The Pan-Asian Screening Network

Drug Screening for Kinetoplastids Diseases

A Training Manual for Screening in Neglected Diseases

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Contents

1. Introduction

1a. Framework

The production of this research manual has been decided as one of the deliverables of the Pan-Asian Network for Drugs for Neglected Diseases from Natural Substances during its first annual meeting in Tokyo (May 2006). The formation of this network was funded by a generous contribution from the Sasakawa Peace Foundation to the Drugs for Neglected Diseases *initiative* (DND*i*).

Team members belonging to the network

CDRI	Central Drug Research Institute, Lucknow, India
FRIM	Forest Research Institute Malaysia, Kepong, Malaysia
IPK	Institut Pasteur, Korea, Seoul, South Korea
KIT	Kitasato Institute, Tokyo, Japan
NITD	Novartis Institute for Tropical Diseases, Singapore
SIMM	Shanghai Institute of Materia Medica, Shanghai, China
MIPN	Malaysian Institute of Pharmaceuticals and Nutraceuticals, Penang, Malaysia
EGU	Eskitis Institute, Griffith University, Brisbane, Australia

1b. Objectives

The objectives of this manual are several:

- To compile in a practical and user-friendly guide the assays available to screen natural products against pathogens responsible for some of the neglected diseases
- To standardize and give recommendations on these screening methodologies and protocols related to these assays
- To describe the principles of good scientific practice related to the preparation, realization and process of these assays and of the obtained data
- To bring information on the sources, storage and handling of material requested to perform these assays
- To list the institutions actively using these assays with mention of their respective expertise and activities and identify centres of reference in this field of research
- To list additional useful key information and references related to these activities

The following flow chart (Figure 1) summarizes the process of screening of natural substances against pathogens responsible for neglected diseases.

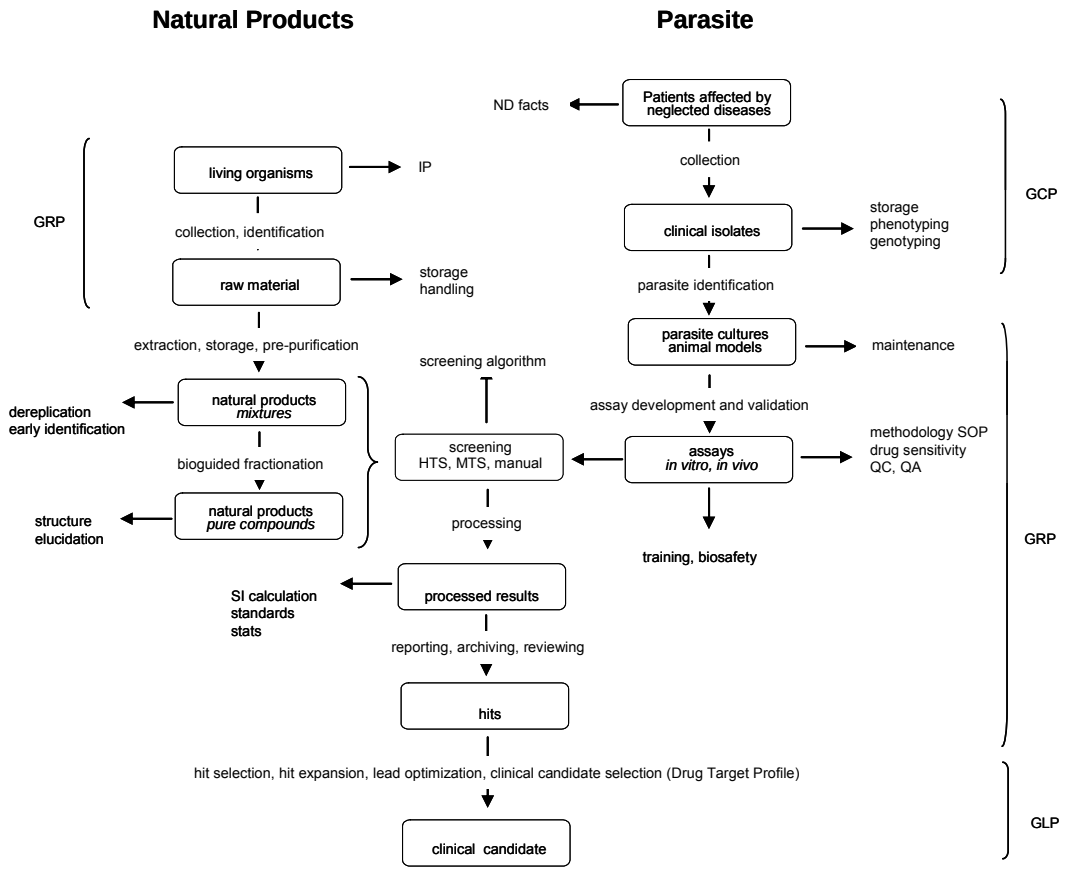

Figure 1. Process for screening natural substances against pathogens responsible for neglected diseases

1c. Preliminary remarks

The focus of this manual is to specifically address the screening of natural products. This includes complex mixtures of unknown composition such as extracts, fractions as well as pure compounds. This document has however been designed in order to address the screening of all kind of samples from both synthetic and natural origin as single molecules or mixtures of compounds. Remarks and recommendations have been made in the text on an ad hoc basis to cover the specificities of types of samples in terms of screening against kinetoplastids. The procedures of obtaining, handling and storing extracts, fractions and pure compounds from material of natural origin for screening purpose are not in the scope of this manual. It is assumed that the samples discussed above have been obtained following state-of-the-art expertise.

A table of abbreviations, a glossary, a list of resources available in the field as well as references and hyperlinks are provided at the end of the document to complement the information enclosed in this manual. Additional questions,

comments as well as feedback can be submitted to the authors using the following contact email address: jrioset@dndi.org.

1d. Distribution of the manual

The manual will be available free-of-charge as an electronic version and eventually as a hard copy. The electronic version will be accessible at the following link www.pan4nd.org. The electronic version will first be released as a pdf and later be made available with hyperlinks to enable a quick and convenient navigation through the document. For any additional questions and requests please use the following contact email address: jrioset@dndi.org.

2. General principles and guidelines

Laboratories involved in the screening of natural substances against neglected diseases must comply with the national regulations related to the specific conduced activities.

They should also follow the international recognized laboratory guidelines listed below.

2a. Good Research Practice

Screening and Efficacy studies which do not contribute to the safety dossier need to be conducted according to Good Research Practice (GRP). By GRP one means that the experimental, technical and administrative procedures employed for collecting, processing and interpreting results of research are appropriate, rigorous, repeatable and auditable. This is essential for any subsequent application for Intellectual Property and in establishing confidence of partners in academia and industry. It also implies that research should be well conducted and recorded, with each study presented as a separate report describing background, experimental protocol, assessments and conclusions. More details than usually presented in a paper are necessary e.g. all data points should be included.

GRP include the following points:

- **Responsibilities**

The research institution is responsible for the overall quality of research conducted within it, including compliance with in-house research and management policies. Managers, group leaders and supervisors have a responsibility to ensure a climate of best scientific practice in the research teams, including a commitment to the development of scientific and technical skills.

The Principal Investigator or Project Leader is responsible for all the work conducted in the project including that of any subcontractors. All staff and students should have defined responsibilities in relation to the project and be aware of these responsibilities.

- **Competence**

All personnel associated with the project must be competent to perform the technical, scientific and support tasks required of them. Personnel undergoing training must be supervised at a level such that the quality of the results is not compromised by the inexperience of the researcher.

- **Project planning**

An appropriate level of risk assessment should be conducted to demonstrate awareness of the key factors that will influence the success of the project and the ability to meet its objectives. There should be a written project plan showing that these factors (including research design, statistical methods and others) have been addressed. Project plans must be agreed in collaboration with all partners/funding bodies taking account of the requirements of ethical committees or the terms of project licences, if relevant. Significant amendments to the plan or milestones must be recorded and approved by the project partners.

- **Quality Control**

The research institution should have planned processes in place to assure the quality of the research undertaken by its scientists. Research projects should be subjected to formal reviews of an appropriate frequency.

The authorisation of outputs shall be as agreed by all project partners in line with signed agreements if any, and subject to senior approval in the organisation, where appropriate. Errors identified after publication must be notified all project partners and agreed corrective action initiated.

Processes and procedures should be regularly reviewed against a policy of continual improvement.

- **Health and Safety**

All research must comply with the relevant Health and Safety regulatory requirements.

- **Handling of samples and materials**

All samples and other experimental materials should be labelled (clearly, accurately, uniquely and durably), and retained for a period to be agreed by partners. The storage and handling of the samples and materials should be as specified in the project plan (or proposal), and must be appropriate to their nature. If the storage conditions are critical, they must be monitored and recorded.

Samples must be readily tracked through the stages of analysis or use, and have designated disposal routes and dates.

- **Facilities and equipment**

The working environment must be appropriate for safe operational equipment, maintenance of sample quality and integrity, and good working practices.

Where special facilities are used (e.g. fume cupboards) they must be regularly checked and maintained.

All equipment must be appropriate for the measurements to be made, calibrated if necessary, and be in good working condition. If critical, there should be contingency plans in case of power failure or other disruption.

- **Documentation of procedures and methods**

All the procedures and methods used in a research project must be documented, at least in the personal records of the researcher. This includes analytical and statistical procedures and the generation of a clear audit trial linking secondary processed information to primary data.

There must be a procedure for validation of research methods as for purpose, and modifications must be traceable through each stage of development of the method.

- **Research/work records**

All records must be of sufficient quality to present a complete picture of the work performed, enabling it to be repeated if necessary.

The Project Leader must ensure the validity of the work by carrying out regular reviews of the records of each scientist.

The location of all project records, including critical data, must be recorded. They must be retained in a form that ensures their integrity and security, and prevents unauthorised modification, for a period to be agreed by the project partners.

This list in not exhaustive and more complete and specific guidelines such as presented by The Wellcome Trust Guidelines on Good Research Practice and the Biotechnology and Biological Sciences Research Council BBSRC Statement on Safeguarding Good Scientific Practice 2000 should be considered as well. These guidelines more particularly refer to 1) Integrity 2) Openness 3) Guidance from professional bodies 4) Leadership and collaboration 5) Supervision 6) Training 7) Primary data/samples 8) Research involving animal 9) Risk of research misuse and 10) Publication practice.

CAVEAT:
Good Research Practice should not be confused with Good Laboratory Practice (GLP). GLP refers to principles providing a framework within which laboratory studies undertaken to assess hazards and risks of pharmaceuticals, agrochemicals, cosmetics, food and feed additives and contaminants, novel foods and biocides to users, consumers and environment. GLP helps assure regulatory authorities that the data submitted are a true reflection of the results obtained during the study and can therefore be relied upon when making risk/safety assessments.

2b. Safety

National regulations for lab safety have to be followed. Usually a specific request should be applied for to the national authority in charge of lab safety anytime a new assay is started, modified or transferred from one site to another.

- Chemical hazard

Chemical hazard has to be considered as a potential risk in a lab. Specific recommendations regarding chemical hazard should be followed whenever they are available (refers to Material Safety Data Sheet). Any lab work conduced with chemicals of unknown nature (e.g. samples to be screened) should be performed as if those chemicals were highly prejudicial to human health and environment.

Additional information is available from the following links:
CDC chemical safety
MSDS online

- Biosafety

Biosafety is a main issue for labs handling protozoan organisms. The following documents provide complete guidelines regarding safety in lab. They include information on chemical, biological, fire and electrical hazard.

WHO biosafety manual (including a useful Safety Checklist, pp. 125-131)

The Centers for Disease Control and Prevention (Atlanta, USA, www.cdc.gov) provide useful links regarding biosafety including general information on biosafety and practical basic procedures to be followed by lab staff in case of biological emergencies, hazard and Biosafety in Microbiological and Biomedical Laboratories (with special mention of parasite agents within this latter document).

- Shipment

The proper way to transport potentially infectious material both nationally and internationally is described in the WHO Guidelines for Safe Transport of Infectious Substances and Diagnostic Specimens.

The American Biological Safety Association website offers links to several useful documents related to biosafety issues including the ones mentioned above.

2c. Ethics

In addition to the guidelines already described in the Good Research Practice section, specific efforts should be made to comply with the following topics:

Research involving animal experiments
All partner institutions will undertake that living vertebrate animals required for use in this project will be handled in accordance with locally existing statutes and generally accepted principles for the human treatment of animals, as embodied in the Amsterdam protocol. In all cases the avoidance of unnecessary suffering will be mandatory. In accordance with the Amsterdam protocol on animal protection and welfare, animal experiments must be replaced with alternatives wherever possible. The 3R principles should be followed i.e. to **reduce** the number of animals, to **refine** the protocols and to **replace** where possible *in vivo* by *in vitro* methods. This particularly applies (pursuant to European Union's Directive 86/609/EEC) to animal experiments involving species which are closest to human beings. Altering the genetic heritage of animals and cloning of animals may be considered only if the aims are ethically justified and the conditions are such that the animals' welfare is guaranteed and the principles of biodiversity are respected.

