *L. infantum* and *T. b. rhodesiense* expressed as IC_{90}s) led to the deprioritization of **1**, **2**, **3**, **5**, and **9**. All four priority hits (**4**, **6**, **7**, and **8**) carried forward into in vivo studies were, to the best of our knowledge, novel in terms of their activity against *T. brucei* subspecies with regard to their respective core 9-substituted-carbazole (**4**), 2-aminoimidazole (**6**), 2-benzyl-4-aminoquinazoline (**7**), and a 6-amidoquinoline (**8**) structures. Unfortunately, none of the four selected hits was able to demonstrate activity in vivo, although this would be a rather ambitious objective to achieve as a direct follow-up from hit identification without related program of chemistry optimization. A panel of in vitro drug action studies was undertaken to better understand possible reasons for those scaffolds failing in vivo and to determine whether they could be overcome by drug development or if they are undesirable from a mechanism of action perspective. Those drug action assays included influence of serum protein binding on in vitro activity, time-to-kill assays using isothermal microcalorimetry, as well as time needed for irreversible effect using drug incubation followed by washout procedures. All four compounds show a high level of protein binding (98.6% or higher), and the in vitro activities as shown were affected by the serum concentration in the medium (**Fig. 2**). Although the free fraction of drug in the in vitro screening assay was not determined, these results suggest that protein binding may be responsible for quite low, possibly suboptimal, concentrations of compound able to exert efficacy in vivo. However, it is also possible that there is a fast dynamic equilibrium of the free fractions between plasma and tissues of action sites. Under such conditions, indeed, even very high percentages of serum binding (>99.9%)—resulting in very low and clearly suboptimal—concentrations of compound in comparison to the respective IC_{90} of the identified hits, may not be enough to explain the lack of efficacy in vivo.[36] It is not possible to be more conclusive at this stage in the absence of measurements of the free fraction of drug in plasma and of data characterizing the nature and kinetics of drug binding to its target site. In addition, the short half-life and the long drug exposure time required to kill the parasites—in conjunction with the serum shift effect described above—are likely to contribute to the lack of observed efficacy of **8** in the acute *T. b. rhodesiense* rodent model. The slow-acting profile of **7** confirmed from the washout experiment (**Fig. 4**), coupled with a static effect rather than a cidal effect and the negative impact of serum concentration on the in vitro *T. brucei* activity, may explain why this compound failed to demonstrate any in vivo activity. The microcalorimetric study of **6** showed that a concentration of 3.6 μM, which is higher than the C_{max} (1.69 μM), was not able to kill the parasites (**Fig. 3**). A suboptimal plasma concentration in vivo with the IC_{90} value being only slightly higher than C_{max} may explain the inefficacy of **6** in the rodent model. The time-to-kill data of **4** demonstrate that a long period of exposure to the compound is required for suppression of parasitaemia (**Fig. 3**; **Suppl. Table S5**), confirmed with the subsequent washout experiment (**Fig. 4**; **Suppl. Table S6**). The relatively high dependency of $IC_{50/90}$ values to the serum concentration and long exposure period required to demonstrate *T. brucei* killing in vitro may be why **4** failed in vivo despite a favorable in vivo PK profile (**Fig. 2**).

Overall, the Open Access Malaria Box has offered a fast, transparent, and cost-effective collaborative framework to advance a set of compounds directly from screening to proof of concept of in vivo efficacy with a clear scientific rationale. The upfront availability of chemical structures devoid of any intellectual property rights, immediate access, and resupply of physical quantities of material for screening and follow-up experiments, as well as the availability of cytotoxicity data related to the compounds and in vivo PK profile, have enabled a quick and rational progression of this interlaboratory collaborative work and greatly facilitated the associated decision-making process. This collaborative framework has also permitted avoiding any work duplication and the concomitant waste of resources by prioritizing the most promising molecules while working on a slower pace or discontinuing any discovery activities on others. Currently, lower priority should be given to those compounds associated with undesirable mechanisms of action, such as static or slow-killing profiles. For other compounds, novel data on structurally related chemical analogs—such as those associated with significantly higher in vitro activity, short onset of drug action, lower protein binding profile, or superior drug exposure in vivo—may lead to a revival of one of more of those active scaffolds in the future.

In summary, the failure to identify to date an in vivo active candidate for kinetoplastid diseases at the end of this

collaborative process may simply reflect the relatively high bar to be cleared without investing more intensively into a medicinal chemistry program, rather than calling into question the adequacy of this specific compound collection and collaborative model to identify new active starting points.

Although the prioritized compounds showed no in vivo efficacy, further investigation of the chemistry of these scaffolds may improve the in vitro activity and/or the PK profiles. Research groups are encouraged to contact the authors should they be interested in these hits. The current literature available regarding hits **1** to **9** (supplemental data, **Supplement A**) and the availability of new data from ongoing screening of the Open Access Malaria Box compounds in a variety of target-based assays may unravel precious indications in the future (e.g., identification of a putative targets or biological pathways related to those actives) and lead to the further development of some of these active scaffolds. In addition, a more detailed analysis of the already published target annotations associated with hits identified from the screening of the Open Access Malaria Box (e.g., via the CHEMBL database) may corroborate the target hypotheses proposed above or identify alternative ones, leading to new avenues of research and development.

In conclusion, the Open Access Malaria Box initiative was able to create an original open collaborative working environment suitable for the discovery and early progression of active candidates for neglected diseases in a timely manner and within an IP-free framework while working with limited resources. The forthcoming Open Access Pathogen Box initiative (www.pathogenbox.org), which will make 400 diverse, druglike molecules with demonstrable activity against a broad panel of neglected diseases available by the end of 2015, is eagerly awaited.

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