Complementary information regarding animal care is available from the following document: Association for Assessment and Accreditation of Laboratory Animal Care

2d. Biodiversity
In addition to the principles and guidelines already cited above, the collection, transport and use for research purpose of material from biological origin should be done in respect with both national regulations of the countries of collection and screening as well as with the principles described in the text of the Convention on Biological Diversity.

3. Neglected Diseases

3a. Introduction

Neglected: "to give not enough care or attention to something that is of responsibility"

The Neglected Diseases are a group of tropical infections that afflict poor people of developing countries in sub-Saharan Africa, Asia, and the Americas. Together, they cause an estimated 500,000 to 1 million deaths annually and cause a global disease burden equivalent to that of HIV-AIDS. HIV/AIDS, tuberculosis and malaria can be added to the group even if they receive much more media attention and research funding. Human African Trypanosomiasis - also known as sleeping sickness - leishmaniases and Chagas disease are considered as the most Neglected Diseases (WHO/IFPMA working group).

3b. Human African Trypanosomiasis

Human African Trypanosomiasis (HAT), also known as sleeping sickness, is caused by two sub-species of the parasite *Trypanosoma brucei*, which are transmitted to humans by tsetse flies (*Glossina* spp.). Sleeping sickness occurs only in sub-Saharan Africa under two forms caused by either *Trypanosoma brucei gambiense* or *Trypanosoma brucei rhodesiense.*

- *Trypanosoma brucei gambiense (T.b. gambiense)* occuring in west and central Africa, represents approximately 95% of the reported cases of sleeping sickness and causes a chronic infection. A person can be infected for months or even years without major symptoms of the disease. When symptoms do emerge, the patient is often already in an advanced disease stage when the central nervous system is affected.
- *Trypanosoma brucei rhodesiense (T.b. rhodesiense)* is found in eastern and southern Africa and represents approximately 5% of the reported cases. This sub-species of the parasite causes an acute infection where first symptoms are observed after a few weeks. The disease develops rapidly and also invades the central nervous system.

Sleeping sickness has two stages. The 1st stage (haemo-lymphatic) entails bouts of fever, headaches, pains in the joints and itching. The 2nd stage (meningo-encephalitic) begins when the parasite crosses the blood-brain barrier and infests the central nervous system. Without treatment, the disease is fatal. Different drugs are needed depending on the parasite species responsible for the infection and the stage of the disease. With regard to treatment, most drugs are old, lack safety and efficacy and are difficult to administer in a rural setting. Human African trypanosomiasis infects 50,000 or more people a year and puts 50 million at risk in sub-Saharan Africa. A safe, effective, and practical 2nd stage drug (preferably for both stages) is needed, as well as an oral treatment for 1st stage.

3c. Leishmaniases

Leishmaniases are parasitic diseases caused by protozoan parasites of the genus *Leishmania* (at least 20 species) that are transmitted to humans by phlebotomine sandfly bites (*Lutzomyia* and *Plebotomus* spp.). Infections can vary from simple cutaneous leishmaniasis to mucocutaneous and fatal visceral leishmaniasis or kala-azar. Leishmaniasis is prevalent in 88 countries throughout the world (72 are in the developing world) and affects more than 12 million people.

Visceral leishmaniasis (also known as kala-azar in India) is the most severe form of the disease and is fatal if left untreated. It affects patients who mostly live in areas where access to health facilities is poor. Visceral leishmaniasis is characterized by prolonged fever, enlarged spleen and liver, substantial weight loss, progressive anaemia, and is complicated by co-infection with other infectious diseases, such as HIV, TB or malaria.

Current first-line treatment options include pentavalent antimonials, which were first used as treatments in the 1940s. However, there are increasing concerns about toxicity, their difficulty of use, as they require the patient to be hospitalized for a 28-day treatment period and the potential emergence of drug resistant parasite (as now seen on the Indian subcontinent). Although very effective, miltefosine, the one of two drugs developed for visceral leishmaniasis in this millenium, is prohibitively expensive and therefore has limited use. Paromomycin has been recently registered for leishmaniasis.

For visceral leishmaniasis, which infects over 500,000 a year, a drug is needed which is safe, affordable, and available as oral short-course (10 days) for all forms of the disease.

4. Screening process

4a. R&D process

The Research & Development pathway from the early discovery stages to the final registration of a drug is described in Figure 2. The screening activities take place at the beginning of this process (second box from the left) following the Basic Science section. Different types of screening can be run taking into consideration the defined screening strategy, the screening models available/envisaged for development as well as the existing knowledge in terms of the known/putative target of interest. The screening can be performed on sub-cellular (e.g. enzymatic or chemical), cellular or supra-cellular models (e.g. tissue or whole animal) using various technical capacities and schemes. This manual will only include low to medium throughput phenotypic screening assays available for Human African Trypanosomiasis and Leishmaniasis. Working as a network, it is important to make sure that methodologies and protocols of screening assays are harmonized between the different institutions of the network. This will enable to compare results generated in different labs and facilitate collaboration and understanding among groups.

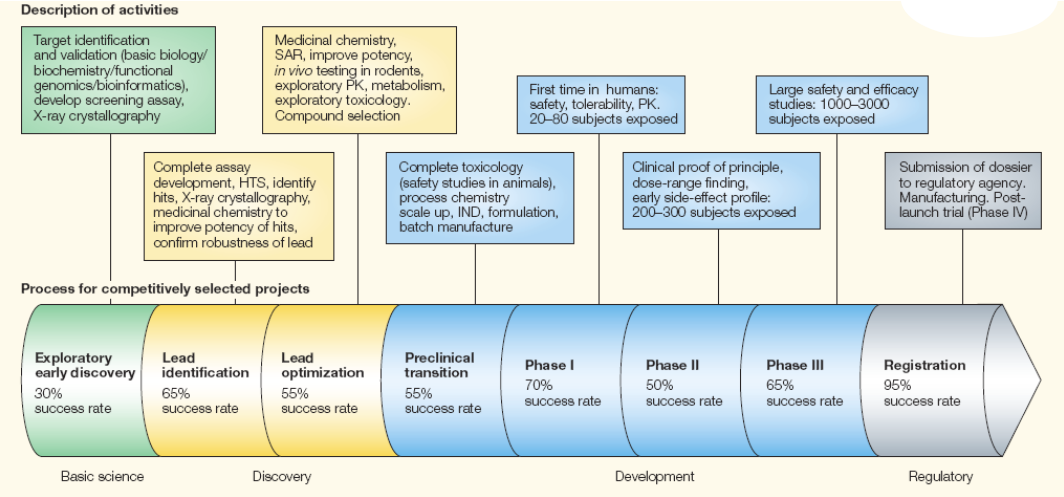

Figure 2. R&D process and success rates of the different stages (Nwaka and Ridley, Nature Reviews Drug Discovery, 2, 919, 2003)

The aim of screening is to identify hits to be evaluated in order to enter Drug Development programmes. Such programmes can typically be sequenced by the following cascade: Hit selection - Hit Expansion - Lead optimization - Clinical Candidate selection. For further details on this process, refer to point 4c.

The criteria of hit selection set by DND*i* is provided below as an example of limited requirements to be reached in order to proceed with the next stage of development towards a drug for Human African Trypanosomiasis and Visceral Leishmaniasis (see Table 1). This document is part of the Project Progression Manual developed by DNDi for Human African Trypanosomiasis and Visceral Leishmaniasis. It is quite important to add that the cut-off values found in this document have been arbitrarily defined and may be reviewed depending on the nature of the screened samples. Mixtures of compounds such as plant extracts and pure compounds should for instance not be considered using the same cut-off values. Also, focused libraries of compounds known or expected to be active against protozoan targets will most probably have to be considered with different (more stringent) IC_{50} values than samples screened randomly.

Table 1. Criteria of hit selection set by DNDi for Human African Trypanosomiasis and Visceral Leishmaniasis.

Hit Selection for Human African Trypanosomiasis and Visceral Leishmaniasis	
Key Activity	Target / Expectation
Efficacy – Whole cell	
Activity against *T.b.brucei* or activity against *L. donovani* amastigotes (in macrophages)	IC50 ≤ 10μM
Selectivity	≥ 10 fold more active against the parasite vs. the mammalian cell line
Mode of action (for hits emerging from a biochemical assay)	Ensure biochemical and whole cell activity correlate
DMPK (*in silico*)	
Lipinski's Rules	These may be used to prioritise hits but should not be used as definitive selection criteria (drug-likeness preferable but not essential)
Structural alerts (metabolism/stability/ reactivity)	Proceed with caution and assay asap to determine extent of perceived problem
Toxicity	
Structural alerts	Proceed with caution and assay to determine extent of perceived problem
Chemical Profile	
Functional activity confirmed on purified/resynthesised compounds	
Chemical Tractability	Acceptable synthetic pathway for compound and/or analogues (<8 steps)
Natural Products	Chemically defined material necessary
Regrowth of broths	Need to obtain sufficient pure material to allow comprehensive characterisation (preferably 100mg+)
Purification/Synthesis	Synthesis depends on complexity of molecule including presence of chiral centres
Freedom to operate	
I.P. Status	Determine status and negotiate license to pursue compounds if patent protected

The Target Product Profiles of the final drugs to be developed for Human African Trypanosomiasis (Tables 2 and 3) and Visceral Leishmaniasis (Table 4) as part of DNDi's mission are also provided on an informative basis. Please note these documents may be subjected to future modifications.

Table 2. Target Product Profile for Human African Trypanosomiasis (stage 1+2 treatment)

1st priority: a safe, effective and practical stage 2 HAT treatment

– To replace current first line treatments
– To improve and simplify the current case management
– Preferably one and the same drug for both stage 1 and 2

Desirable	**Acceptable: improvement to current Standard Treatments**
Effective against stage 1 and 2	Effective against stage 2
Broad Spectrum (*T.b. gambiense* and *T.b. rhodesiense*)	Efficacy against *T.b. gambiense* only
Clinical efficacy > 95% at 18 months follow up	Clinical efficacy no worse than current treatments
Effective in melarsoprol refractory patients	Effective in melarsoprol refractory patients
Safe during pregnancy and for lactating women	Safe during pregnancy and for lactating women
<0.1% drug related Mortality	1% drug related mortality
Formulation adapted to adults and children	
No monitoring for adverse effects	Weekly simple lab testing (field testing)
< 7 days p.o. once daily (DOT)	< 20 days p.o. (DOT)
< 7 days i.m. once daily	< 20 days i.m.
	< 5 days i.v. if no toxicity
Stability in zone 4 for > 3 years	Stability in zone 4 for > 12 months
Cidal	
Multitarget	Unique target (but not uptake via P2-transporter only)
< 30 € / course* (only drug cost)	< 100 €* / course
	< 200 €* / course ok if very good on other criteria

*It is not expected that patients pay this, rather donor agencies. Considering that per year some 20-50,000 patients might require treatment, this is still realistic

2nd priority: a simple stage 1 HAT treatment

– To increase access to treatment and coverage of HAT cases,
– To be used at the local health centre level
– Is dependent on the availability of a simple diagnostic field test

Table 3. Target Product Profile for Human African Trypanosomiasis (stage 1 only): mass screening + treatment campaigns*

	Desirable	Acceptable: improvement to current Standard Treatments
Target Label	Effective against stage 1 and 2	Effective against stage 1
Species	Broad spectrum (*T.b. gambiense* and *T.b. rhodesiense*)	Efficacy against *T.b. gambiense* only
Clinical Efficacy	Clinical efficacy > 95% at 18 months follow up	Clinical efficacy no worse than current treatments
Safety and Tolerability	No monitoring for adverse effects; 0% drug related mortality	No monitoring for adverse effects; 0% drug related mortality
Contraindications	Safe during pregnancy and for lactating women	Safe during pregnancy and for lactating women
Formulation	Formulation adapted to adults and children	Formulation adapted to adults and children
Treatment Regimen	Single dose p.o. or i.m. < 7 days i.m. once daily	2-3 daily doses p.o. or i.m.
Stability	Stability in zone 4 for > 3 years	Stability in zone 4 for > 12 months
Mechanism of action	Multitarget; Cidal	1 target but resistance not readily inducible; Cidal
Cost of drug	< 10 € / course* (only drug cost)	< 30 €* / course

* Relies on availability of easy field diagnostic test

Table 4. Target Product Profile for Visceral Leishmaniasis

	Desirable	Acceptable: improvement to current Standard Treatments
Target Label	VL and PKDL	VL
Species	All species	*L. donovani* (covers most endemic areas)
Distribution	All areas	Either India or Africa
Target Population	Immunocompetent and immunosuppressed Adults and children	Immunocompetent Children
Clinical Efficacy	> 95%	> 90%
Resistance	Active against resistant strains	Not active against resistant strains
Safety and Tolerability	No adverse effects requiring monitoring	1 monitoring visit in mid-point
Contraindications	None	Pregnancy/lactation
Interactions	None - Compatible for combination therapy	None that prevent appropriate anti-HIV, Malarial or TB therapy
Formulation	Oral / im depot	Oral / im / iv

Treatment Regimen	Max 1/day for 10 days po or 3 shots over 10 days	2/day≤21days or 1 shot/day over 10 days
Stability	3 years in zone 4	2 years in zone 4
Cost of drug	< $10 / course (20$ in 7 years)	< $50 / course

4b. Screening algorithm

A simple screening algorithm developed by the Swiss Tropical Institute and mostly based on efficacy and selectivity criteria is proposed below (see Figure 3). The values of selection points (cut-offs) can be adjusted to the group of samples to be tested (e.g. random screening vs. screening of chemical analogues). The main issue is to get good activity discrimination. For unknown, crude extracts the cut-off values can be less stringent (e.g. >50% inhibition at 20μg/ml or >30% inhibition at 10 μg/ml). Only the most active samples should advance to the next screening model (<15% of the total sample collection, depending on the nature of the samples). If necessary the cut-offs can be tightened. For pure compounds of specific interest or analogues of compounds previously characterized with known activity, the preliminary step of single-concentration screening – known as Medium Throughput Screening or MTS on Figure 3 - can be skipped and their screening started straight with the serial drug dilution assay. Within a lead optimisation program, *in vitro* active compounds can be tested directly in the stringent STIB900 acute mouse model (STIB795 model omitted).

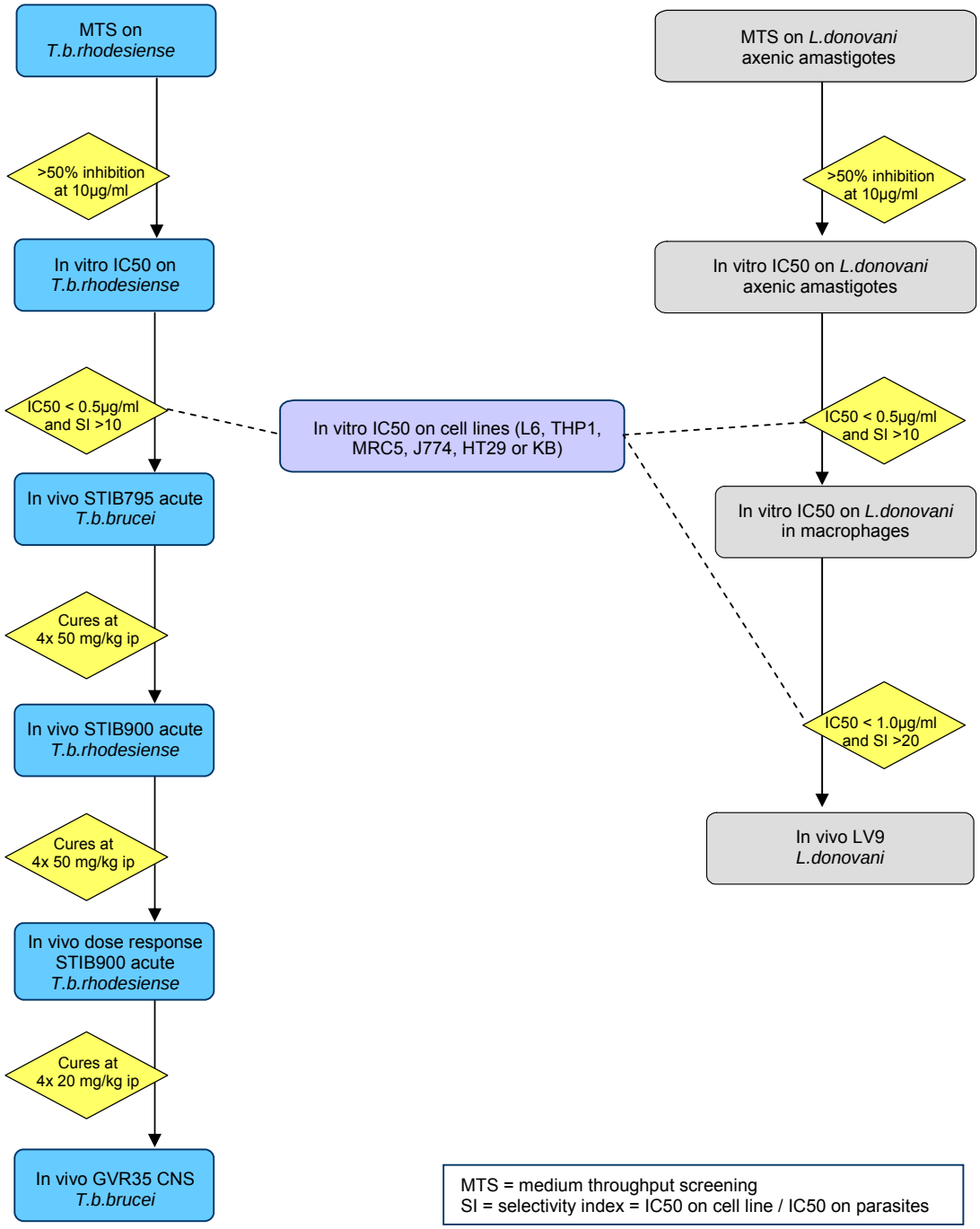

Figure 3. Screening algorithm for use in the frame of the Pan-Asian Network

4c. Drug Development programme: From Hit to Drug Candidate

The preclinical Drug Development from a hit to a drug Candidate (Hit selection - Hit Expansion - Lead optimization - Drug Candidate selection) is a complex, multidisciplinary process that will not be detailed in the frame of the manual.

The preclinical Drug Development activities will be assisted by a Project Progression Manual that will set key activities, timeframes, decision points, Values for Project Progression for a defined Target Product Profile. Decision matrices that include ideal and acceptable values together with values for comparator drugs will be used to facilitate assessment and review of chemical entities of interest with respect to the Target Product Profile and current drugs.

Human African Trypanosomiasis
A Human African Trypanosomiasis Project Progression Manual and associated decision matrices have been designed by DNDi for HAT. It can be obtained from DND*i* upon request. To proceed, please contact jrioset@dndi.org).

Leishmaniasis
A Leishmaniasis Project Progression Manual and associated decision matrices have been designed by DNDi for VL. It can be obtained from DND*i* upon request. To proceed, please contact jrioset@dndi.org).

5. Assays

To ensure that the assays will be conducted in a same and standardized way within the network it has been decided to opt for one specific protocol for each of the listed assay. Most of these assays have been developed, validated and are routinely used over several years for drug screening purposes by the Parasite Chemotherapy Group (Prof Reto Brun) at the Swiss Tropical Institute (Basel, Switzerland). Dr Vanessa Yardley from the Department of Infectious and Tropical Diseases (London School of Hygiene and Tropical Medicine) has complemented the described *Leishmania* assays with her long-standing expertise in the field.

The assays listed below are shortly described in terms of experimental under sections 5a (Human African Trypanosomiasis) and 5b (Leishmaniasis). The associated Standard Operating Procedures (SOPs) are gathered in Chapter 9. (Appendices) together with specific recommendations that aim to provide useful practical information in order to conduct such assays.

Main assays

The *in vitro* assays described below are growth inhibition tests in the presence of a serial dilution of samples (pure compound or mixture) with IC_{50} determination. This can be achieved by dilution of the sample in a medium suitable to support growth of the parasite, incubating the parasites for at least 2-3 generations and determining growth inhibition. For totally unknown natural products, it is advisable to test the samples first at only 1 or 2 concentrations (e.g. 2μg/ml and 10μg/ml). This Medium Throughput Screening (MTS) assay allows screening in a large scale of 88 or 44 compounds per plate (including control wells).

5a. Human African Trypanosomiasis

5a.1. *In vitro* assays (whole cell)

Primary screen

Trypanosoma brucei spp. strains are used in this study. *T.b. rhodesiense* STIB 900**,** a clone of a population isolated in 1982 from a patient in Tanzania and known to be susceptible to all currently used drugs is used for the primary *in vitro* screening.

The bloodstream form trypomastigotes are maintained in MEM medium with Earle's salts supplemented with 25 mM HEPES, 1g/l additional glucose, 10ml/l MEM non-essential amino acids (100x), 0.2 mM 2-mercaptoethanol, 1mM Na-pyruvate, 0.5mM hypoxanthine and 15% heat inactivated horse serum (See 9. Appendices).

All cultures and assays are conducted at 37°C under a humidified atmosphere of 5% CO_2 in air.

Drug sensitivity assays

Stock drug solutions are prepared in 100% dimethylsulfoxide (DMSO) at 10 mg/ml, and heated or sonicated if necessary. The stocks are kept at –20°C. For the assays, the compound is further diluted to the appropriate concentration using complete culture medium.
Assays are performed in sterile 96-well flat-bottom microtiter plates, each well containing 100 μl of parasite culture (2 x 10^3 bloodstream forms) with or without serial drug dilutions. The highest concentration for the test compounds is 90 μg/ml. Each drug is tested in duplicate. After 70 hours of incubation the plates are inspected under an inverted microscope to assure good growth and motility of the controls. 10μl of Alamar Blue® (12.5mg resazurin dissolved in 100ml ddH_2O) are added to each well and the plates incubated for another 2-5 hours. Then the plates are read with a fluorescence plate reader using an excitation wave length of 536 nm and an emission wave length of 588 nm (optional, filter set 530nm/590nm). Data are transferred into a graphic software (e.g. Excel®, Prism®, SoftmaxPro®), sigmoidal inhibition curves

determined (e.g. by 4-parameter model) and IC_{50} values calculated (SOP see 9. Appendices).

In the primary screen with *T.b. rhodesiense* strain STIB900, the compounds are tested at 7 concentrations (drug concentration range from 90 μg/ml to 0.123 μg/ml in 3-fold dilutions). For active compounds the dilutions are adjusted to lower concentrations in further assays.

If the IC_{50} is >5μg/ml, the compound is classified as inactive
If the IC_{50} is 0.5-5μg/ml, the compound is designated as moderately active
If the IC_{50} is <0.5μg/ml, the compound is classified as active

The standard drug is melarsoprol, which is run in the same assay; the IC_{50} for melarsoprol is 2.1 ng/ml. Optionally, a 2nd standard drug is used e.g. pentamidine, suramin.

Secondary screen

Active compounds (IC_{50} <0.5 μg/ml and *in vivo* efficacy) are tested against different trypanosome strains to gather additional information following the same protocol as described above. For some strains, minor modifications are necessary as initial trypanosome inoculum size or medium supplementation according to Baltz or Hirumi. Please note, *T.b. gambiense* requires human serum (15% human serum and (optional) 5% FCS).

Other trypanosomes of interest for screening
T. b. brucei STIB 950, a clone of a population isolated in 1985 from a bovine in Somalia is resistant to diminazene, isometamidium and quinapyramine (mdr).
T. b. brucei GUTat3.1 (restabilated as STIB764) originally TREU 667
T. b. brucei S427 was isolated from *G. pallidipes* in Uganda 1960 (derivatives are, BS221, STIB795).
T.b. gambiense STIB 754, a derivative of strain TH1/78E (031) isolated in 1978 from a patient in Ivory Coast is known to be sensitive to all drugs used.
For certain questions, specific knockout strains can be added to the secondary screening. For example, the AT1KO, a P2 aminopurine transporter knockout strain (of BS221) can be used for drug uptake studies.

5a.2. *In vivo* assays

Parasite and animal strains for acute mouse models

T. b. brucei STIB 795 is a derivative of strain S427 which is highly virulent for mice but easy to cure. *T.b. rhodesiense* STIB 900 is a clone of a population isolated in 1982 from a patient in Tanzania. Although this population is sensitive *in vitro* to all known drugs with comparable IC_{50} values, this mouse model is very difficult to cure. For both strains, female NMRI mice, weighing 20-25g are used and maintained at 22°C and 70% relative humidity. The *T.b.*

gambiense STIB 754 acute mouse model is only rarely used. It is not an easy model as immunodeficient mice (SCID) or immunosuppressed mice (e.g. BALB/c with cyclophosphamide) are required.

General experimental procedure

Assessment of the highest tolerated dose (HTD): The HTD is assessed by applying the compound to a single mouse i.p. or p.o. with increasing doses starting at 20 mg/kg up to max 150 mg/kg within 6 hours. As soon as any toxic symptoms are observed, the administration of more sample is stopped and the total dose recorded (see 9. Appendices).

Infection: The bloodstream forms come from a stock of cryopreserved stabilates containing 10% glycerol. The trypanosomes are suspended in PSG (phosphate-saline-glucose) 6:4 (Lanham & Godfrey, Exp. Parasitol. 28, 521-534, 1970). Each mouse is injected intraperitoneally with a volume of 0.25 ml (trypanosomes concentration depends on the strain) on day 0. Untreated control mice die typically between day 7-10 post-infection. Routinely, 4 mice are used per experimental or control group.

Compound preparation: Compounds are prepared at appropriate concentrations in 100% DMSO and are further diluted in distilled H_2O to a final DMSO concentration of 10%, unless a more appropriate solvent is recommended by the supplier. The total amount of solvent administered has to be below toxicity (10% for DMSO i.p.).

Evaluation endpoint:
Three different endpoints can be evaluated, the mean relapse day (MRD), the mean survival day (MSD) and the number of cured mice (cured/infected).
After drug treatment, parasitaemia of all animals is checked by tail blood examination on day+7, day+10, then twice a week until day+30 followed by once a week until day+60 post infection. The parasitaemia is recorded based on a subjective scale ranging from (+) = 1 trypanosome/20 fields to +++ = >100 trypanosomes/field with a 200x magnification. Aparasitaemic mice at day +30 (STIB 795) or day +60 (STIB 900) are considered as cured and then euthanized.

All mice dying before day +30 (STIB 795) or day +60 (STIB 900) are recorded, the surviving mice euthanized and the MSD calculated. To reduce the severity code of animal experiments the MRD can be evaluated to replace the MSD. All mice are euthanized once parasitaemia has reappeared and the day of parasitaemia relapse is recorded to calculate the MRD.

5a.2.1. *Trypanosoma b. brucei* STIB 795 in NMRI mice

Treatment regimen:
Compounds are daily administered i.p. or p.o. in a total volume of 10 ml/kg body weight from day+3 to day+6 of the experiment at 50% of the highest tolerated dose (HTD).

Activity criteria:

Not active:	parasitaemia remains
Moderately active:	parasitaemia disappears temporarily
Active:	≥50% of mice aparasitaemic for 30 days

Table 5. STIB 795 *in vivo* activities of melarsoprol and pentamidine

standard drug	**dose mg/kg** days 3-6	**route**	**cured/infected**	**MSD***
Melarsoprol	4x 0.5	i.p.	0/4	>30
	4x 1	i.p.	2/4	>30
	4x 2	i.p.	4/4	>30
Pentamidine isethionate	4x 0.5	i.p.	0/4	>30
	4x 1	i.p.	3/4	>30
	4x 2.5	i.p.	4/4	>30

*MSD: mean survival days post infection

5a.2.2. *Trypanosoma b. rhodesiense* STIB 900 in NMRI mice

Primary screening

Treatment regimen:
Compounds are daily administered i.p. or p.o. in a total volume of 10 ml/kg body weight from day+3 to day+6 of the experiment at 50% of the highest tolerated dose (HTD).

Activity criteria:

Not active:	parasitaemia remains
Moderately active:	parasitaemia disappears temporarily
Active:	≥50% of mice aparasitaemic for 60 days

Secondary screening

Dose response and minimum curative dose (MCD). The MCD is determined by lowering the daily doses of the 4 day treatment schedule (dose response). Size of the groups and activity criteria are the same as above.

Table 6. STIB 900 *in vivo* activities of melarsoprol and pentamidine

standard drug	dose mg/kg days 3-6	route	cured/infected	MSD*
Melarsoprol	4x 0.5	i.p.	0/4	19.5
	4x 1	i.p.	2/4	>51.5
	4x 2	i.p.	4/4	>60
Pentamidine isethionate	4x 5	i.p.	1/4	>38
	4x 20	i.p.	2/4	>57.5

*MSD: mean survival days post infection

Within the secondary screening alternative dosing regimes, single applications and different routes of application (oral vs parenteral, i.p. vs i.v.) can be studied.

5a.2.3. *Trypanosoma b. gambiense* STIB 754 in SCID mice

Females SCID (severe combined immunodeficient) mice approximately 4 weeks old and weighing >15 g are used. The untreated control mice normally die between day 20 and 30 post-infection and the monitoring period is extended to 90 days. Alternatively, immunosuppressed Balb/c mice can be used. They are injected with 200 mg/kg cyclophosphamide 3 days before infection and on day 4, 11 and so on post-infection.

Treatment regimen:
Compounds are daily administered on 4 consecutive days i.p. or p.o. in a total volume of 10 ml/kg of body weight from day +8 to day+11 of the experiment at 50% of the highest tolerated dose (HTD).

Activity criteria:

Not active:	parasitaemia remains
Moderately active:	parasitaemia disappears temporarily
Active:	≥50% mice aparasitaemic for 90 days

5a.2.4. Parasite and animal strains for CNS mouse model

T. b. brucei GVR 35 leads to a CNS infection in mice before day 21 post-infection (Jennings and Gray, 1983). Female NMRI mice, weighing 20-25g are used and maintained at 22°C and 70% relative humidity for the CNS mouse model.

General experimental procedure

Infection: The bloodstream forms come from a stock of cryopreserved stabilates containing 10% glycerol. The stabilate is thawed rapidly and suspended in PSG (phosphate-saline-glucose) 6:4 (Lanham & Godfrey, Exp. Parasitol. 28, 521-534, 1970) to obtain a trypanosome concentration of 8 x 10^4/ml. All mice are infected intraperitoneally with 0.25 ml (equivalent to 2x10^4

tryps) on day 0. Control mice treated with 1 x 40mg/kg ip diminazene aceturate first clear parasitaemia, later relapse and die between day 40-60 post-infection. Routinely, 5 mice are used per experimental or control group.

Treatment regimen:
Compounds are daily administered i.p. or p.o. in a volume of 10 ml/kg of body weight from day+21 to day+25 or alternatively from day+21 to day+30 post infection at 50% of the highest tolerated dose (HTD).

Evaluation endpoint: The parasitaemia of all animals is checked by tail blood examination on day+28 (or +32 for a 10-day treatment) until day 60 twice a week followed by once a week until day+180. The parasitaemia is recorded based on a subjective scale ranging from (+) = 1 trypanosome/20 fields to +++ =>100 trypanosomes/field with a 200x magnification. After detection of parasitaemia, mice are euthanized and the day of parasitaemia relapse recorded to calculate the mean relapse day (MRD) or the mice are kept alive until their death to calculate the mean survival day (MSD). At day+180 aparasitaemic mice are considered as cured and then euthanized.

Activity criteria:

Not active:	relapses <20 days later than the MRD of diminazene control
Moderately active:	relapses >20 days later than the MRD of diminazene control but < 40% of mice aparasitaemic by day +180
Active:	≥ 60% of the animals aparasitaemic until day +180

5b. Leishmaniasis

5b.1. *In vitro* assays (whole cell)

5b.1.1. *Leishmania donovani* MHOM/ET/67/HU3 axenic form

Parasite and cell cultures
The *Leishmania donovani* strain MHOM/ET/67/HU3 (obtained from Dr. S. Croft, London School of Hygiene and Tropical Medicine) is used. The strain is maintained in the Syrian Golden hamster or RAG1.B6 mice. Amastigotes are collected from the spleen of an infected animal. Amastigotes are grown in axenic culture at 37°C in SM medium (Cunningham I., *J. Protozool*. 24, 325-329, 1977) at pH 5.4 supplemented with 10% heat-inactivated fetal calf serum (FCS) under an atmosphere of 5% CO_2 in air.

Drug sensitivity assays
Stock drug solutions are prepared in 100% dimethylsulphoxide (DMSO) unless otherwise suggested by the supplier at 10 mg/ml, and heated or sonicated if necessary to dissolve the sample. After use the stocks are kept at -20°C. For the assays, the compound is further diluted to the appropriate

concentration using complete medium. The DMSO concentration in the wells with the highest drug concentration should not exceed 1%.

Assays are performed in 96-well flat-bottom microtiter plates (Costar, Corning Inc.), each well containing 100 μl of culture medium with 10^5 amastigotes from axenic culture with or without a serial drug dilution. Before the amastigotes are counted in a CASY cell analyzing system, the parasite culture is passed twice through a 22 gauge needle to break up clusters of amastigotes. In the serial drug dilution assay, seven 3-fold dilutions are used, covering a range from 90 μg/ml to 0.123 μg/ml. Each drug is tested in duplicate. Active or moderately active compounds are tested twice for confirmation. After 70 hours of incubation, the plates are inspected under an inverted microscope to assure growth of the controls and sterile conditions. A volume of 10 μl of Alamar Blue® (12.5 mg resazurin dissolved in 100 ml distilled water) is then added to each well and the plates are incubated for another 2-5 hours. Then the plates are read with a microplate fluorometer using an excitation wave length of 536 nm and an emission wave length of 588 nm. Data are analyzed using appropriate microplate reader software. Decrease of fluorescence (i.e. inhibition) is expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. The IC_{50} value is calculated from the sigmoidal inhibition curve by the software program.

Primary screening

The compounds are tested in duplicate at seven concentrations (drug concentrations ranging from 90 μg/ml to 0.123 μg/ml in 3-fold dilutions).
If the IC_{50} is >5 μg/ml, the compound is classified as inactive.
If the IC_{50} is 0.5 - 5 μg/ml, the compound is classified as moderately active.
If the IC_{50} is <0.5 μg/ml, the compound is classified as active.

Miltefosine is used as the reference drug and shows an IC_{50} value of 0.175 μg/ml.

Secondary screening

Active compounds are tested in a macrophage assay infected with intracellular amastigotes (see below point 5.b.1.2.).

5b.1.2. *Leishmania donovani* MHOM/ET/67/HU3 intracellular form

The assay with *Leishmania donovani* intracellular forms can be performed in different ways using different parasite forms and different host cells. Three parasite forms can in principle be used: amastigote forms from axenic culture, amastigotes isolated from the spleen of an infected hamster or promastigotes from 27°C culture. Infective (metacyclic) promastigotes only appear in late stationary phase in culture after several days without medium change. Amastigote forms result in higher and more reproducible infections.

As host cells primary macrophages from mouse or hamster can be used as well as macrophage-like cell lines (e.g. P388D, J744). The latter have the advantage of being available as continuous culture with the drawback of

undergoing cell division which leads to a distribution of the parasites among the daughter cells. In principle, any combination of a parasite form with a host cell can be established in to a workable system.

a) *Leishmania donovani* MHOM/ET/67/HU3 intracellular form (infection using parasite and host cell collected from animal)

Parasite and cell cultures:

Leishmania donovani MHOM/ET/67/HU3 is used. The strain is maintained in the hamster or RAG1.B6 mouse. Amastigotes are collected from the spleen of an infected animal and spleen parasite burden is assessed using the Stauber technique or amastigotes are counted in a Thoma™ counting chamber after 1:100 dilutions in Trypan blue. Primary peritoneal mouse (CD1 or BALB/c) macrophages are collected 1 day after macrophage production stimulation with an i.p injection of 2ml of 2% starch in distilled water. All cultures and assays are conducted at 37°C under an atmosphere of 5% $C0_2$.

Drug sensitivity assays

Compound stock solutions of 20 mg/ml compound stock solutions are prepared as advised by the supplier, otherwise dissolved in 100% DMSO. Stock solutions are kept at –20°C. The compounds are pre-diluted to 1mg/ml. Assays are performed in sterile 16-well tissue culture slides, each well containing 100 μl of the compound dilutions together with 100 μl of macrophage/parasite inoculum ($4x10^5$ macrophages/ml and $1.2x10^6$ parasites/ml). The inoculum is prepared in RPMI-1640 medium supplemented with 10% heat inactivated FCS. After 96 hours of drug incubation, parasite growth is microscopically assessed after staining the cells with a 10% Giemsa solution. The results are expressed as % reduction in parasite burden compared to control wells (100% parasite growth) and the IC_{50} calculated by linear regression analysis.

The compounds are tested in duplicate at 2 concentrations (30, 10 μg/ml).

If the IC_{50} is >10μg/ml, the compound is classified as inactive
If the IC_{50} is 3-10μg/ml, the compound is designated as moderately active
If the IC_{50} is <3μg/ml, the compound is classified as active and further evaluated with an extended dose range adjusted as appropriate.

The standard drug is miltefosine, run in the same assay; its IC_{50} is 0.33 μg/ml.

b) *Leishmania donovani* MHOM/ET/67/ HU3 intracellular form (infection using promastigotes to infect macrophages)

If a source of *Leishmania* amastigotes in unavailable, cultured promastigotes can be used to infect host cells. *In vitro*, the host cells are often less permissive to promastigote invasion and tend to achieve lower levels of infection after 24 hours and do not sustain an infection for the duration of the assay. If possible, before starting drug sensitivity assays, evaluate different host cells, infection ratio's etc in order to achieve the optimum level of infection. Promastigote infectivity declines with passages (sub-culture) over time. Where possible, primary isolate cultures should be used, namely promastigotes recently derived from a clinical (or animal model) sample. Stationary phase (long, slender) promastigotes being closer to metacyclogenesis, are also more likely to successfully enter the host cells and establish a persistent infection.

Adherent macrophages are infected with late-stage promastigotes at a pre-determined ratio and incubated at 37ºC, 5% CO_2 in air, for 24 hours. After 24 hours the infected cells should be gently washed with cold (4ºC) culture medium w/o serum. The overlay is removed and 100μl of cold medium gently dispensed and withdrawn 2-3 times. This dislodges the majority of any extracellular promastigotes. Finally, 100μl of culture medium with serum is added prior to the administration of drug. At this point a slide is fixed (100% methanol) and stained (10% Giemsa, 10 minutes) to determine the level of infection at 24 hours. To the remaining slides, a 3 fold drug dilutions is added and the cells incubated for 96 hours at 37°C, 5%CO_2. The endpoint is evaluated microscopically after the cells are fixed and Giemsa stained.

5b.2. *In vivo* assays

5b.2.1. *Leishmania donovani* (strain MHOM/ET/67/HU3 in BALB/c mice,

Parasite and animal strains

The sodium stibogluconate sensitive *Leishmania donovani* (strain MHOM/ET/67/HU3, also known as LV9 or L82) is used. Female BALB/c mice (20g), specific pathogen free and Syrian hamsters - *Mesocricetus auratus* - are supplied by accredited suppliers and maintained under Category 3 conditions.

General experimental procedure

Infection: *L. donovani* amastigotes are isolated from the spleen of a heavily infected donor animal. Parasite burden is assessed on a Giemsa stained spleen impression smear using the Stauber method [J. Protozool. 5(4), 269-273 (1958)] and by Thoma™ counting chamber. An inoculum containing 1.0 x 10^8 amastigotes/ml in RPMI1640 is prepared. Animals are infected intravenously (tail vein) with a 0.2 ml bolus (equivalent to 2.0 x 10^7 amastigotes) on day 0. Infected mice are randomly assorted in to groups of

five. On day 7 post infection one mouse is sacrificed, liver smears taken, methanol fixed and Giemsa stained and infection assessed.

Evaluation endpoint: On day 14 post-infection of methanol fixed, 10% Giemsa-stained liver impressions smear for determination of the total parasite burden.

Primary screening

Drug is prepared in 10% DMSO/PBS. Insoluble compounds are sonicated and/or ball-milled to reduce particle size.

Mice are treated intraperitoneally once a day for 5 days on days 7-11 of infection (5 animals/group) with 50 mg/kg/day (unless previous toxicity data indicate that a lower dose is preferable). One group of 5 mice is treated with the drug vehicle only; one other group of 5 mice is treated with 15 mg/kg sodium stibogluconate (SbV) on 5 consecutive days by the s.c route. Groups of mice are weighed before and after treatment and the % weight change noted. On day 14 post-infection, three days after the completion of treatment, parasite burdens are determined microscopically on Giemsa-stained liver smears. The number of amastigotes/500 liver cells is counted microscopically (5x100, oil immersion). The results are expressed as Leishman Donovan Units (LDU): mean number of amastigotes per liver cell x mg liver [Bradley & Kirkley, Clin. Exp. Immunol. (1977), 30, 119-129].The LDU of drug treated groups is compared with that of the untreated group and the % inhibition calculated.
For the average level of infection, the standard drug treatment of 15mg SbV/kg s.c. x 5 days (Pentostam®) should give around 50% reduction of liver parasite load.

Secondary screening

Compounds with good activity in the primary screen are further evaluated in a secondary evaluation .The compounds and the standard drug SbV are repeated over the appropriate range at least over 3 dose levels in a 3 fold dilution series.(45, 15 and 5 mg/kg for the standard). Data are analysed by Microsoft Excel Fit or a similar software and ED_{50} and ED_{90} (with 95% confidence limits) determined.

5c. Cytotoxicity assays

Cell cultures

The Swiss Tropical Institute uses the L-6 cell line (rat skeletal myoblasts) for cytotoxicity determination. The L-6 cells are also used as host cells for *Trypanosoma cruzi*. The cells are grown in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum in T-25 tissue culture flasks at 37°C in 5% CO_2 in air. The cultures are subpassaged 2-3 times a

week, using trypsin to detach the cells, and split in a 1:3 or 1:10 ratio, depending on the density of the parent culture. Aliquots are cryopreserved at a low passage number to act as a stock.

Drug sensitivity assays

Stock drug solutions are prepared in 100% dimethylsulphoxide (DMSO) at 10 mg/ml, and heated or sonicated if necessary to dissolve the sample. After use the stocks are kept at –20°C. For the assays, the compound is further diluted to the appropriate concentration using complete medium. The DMSO concentration in the wells with the highest drug concentration should not exceed 1%.

Assays are performed in 96-well microtiter plates, each well receiving 100 μl of complete medium with 4 x 10^3 cells. After 24 hours, the medium is removed from row H and replaced with fresh medium (150 μl) containing the highest drug concentration. Serial drug dilutions are prepared by transferring 50 μl from wells of row H to wells of row G. After gentle mixing 50 μl from row G are transferred to row F, and so on. The highest concentration for the test compounds is 90 μg/ml. Seven 3-fold dilutions are used, covering a range from 90 μg/ml to 0.123 μg/ml. Each drug is tested in duplicate. After 72 hours of incubation the plates are inspected under an inverted microscope to assure growth of the controls and sterile conditions. Then 10 μl of Alamar Blue® (12.5 mg resazurin dissolved in 100 ml distilled water) are added to each well and the plates are incubated for another 1.5- 2 hours. Then the plates are read with a fluorescence plate reader using an excitation wave length of 536 nm and an emission wave length of 588 nm. The IC_{50} values are determined using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA) or a similar software (SOP see 9. Appendices).

As an alternative, the endpoint can be determined with the use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and formazan formation. In a first step a 5 mg/ml stock solution of MTT is prepared in distilled sterile water. At the end of the 72 hour incubation period of the mammalian cells with the serial drug dilution, 25 μl MTT are added to each well and the plate incubated for 2 hours at 37°C. Then the supernatant is removed and 150 μl/well DMSO is added. The plate is put on a shaker for 15 min and then read for absorbance at 544 nm.

The MTT assay is more labour-intensive as compared to the Alamar Blue® assay. However, as the drug is removed from the cells no extra blank is needed enabling to test 6 instead of 4 compounds per plate, and a photometer is sufficient to read the assay (fluorescence scanner for the Alamar Blue® assay).

Activity criteria:
If the IC_{50} is ≥ 90 μg/ml, the compound is classified as not cytotoxic
If the IC_{50} is 2 - 89 μg/ml, the compound is classified as moderately cytotoxic
If the IC_{50} is < 2 μg/ml, the compound is classified as cytotoxic

With the IC_{50} values for the mammalian cells and for a given parasite, a selectivity index (SI) can be calculated:

$$SI = \frac{IC_{50} \text{ for mammalian cell line}}{IC_{50} \text{ for protozoan parasite}}$$

The standard drug is podophyllotoxin which is run in the same assay; the IC_{50} for L-6 cells is 6-8 ng/ml.
For compounds that showed activity against a protozoan parasites and no cytotoxicity on L-6 cells, additional human and animal cell lines can be used to obtain a more complete picture of cytotoxicity. Such cell lines are tested using the same protocol. Slight adaptations regarding culture medium, the seeding density and test duration according to the growth behavior of the cell line, incubation time in resazurin, etc. have to be made as required.
Suggestions of additional cell lines to be employed for cytotoxicity determination:

MRC-5, fetal human lung fibroblast; MEM with 15% FBS
THP-1, human acute monocytic leukemia cell line; RPMI 1640 with 10% FBS
HT-29, human bladder carcinoma cell line; MEM with 10% FBS
Vero, African green monkey kidney epithelial cells; MEM with 5% FBS
KB, human epithelial carcinoma cell line; MEM with 10% FBS
J774, mouse macrophage derived cell line; RPMI 1640 with 10% FBS

5d. Validation of assays

Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement in a given biological matrix is reliable and reproducible for the intended use. The fundamental parameters for this validation include (1) accuracy, (2) precision, (3) selectivity, (4) sensitivity, (5) reproducibility, and (6) stability. Robustness of assays should also be considered as a key point. Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended applications. The acceptability of data corresponds directly to the criteria used to validate the method. Biological assays can be approached from the same standpoint as physical or chemical assays, as long as the inherent variability of biological systems is taken into account.

The validity of a given developed method/bioassay should be established and verified by laboratory studies, and documentation of successful completion of such studies should be provided in the assay validation report. General and

specific SOPs and good record keeping are an essential part of the validation process. The following structure can be used for this purpose (1) summary information, (2) method development and establishment, (3) reports of the application of any methods to routine sample analysis, and (4) other information applicable to method development and establishment and/or to routine sample analysis.

The general FDA guidelines for the Bioanalytical Method Validation can be used as a basis document for the validation of bioassays (see Guidance for Industry: Bioanalytical Method Validation. US Food and Drug Administration web site).

The International BioPharma website provides a useful and critical piece of information based on the aforementioned guidelines.

The Eli Lilly Assay Guidance Manual provides a comprehensive practical support for reviewing the different parameters during the process of validation.

5e. Quality control

Each *in vitro* assay is inspected visually under the microscope for viability and density of the parasites, for solubility of the compounds and for contaminations. The assays should be performed according to the SOP. The quality of each *in vitro* Alamar Blue® assay can be assessed by the IC_{50} of a corresponding standard drug, the signal/background ratio of the control wells and the time of resazurin conversion. Parameters and the expected corresponding values of the antitrypanosomal *in vitro* assay against STIB900 are summarized as example in Table 7. For each parameter, a range has to be given. The limits have to be set up to ensure data reproducibility but in a way that most assays are able to fulfil the conditions. The standard drug activity can vary around the mean activity +100% / -50%.

Table 7. Typical values of parameters considered for quality control

Assay	STIB900	range
signal positive control (RFU)	5000	>4000
signal negative control (RFU)	500	<600
positive/negative	10	>8
time of Resazurin conversion (h)	4	<6
IC50 Melarsoprol (ng/ml)	2.2	1.1 - 4.4
IC50 Pentamidine (ng/ml)	1.9	1.0 - 3.8

Slight discrepancies of the individual parameters between different laboratories are acceptable. The activity of the experimental samples has to be seen in relation to the standard drug. If in one laboratory, the IC_{50} of the standard drug is slightly but reproducibly different e.g. higher, it is expected that the IC_{50} of the experimental sample is slightly higher as well. Each unknown sample should be tested at least twice and once more retested if the IC_{50} values differ by more than 3 times. Quality control relates also to the

parasite. The strain characteristics can be maintained as described in chapter 5f.4. (Maintenance of strain characteristics). The quality of the various reagents/products necessary to run a specific assay have also to fit with the defined standards of quality for such products. This can be ensured on a practical way by adhering to expiration dates of such products as well as by complying with the use and storage recommendations provided by the supplier.

5f. Supporting material and methods

5f.1. Storage of compounds/extracts/fractions

Powder compounds: Synthetic compounds are usually stable if stored in the dark in a dry and cool place. It is advisable to store all samples and especially mixtures from natural origin such as extracts and fractions at -20°C. The containers should be air-tight to avoid binding of water and excessive contact with oxygen.

DMSO stock solutions: Stock solutions in pure DMSO (e.g. at 10 mg/ml) can be stored at room temperature provided they are air-tight. For long term storage -20°C is advisable (make sure the sample is properly mixed after thawing and the compound is back in solution).

Solutions in water or culture medium: Synthetic compounds or natural products in solution in water or in culture medium (without serum) are usually not stable at room temperature and may not be stable at 4°C. They should therefore be stored at -20°C and not submitted to repetitive freeze-thawing unless it is known these compounds (e.g. a standard drug) are not affected by the process. Compounds in a solution of culture medium containing serum should be prepared freshly prior to each assay and should not be stored.

For *in vitro* assays as well as for animal experiments the compound solutions should be prepared freshly before use. For multiple administrations it can be considered to prepare the compound solution every second day and store it in between in the refrigerator. Longer storage than 24 hours is not recommended unless it is known that a compound is stable in solution in a given vehicle.

5f.2. Preparation of stock solutions of standards and samples to be tested

In vitro solutions:
Pure compound or extract solutions are prepared as advised by the supplier. If not specified use DMSO. Prepare stock solutions of 10 mg/ml in 100% DMSO and keep aliquots at -20°C. If needed prepare also lower concentrated solutions in DMSO by 1:10 dilution steps. Since DMSO is toxic to cells, care has to be taken not to exceed a final concentration of 1% DMSO (or 1%

ethanol) in the assay. Routinely start with 90μg/ml and adjust to lower starting concentration in further assays if the compound shows activity.

In analogy, most standard drugs are dissolved in 100% DMSO as 10 mg/ml stock solutions.

Pentamidine isethionate can be obtained from Sigma-Aldrich (order number P0547) and is soluble in DMSO.
Suramin (Germanin®/Germanine®) is produced by BAYER. Stock solutions can be prepared in H_2O or DMSO.
Diminazene aceturate can be obtained from Sigma-Aldrich (order number D7770) and is soluble in DMSO.
Miltefosine and amphotericin B are used for the antileishmania screening and dissolved in DMSO.
The preparation of melarsoprol stock solution is different due to solubility issues. Use Arsobal® which is an already prepared solution of melarsoprol (36mg/ml) in propylene glycol. Add 10 μl of Arsobal® to 5 ml of sterile water and store in 100 μl aliquots at -20°C (72 μg/ml). For the trypanosome assay, dilute this stock solution 1:10 (i.e. 10 μl melarsoprol stock + 90 μl culture medium). Further dilute 1:50 (i.e. 5 μl + 250 μl culture medium) which gives you a final drug starting concentration of 72 ng/ml (after trypanosome suspension is added 1:1).

Eflornithine (DFMO) and Pentostam (sodium stibogluconate) are less appropriate as standard drugs as both are *in vitro* only moderately active.

Pure compounds can be set up also as mM or μM. However this is impossible when extracts or fractions are screened and for pure compounds this is only possible if the exact molecular weight is known. Additionally, it is simpler to set up the dilutions in mg/ml. During data processing, it is advisable, to convert the activity from μg/ml into μM with a known molecular weight and report both.

In vivo vehicles:

10% DMSO
When not soluble in H_2O, dissolve compound first in 100 % DMSO followed by a 10-fold dilution in distilled H_2O to a final concentration of 10 % DMSO.

Tween/EtOH
Dissolve compound in Tween80 / Ethanol (70/30) followed by a 10-fold dilution in distilled H_2O to a final concentration of 7% Tween 80 and 3% Ethanol.

Standard Suspension Vehicle (SSV)
SSV is prepared with 5g Carboxymethyl cellulose, 4ml Tween 80, 5ml Benzyl alcohol and 1000ml NaCl 0.9%. SSV is stable for 3 week at 4°C.

5f.3. Storage of strains and cells

A population of trypanosomes can be cryopreserved alive as a stabilate in liquid nitrogen (or at -80°C for 2-3 years only).

Preparation of cryostabilates of trypanosomatids

16% DMSO is mixed with cell culture medium containing 20% serum. It serves as cryomedium. Chilled cryomedia is added slowly drop by drop to the same volume of a log phase cell culture (< $2x10^6$ trypanosomes/ml) to obtain a final concentration of 8% DMSO. 0.5 ml are transferred into cryo-ampoules. As DMSO harms the cells, cryo-ampoules should be kept on ice. Ampoules are frozen with a cooling device e.g. “Mr Frosty” cryocontainer Nalgen ref 5100-0001 (www.VWR.com) or Handi-Freeze Freezing Tray (Taylor-Wharton) directly in liquid nitrogen.

If using glycerol as cryo-protectant, the final concentration should be 10%. Again, 0.5 ml are transferred into cryo-ampoules and frozen using Handi-Freeze Freezing Tray or “Mr Frosty” at -80°C overnight and then the ampoules transferred to liquid nitrogen storage.

For *Leishmania* amastigotes
After centrifugation at 3100 rpm for 15 min the amastigote pellet is resuspended in 1 ml of fresh medium + 20% HIFCS + 10% glycerol (or 8% DMSO), 0.3 – 0.5 ml filled into cryo-ampoules and frozen as described for the trypanosomes.

Thawing of cryostabilates

The ampoule is quickly thawed in a 37°C waterbath. A small portion of the cell solution is placed into complete growth medium (of mammalian cells the entire volume) to get a final DMSO concentration of max 1%. The remaining cell solution is put in an Eppendorf tube and centrifuged at 800 to 1200 rpm for 10 min (*Leishmania* amastigotes 3100 rpm, 15 min). The supernatant is discarded and the cells resuspended in complete growth medium. Viable cells should be counted. After one day the medium is replaced or the cells subpassaged.

5f.4. Maintenance of strain characteristics

Maintaining a cell line of mammalian cells in culture has an influence on growth characteristics, life expectancy and genetic characteristics through selection and mutations. A change in growth characteristics may change the susceptibility to toxic compounds or may alter its behaviour as a host cell. A cell line with a finite life span will eventually stop growing because the number of generations it can go through is reached. And finally, we have to encounter mutations which also can change the behaviour of our cell line.

To make sure that we are always using the cell line in a comparable state (with the same characteristics) we should discontinue a line after 3-6 months in continuous culture and go back to the same set of cryopreserved samples. This set should be of a low passage number (a finite cell line has a certain no. of generations to go (e.g. 50) before it stops growing) and large enough to last for a few years (e.g. 30 cryostabilates). If a new cell line is acquired from outside, one should make sure to obtain it from a reliable source (e.g. American Type Culture Collection, ATCC) because contamination or mix up of cell lines is a common event.

For trypanosomes and leishmanias the same procedure has to be followed: Going back to a stock of cryostabilates after 3-6 months of continuous culture.

Maintaining trypanosomes and leishmania in rodents usually leads to an increase of virulence. Mutations and selection processes may lead to a change in the phenotype. To avoid this, it is recommended to go back to an original population which is stored as cryostabilates.

5f.5. Media for *Trypanosoma* culture and assays

Two different media (A and B) are proposed as used in different labs for different *T. b. brucei* and *T.b. rhodesiense* strains. Please note, that *T.b. gambiense* requires human serum (15% inactivated human serum and optional plus 5% FCS). All media and sera have to be of validated quality.

Table 8. HMI-18 medium (A)

Constituent	Source	for 500ml	for 1000ml
Modified Iscove's medium	Gibco 42200022	8.84 g	17.68 g
0.05 mM bathocuproine	Sigma B-1125	14 mg	28 mg
1.5 mM l-cysteine	Sigma C-8152	91 mg	182 mg
1.0 mM hypoxanthine	Sigma H-9636	68 mg	136 mg
0.16 mM thymidine	Sigma T-1895	19.4 mg	38.8 mg
1.0mM sodium pyruvate	Sigma	55 mg	110 mg
35 mM sodium bicarbonate	Gibco	1500 mg	3 g
15% heat-inactivated FCS			
0.2 mM 2-mercaptoethanol	Sigma	5 ml[1]	10 ml[1]

Make up to required volume with ddH_2O and adjust to pH 7.3 with 1N NaOH.
[1]stock solution: 14 µl of 2-mercaptoethanol in 10 ml ddH_2O. Make fresh each time.

Store medium at 4°C up to 2 weeks.

Optional medium additives (for less well adapted strains)

Constituent	Source	for 500 ml	for 1000 ml
HMI-18	as above	as above	as above
Glucose	Sigma G-7021	500 mg	1000 mg
MEM non-essential amino acids	Gibco 043-01140	5.0 ml	10.0 ml

Table 9. BMEM-medium (B)

BMEM

Constituent	Source	for 100 ml
MEM	See below	83 ml
Baltz Supplement	See below	1 ml
Heat-inactivated horse serum		15 ml
2-mercaptoethanol solution	See below	1 ml

The medium must sit at 4ºC for 24 hours or 37ºC for 2 hours to detoxify the 2-mercaptoethanol before use. Store the medium at 4ºC for up to 2 week or at -20ºC for up to 6 months.

MEM

Constituent	Source	for 10 litre
MEM powder	Gibco 61100	97.5 g
HEPES	Sigma	60 g
Glucose	Fluka	10 g
NaHCO3	Merck	22 g
MEM non-essential amino acids	Gibco 11140	100 ml
dd H2O		9.9 litre

Adjust pH to 7.4 with 4N NaOH and filter sterilize. Store at 4°C in 0.5 or 1L bottles (up to 6 month).

Baltz supplement

Constituent	Source	For 250 ml
Hypoxanthine	Fluka	1.7 g
1N NaOH		Small increments
Na-Pyruvate	Merck	2.75 g
ddH2O		to 250ml

Add small increments (use as little as possible) of 1N NaOH to dissolve 1.7 g Hypoxanthine (until transparent). After dissolving add Na-Pyruvate and ddH2O. Filter sterilize and store in glass bottles at -20°C in 5 ml aliquots.

Mercaptoethanol solution

Constituent	Source	10ml
ddH2O		10 ml
2-mercaptoethanol	Fluka	14 ul

Prepare 2-mercaptoethanol dilution freshly or keep max for 2 days at 4°C.

Medium for *T.b. gambiense* can be prepared with human serum in analogy to media A and B. For example BMEM with 15 % inactivated human and 5 % inactivated FCS:

Table 10. BMEM with human serum (for *T.b. gambiense*)

Constituent	Source	for 100 ml
MEM	See above	78 ml
Baltz Supplement	See above	1 ml
Heat-inactivated human serum		15 ml
Heat-inactivated FCS		5 ml
2-mercaptoethanol solution	See above	1 ml

The medium must sit at 4ºC for 24 hours or 37ºC for 2 hours to detoxify the 2-mercaptoethanol before use. Store the medium at 4ºC for up to 1 week (human serum expires fast) or at -20ºC for up to 6 months.

5f.6. Media for *Leishmania* culture and assays

SM-Medium
Leishmania promastigotes and axenic amastigotes are cultivated in SM culture medium adjusted to pH 7.3 (buffered by HEPES) and pH 5.4 (buffered by MES), respectively, plus 10% heat inactivated FCS (SM medium see appendices). Axenic amastigotes are maintained at 37°C and the promastigotes at 25-27°C.

5f.7. Antibiotic mix

Mäser Mix (100x)
100mg Chloramphenicol are dissolved in 1.4ml 70% EtOH and subsequently added to 600mg Penecillin, 1.0g Kanamycin, 500mg Fluorocytosin and 98.6ml ddH2O.
The antibiotic mix is sterile filtered and stored in 1ml aliquots. It can be used for trypanosomes and for leishmania at a concentration of maximum 1%.

5g. Processing of data

The MTS or serial drug dilution assays can be evaluated with the fluorescent reader software e.g. SoftmaxPro or Prism. The 4-Parameter mathematical model fits a dose–response curve to the data (see Figure 4) and is a good discriminator to estimate the activity of the compound. Alternatively, Excel can be used to calculate the IC-50 by linear interpolation (Huber & Koella, Acta Tropica, 55 (1993) 257-261).

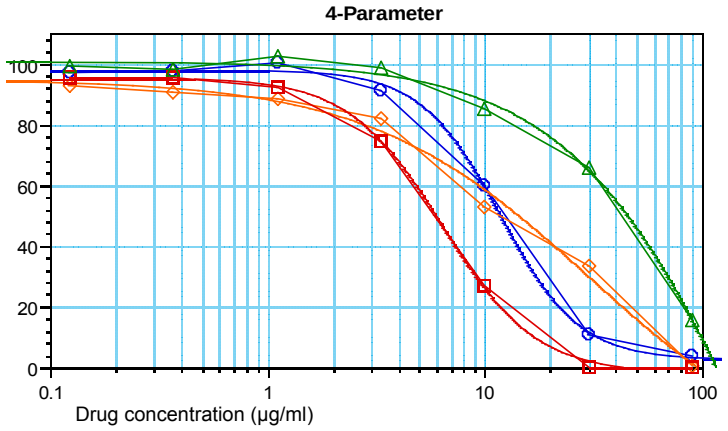

Figure 4. Typical sigmoidal inhibition curve obtained by a serial drug dilution assay.

Each compound is tested in duplicates. Reported data of active or moderately active compounds is the average value of at least two individual assays.

Table 11. Example of an *in vitro* raw data template

Supplier, received on 10.09.08

All values as: µg / mL

parasite	standard drug	Run 1	Run 2	Run 3	Average standard
T.b.rhodiense:	melarsoprol	0.0027	0.0018	0.0022	0.002
L.d. axenic:	miltefosin	0.123	0.162	0.118	0.134
Cytotoxicity:	podophyllotoxin	0.008	0.006	0.008	0.007

	T. b. rhodesiense				L.d axenic				Cytotoxicity			
	15.09.2008	19.09.2008	26.09.08	Average	16.09.2008	23.09.2008	30.09.08	Average	15.09.2008	22.09.2008	3	Average
drug	IC-50	IC-50	IC-50	IC-50	IC-50	IC-50	IC-50	IC-50	IC-50	IC-50	IC-50	IC-50
535.08	0.005	0.006	0.008	0.006	0.261	0.611	0.345	0.406	18.9	27.6	-	23.3
536.08	0.047	0.043	0.072	0.054	0.123	0.258	0.112	0.164	2.3	0.8	-	1.6
536.08	3.0	3.1	-	3.060	11.45	15.18	-	13.315	67.7	72.3	-	70.0
536.08	1.63	1.37	-	1.500	23.12	31.29	-	27.207	>90	>90	-	>90

5h. Archives of data and reporting

The final report should include the following information:

1. Identification of the Study, the Test Item and Reference Item
   a) A descriptive title
   b) Identification of the test item by code or name
   c) Identification of the reference item by name
   d) Characterisation of the test item including source, purity, stability and homogeneity

2. Information Concerning the Test Facility
   a) Name and address of any test facilities and test sites involved
   b) Name and business address of the Principal Investigator(s) and the phase(s) of the study delegated, if applicable
   c) Name and business address of scientists having contributed reports to the final report

3. Dates
   Experimental starting and completion dates

4. Study Plan
   a) Aims and summary of experiment
   b) Description of methods and materials used (reference to SOP)

5. Results
   a) A summary of results
   b) All information and data required by the study plan (Raw data from which the calculations and conclusions are made);
   c) A presentation of the results, including calculations and determinations of statistical significance
   d) An evaluation and discussion of the results and, where appropriate, conclusions

6. Archives of raw data
   The location of the raw data (e.g. laboratory notebook, references, computer files) has to be clearly indicated. These documents should be named in systematic and a self-explaining manner.

The report could be made up a Table of Results (see reporting template of Table 12) and an accompanying document covering the other points mentioned above.

Table 12. Example of a reporting template

In Vitro Screening Results

Parasite:	*strain:*	*stage:*	*reference drug:*
T. b. rhodes	STIB 900	trypomastigotes	Melarsoprol
T. cruzi	Tulahuen C4	amastigotes	Benznidazole
L. donovani	MHOM-ET-67/L8	amastigotes	Miltefosine
P. falciparur	K1	IEF	Chloroquine
Cytotoxicity	L6		Podophyllotoxin

active, suggested for in vivo study
boarderline activity
active, but cytotoxic
repeats ongoing, pending
active extract, suggested for further purification

name of project **tested by (contact address)**
name of PI **location of raw data**

number of replicates: x
All values as: µg / mL

[a] not soluble in DMSO [b] precipitates out in a not tested

Supplier	Arriving Date	ID No	External No.	T.b.rhod.		L.don. axen.		L. don. inf. mac.		Cytotox. L6		Comments
				IC-50	ref drug	IC-50	ref drug	IC-50	ref drug	IC-50	ref drug	
ABC	26/11/2006	PC1	1	12.5	0.004	>30	0.10	>28	0.006	>30	0.006	a
ABC	26/11/2006	PC2	2	>30	0.004	>30	0.10	>29	0.006	>30	0.006	a
ABC	26/11/2006	PC3	3	>30	0.004	>30	0.10	>30	0.006	2.5	0.006	
ABC	26/11/2006	PC4	4	>30	0.004	>30	0.10	>30	0.006	>30	0.006	
ABC	26/11/2006	PC5	5	>30	0.004	>30	0.10	>30	0.006	>30	0.006	
ABC	26/11/2006	PC6	6	0.1	0.004	6.25	0.10	3.45	0.006	>30	0.006	
ABC	26/11/2006	PC7	7	1.5	0.004	5	0.10	2.5	0.006	0.5	0.006	
ABC	26/11/2016	PC8	8	>30	0.004	>30	0.10	>30	0.006	>30	0.006	
ABC	26/11/2006	PC9	9	>30	0.004	>30	0.10	>30	0.006	>30	0.006	
ABC	26/11/2006	PC10	10	>30	0.004	>30	0.10	>30	0.006	>30	0.006	b
ABC	26/11/2006	PC11	11	>30	0.004	>30	0.10	>30	0.006	>30	0.006	
ABC	26/11/2006	PC12	12	0.01	0.004	0.01	0.10	>30	0.006	>30	0.006	
ABC	26/11/2006	PC13	13	>30	0.004	>30	0.10	>30	0.006	>30	0.006	
ABC	26/11/2006	PC14	14	>30	0.004	>30	0.10	>30	0.006	2.4	0.006	
ABC	26/11/2006	PC15	15	>30	0.004	>30	0.10	>30	0.006	10.2	0.006	

6. List of resources

The list of resources listed below is not meant to be comprehensive. Further websites, documents, references and other types of information of interest can be located from the following documents and links.

6a. Websites

Neglected Diseases

DNDi Facts on ND

WHO Tropical Diseases Research (TDR)

Sleeping sickness

Center for Disease Control and Prevention (CDC)

Doctors Without Borders/Medecins sans Frontieres

Kinetoplastid Biology and Disease online

Pathology of African Trypanosomiasis (Sleeping Sickness)

The Programme Against African Trypanosomiasis (PAAT)

World Health Organization (WHO)

WHO Tropical Disease Research (TDR)

WHO Control of Neglected Tropical Diseases (NTD)

Wikipedia

Leishmaniasis

Center for Disease Control and Prevention (CDC)

Doctors Without Borders/Medecins sans Frontieres

Kinetoplastid Biology and Disease online

World Health Organization (WHO)

WHO Tropical Disease Research (TDR)

WHO Control of Neglected Tropical Diseases (NTD)

Wikipedia

6b. Databases

Chapman & Hall Dictionary of Natural Product

ChemIDplus

Low Hanging Fruit

Napralert

PubMed

Scirus

The International Plant Names Index

Traditional Chinese Medicine Information Database

6c. Journals

Acta Tropica

American Journal of Tropical Medicine and Hygiene

Annals of Tropical Medicine and Parasitology

Disease Outbreak News, WHO

Drug Discovery Today

Journal of Antimicrobial Chemotherapy

Journal of Ethnopharmacology

Journal of Natural Products

Nature (issue dedicated to Neglected Diseases)

Nature Review Drug Discovery

Natural Product Updates

Natural Product Report

Phytochemistry

Planta Medica

PLoS Neglected Diseases

Tropical Diseases Bulletin

Tropical Medicine and International Health

6d. Images

TDR database

Links to pictures of Sleeping Sickness (Hardin MD/Univ of Iowa)

6e. Actors in the field of Neglected Diseases

DNDi

Drug Discovery at Dundee

FIND Diagnostics

Gates Foundation Neglected Diseases

LSHTM

MMV

Neglected Tropical Diseases Coalition

Pan-Asian Screening Network

STI

TB Alliance

TDR

The Tropical Diseases initiative

WHO Tropical Diseases

Wikipedia Neglected Diseases

6f. Articles of interest

General

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Drug Discovery and Development

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Croft, S. L., Seifert K., Yardley V. Current scenario of drug development for leishmaniasis. *Indian J Med Res.* 2006. 123, 399-410.

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Natural Products and Neglected Diseases

Fournet, A. and Muñoz V. Natural products as trypanocidal, antileishmanial and antimalarial drugs. *Curr Top Med Chem*. 2002. 2, 1215-1237.

Hoet, S., Opperdoes F., Opperdoes F., Brun R., Quetin-Leclercq J. Natural products active against African trypanosomes: a step towards new drugs. *Natural Product Reports*. 2004. 21, 353-364.

Ioset, J.-R. Natural Products for Neglected Diseases: A Review. *Current Organic Chemistry*, 12, 8, 2008. 643-666.

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Treatments

Croft, S. L. and Coombs G. H., Leishmaniasis - current chemotherapy and recent advances in the search for novel drugs. *Trends in Parasitology*. 2003. 19, 502-508.

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7. Glossary

This glossary gathers several relevant definitions taken from various scientifically recognized sources. The alphabetic order has been followed.

Accuracy
A measure of the closeness of agreement of a measured test result obtained by the analytical method to its theoretical true (or accepted reference) value.

Acute Toxicity test
A method used to determine the concentration of a substance that produces a toxic effect on a specified percentage of test organisms in a short period of time (e.g., 96 hours). As a general guideline, death is the measure of toxicity.

ADME
The characteristics of a substance in terms of Absorption, Distribution, Metabolism and Excretion.

Analog
A drug whose structure is related to that of another drug but whose chemical and biological properties may be quite different.

Assay
An experimental test, measurement or analysis to determine whether compounds have the desired effect either in a living organism, outside an organism, or in an artificial environment.

Bioavailability
The percentage of drug that is detected in the systemic circulation after its administration. Losses can be attributed to an inherent lack of absorption/passage into the systemic circulation and/or to metabolic clearance. Detection of drug can be accomplished pharmacodynamically (quantification of a biological response to the drug) or pharmacokinetically (quantification of actual drug concentration). Oral bioavailability is associated with orally administered drugs.

Biological matrix
A discrete material of biological origin that can be sampled and processed in a reproducible manner. Examples are blood, serum, plasma, urine, feces, saliva, sputum, and various discrete tissues.

Blank
A sample of a biological matrix to which no analytes have been added that is used to assess the specificity of the bioanalytical method.

Chronic toxicity
An adverse effect that lingers or continues for a relatively long period of time. A chronic effect can be lethality, growth, reduced reproduction, etc.

Clinical trials
The last phase in the development process for new drugs in order to determine the effect on patients in real life studies.

Coefficient of Variation (CV)
The standard statistical measurement of the relative variation of a distribution or set of data, defined as the standard deviation divided by the mean. Coefficient of variation is a measurement of precision within and among laboratories.

Compound
A chemical substance that is analyzed for a specific activity.

Confidence limits or interval (CI)
The limits or interval within which, at some specified level of probability, the true value of a result lies. Typically LC50 values are reported with 95 % confidence limits.

Cross-validation
A comparison of the validation parameters of two methods or assays

Culture Passages or Subcultures
A passage or subculture is simply the transfer of established microorganism from a growing culture to fresh media. The subsequent growth on the fresh media constitutes a passage or subculture. Growing a reference culture or reference stock culture from its preserved status (e.g. frozen or lyophilized) is not a passage or subculture. The preserved microorganism is not in a stage of established growth until it is thawed or hydrated and grown for the first time.

Dose Response Curve
A mathematical representation of the response of test organisms to different concentrations of a toxicant/effluent.

Drug

An active ingredient that is intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment or prevention of a disease, or to affect the structure or any function of the human body, but does not include intermediates used in the synthesis of such ingredient *(FDA)*.

Drug Candidate
A compound that has successfully completed all steps of preclinical development and therefore fulfills criteria to enter clinical trials.

Drug candidate Selection
The decision to proceed to clinical evaluation of one compound or a small number of compounds from the same chemical class.

Drug development process
- Discovery: Identification of a biological, genetic or protein target linked to a particular disease; subsequent lead identification of a potential drug that interacts with the target to help cure the disease or halt its progression.
- Pre-clinical Phase: Comprehensive *in vitro* and animal testing of the drug candidate to establish its target specificity, toxicity in various doses and pharmacokinetics.
- Clinical Phase I: Human trials conducted to demonstrate safety and effectiveness (efficacy); tests with paid, healthy volunteers to establish dosage, side effects and pharmacokinetics.
- Clinical Phase II: Trials with small numbers of patients conducted to identify drug performance characteristics (optimal dosing, administration, key indication).
- Clinical Phase III: Pivotal trials conducted with larger patient populations to establish efficacy and provide additional safety information.
- Approval: Data is analyzed and submitted for regulatory review. The U.S. submission to the FDA is called an NDA (New Drug Application) or BLA (Biologic License Application); the European submission to the EMEA (European Medicines Evaluation Agency) is called an MAA (Marketing Authorization Application). After stringent analysis and review of the submission, the regulatory agency provides final approval.

EC50/IC50
The concentration of a defined test substance that effects 50 percent of a test population during continuous exposure over a specified period of time, for instance to inhibit the growth of a specific microorganism. Strictly talking, the IC50 refers to the concentration of the test substance that is required to inhibit 50 percentage of the function of an enzyme or growth of cells. However IC50 is currently used instead to EC50 to describe other types of systems.

Enzyme
Any of the numerous proteins or conjugated proteins produced by living organisms and functioning as biochemical catalysts.

Exposure time
Length of time a test organism is exposed to a test solution.

Full validation
Establishment of all validation parameters to apply to sample analysis for the bioanalytical method for each analyte.

Guideline
A document developed through the consensus process describing criteria for a general operating practice, procedure, or material for voluntary use. A guideline may be used as written or modified by the user to fit specific needs.

Hit(s)
An individual or small numbers of structurally related molecules that have established anti-pathogen activity regardless of other important drug discovery considerations

Hit Explosion/Hit Expansion
Process of establishing structure-activity relationships around a hit by preparing new libraries or series of analogues using related building blocks and/or scaffolds to those employed in the preparation of that hit.

Hit Selection
The decision to proceed to *in vivo* studies and hit expansion based on identification of a single compound from library screening or literature search

High-throughput screening (HTS)
A technique to analyze a large number of compounds in biological assays in parallel in order to identify molecules with specific biological effects from large compound libraries.

Inhibitors
Agents that block or suppress the activity of enzymes such as proteases.

In vitro
Literally, "in glass" meaning in the test tube. A method by which certain processes or complete scientific experiments are carried out in test tubes or Petri dishes.

In vivo
Literally, "in life" meaning in a living cell or organism. An experiment on a living, multi-cell organism, such as an animal or plant.

IC50/EC50
The concentration of test substance that effects 50 percent of a test population during continuous exposure over a specified period of time, for instance to inhibit the growth of a specific microorganism. Strictly talking, the IC50 refers to the concentration of the test substance that is required to inhibit 50 percentage of the function of an enzyme. However IC50 is currently used instead to EC50 to describe other types of systems.

LC50
Lethal concentration of a substance killing 50 percent of an exposed organisms at a specific time interval.

Lead Selection
The decision to proceed with chemical optimization based on review of a series of chemical analogues of the ‘Hit’.

Leads
Molecules within a series that display a more substantial structure-activity relationship (SAR) around a given hit, coupled with important factors such as selectivity and pharmacokinetic and/or *in vivo* data.

Lead discovery
The process of identifying active new chemical entities, which by subsequent modification may be transformed into a clinically useful drug.

Lead Optimization
The synthetic modification of a biologically active compound, to fulfill stereo electronic, physicochemical, pharmacokinetic and toxicological clinical usefulness.

Lethal Concentration (LC)
Toxicant concentration producing death of test organism. For example, a 96 hr LC50 would be the test concentration killing 50% of exposed organisms after 96 hours of exposure.

Limit of detection (LOD)
The lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise.

Lipinski's Rule of 5
Set of criteria for predicting the oral bioavailability of a compound on the basis of simple molecular features (Molecular Weight <= 500, clogP <= 5.0, H bond donors <= 5, H bond acceptors <=5, Free-rotation bonds <= 10). Often used to profile a library or virtual library with respect to the proportion of drug - like members which it contains. An algorithm, developed by Christopher A. Lipinski (of Pfizer) and colleagues, in which many of the cutoff numbers are five or multiples of five. There are actually four rules, and Pfizer has developed a additional number of criteria for adoption of lead candidates.

Lipophilic
Capable of combining with or dissolving in lipids.

Lipophilicity
The affinity of a molecule or a moiety for a lipophilic environment. It is commonly measured by its distribution behavior in a biphasic system, either liquid-liquid (e.g., partition coefficient in 1-octanol/water) or solid/liquid (retention on reversed-phase high performance liquid chromatography (RP-HPLC) or thin-layer chromatography (TLC) system).

Matrix effect
The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample.

Medicinal Chemistry
A chemistry-based discipline, also involving aspects of biological, medical and pharmaceutical sciences. It is concerned with the invention, discovery, design, identification and preparation of biologically active compounds, the study of their metabolism, the interpretation of their mode of action at the molecular level and the construction of structure-activity relationships.

Method
A comprehensive description of all procedures used in sample analysis.

Micromolar (μM)
A concentration representing one millionth of a mole.

Minimal Inhibitory Concentration (MIC)
The lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test.

Moieties
Chemical compounds or functional groups or portions of those compounds.

Optimization
The process of developing an assay (prior to validation) wherein the variables affecting the assay are elucidated (e.g., antibody concentration, incubation time, wash cycles, etc.). This process is ideally carried out using a multi-variant factorial approach where the inter-dependence between multiple variables/parameters can be taken into account.

Optimized Lead Selection
The decision to proceed to GLP preclinical toxicology and GMP pharmaceutical development of one compound or a small number of compounds from the same chemical class.

Partial validation
Modification of validated bioanalytical methods that do not necessarily call for full revalidation.

Pharmacokinetics (PK)
The study of absorption, distribution, metabolism and excretion (ADME) of bioactive compounds in a higher organism.

Pharmacology
The science of studying both the mechanisms and the actions of drugs, usually in animal models of disease, to evaluate their potential therapeutic value.

Pharmacophore
A pharmacophore is the ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target structure and to trigger (or to block) its biological response. A pharmacophore does not represent a real molecule or a real association of functional groups, but a purely abstract concept that accounts for the common molecular interaction capacities of a group of compounds towards their target structure.

Potency
Refers to the concentration of an agent (drug) at which it inhibits an enzyme to a defined extent, i.e. IC50 is the concentration at which an inhibitor blocks the activity of an enzyme by 50 per cent.

Precision
The closeness of agreement (*degree of scatter*) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

Quality control sample (QC)
A spiked sample used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch.

Quality control
Quality control is a process employed to ensure a certain level of quality in a product or in a process. The basic goal of quality control is to ensure that products or processes meet defined requirements.

R&D
Research and development

Reference Compound
A compound that is routinely run in the same manner as the test compounds in every run of the assay. This term does not refer to the plate controls used to define the maximum and minimum responses, and they may or may not be a "literature standard" or "reference" compound.

Reference Culture
A reference culture is a microorganism preparation that is acquired from a culture type collection such as ATCC®, CIP or NCTC.

Reference Stock Culture
A reference stock culture is a microorganism preparation that is derived from a reference culture. Guidelines and standards outline how reference stock cultures must be processed and stored. In-house laboratory preparations and commercial preparations can meet reference stock culture criteria.

Repeatability
Repeatability is the *precision* of repeated measurements within the same analytical run under the same operating conditions over a short interval of time. It is also termed intra-assay or intra-batch precision.

Replicate
Repetition of a specific assay 2 or more times with a defined sample using the same experimental conditions

Report
A document that has not been subjected to consensus review

Reproducibility
It represents the precision of specific assay/method under different operating conditions (different labs, technicians, instruments, …)

Reproducibility (Lab to Lab)
Reproducibility across labs expresses the precision between laboratories. It is useful for assessing the "transferability" of an assay and/or the validity of comparing results from samples that are run in two or more laboratories.

Robustness/Ruggedness of the Assay
Robustness is a measure of the capacity of the assay to remain unaffected by small, but deliberate changes in method parameters and provides an indication of its reliability during normal run conditions.

Sample
A generic term encompassing controls, blanks, unknowns, and processed samples to be assayed in a defined test.

Scaffold
Core portion of a molecule common to all members of a group of chemicals (series, library, …)

Signal to Noise Ratio
The signal (max minus min) divided by an estimate of variability (or noise) such as the standard deviation of the max controls or the standard deviation of max minus min. There is some disagreement over the meaning of this term.

Selectivity (selective index=SI)
The selectivity index (SI) is the ratio of IC_{50} values measured on the mammalian cells and for a given parasite (IC_{50} for mammalian cell line/IC_{50} for protozoan parasite).

Stability
The chemical stability of a sample in a given matrix under specific conditions for given time intervals.

Standard
A document developed through the consensus process that clearly identifies specific, essential requirements for materials, methods, or practices for use in an unmodified form. A standard may, in addition, contain discretionary elements, which are clearly identified.

Structure - Activity Relationship (SAR)
An analysis which defines the relationship between the structure of a molecule and its ability to affect a biological system.

Susceptibility threshold
Lowest *in vitro* concentration at which most organisms are still considered susceptible. Organisms that do not grow at this concentration or at lower concentrations are reported as susceptible.

Targets
Molecules that are vital for the parasite, e.g. enzymes. Effective drugs bind to such targets influencing their biological function leading to the death of the parasite.

Toxicology
The investigation of toxic characteristics of potential drug candidates.

Toxicity
Adverse effect to a test organism caused by a substance. Toxicity is a resultant of concentration and time, modified by variables such as temperature, chemical form, and availability.

Toxicity test
A measure of the toxicity of a chemical or an effluent using living organisms by determining the degree of response (survival, reproduction, growth, etc.) of an exposed organism to the chemical or effluent.

Validation
Validation includes all the laboratory investigations that demonstrate that the performance characteristics of an assay are suitable and reliable for its intended use. It describes in mathematical and quantifiable terms the performance characteristics of an assay.

Verification
Verification is a process by which selected inspection, measurement, and testing is performed to determine that the objectives of the validation process have been met.

8. References

American Biological Safety Association

Association for Assessment and Accreditation of Laboratory Animal Care

Biosafety in Microbiological and Biomedical Laboratories

Biosafety in Microbiological and Biomedical Laboratories with special mention of parasite agents within

Biotechnology and Biological Sciences Research Council BBSRC Statement on Safeguarding Good Scientific Practice 2000

CDC chemical safety

CDC Biological hazard

Convention on Biological Diversity

Eli Lilly Assay Guidance Manual (Version 4.1, 2005)

European Union's Directive 86/609/EEC

General information on biosafety and practical basic procedures

Good Laboratory Practice (GLP)

International BioPharma

Material Safety Data Sheet

MSDS online

Neglected Diseases

US Food and Drug Administration

Wellcome Trust Guidelines on Good Research Practice

WHO biosafety manual (including a useful Safety Checklist, pp 125-131)

WHO Guidelines for Safe Transport of Infectious Substances and Diagnostic specimens

WHO/IFPMA working group

9. Appendices

9.1. SOP *Drug Preparation*: Stock Solution for *in vitro* Screening

Standard solvent:
DMSO

Standard concentration:
10mg/ml

Procedure:

1. 1-2 mg compound is exactly weighed out into an appropriate vial e.g. Eppendorf tube.
2. The appropriate quantity of DMSO is added to achieve a 10mg/ml stock solution.
3. If required, the sample is put on a plate shaker over night at ambient temperature and at low rotation rate.
4. If required, the sample is vortexed for 1-2 minutes.
5. If required, the sample is sonicated for 30 minutes
6. If the compound should still be insoluble, the sample is warmed up to maximally 60°C.
7. The stock solution is stored at -20°C.
8. For the assays the stocks are warmed up to room temperature, vortexed and an aliquot removed for the assay.
9. The vial with the stock solution is moved back to -20°C

9.2. SOP *In vitro* sensitivity assays: African Trypanosomes (Alamar Blue® assay)

Standard parasite strains:
Trypanosoma brucei rhodesiense; STIB 900
Trypanosoma brucei gambiense; STIB 930
Trypanosoma brucei brucei (mdr); STIB 950

Standard drug:
Melarsoprol™ (sanofi-Aventis, France; main standard for STIB 900)
Pentamidine (Pentacarinat™, Rhone-Poulenc, France)
Suramine (Geramine™, Bayer, Germany)

Standard conditions:
Medium: ***T.b. rhodesiense*, *T.b. brucei***
Per 100mL:
85 mL MEM with Earles's salts including 0.2 mM 2-mercaptoethanol, 1 mM Na-pyruvate, 0.5 mM hypoxanthine and 15mL heat-inactivated horse serum.

T.b. gambiense
80 mL MEM with Earles's salts including 0.2 mM 2-mercaptoethanol, 1 mM Na-pyruvate, 0.5 mM hypoxanthine and 15 mL normal human serum plus 5mL heat-inactivated fetal bovine serum.

Plates: Costar™ 96-well microtitre plates

Incubation: 37°C, 5% CO_2

Definition of test score:

no activity (no repeat):	IC50 > 5 μg/ml
moderate activity (repeat):	0.5 μg/ml < IC50 < 5 μg/ml
high activity (repeat):	IC50 < 0.5 μg/ml (for active series<0.1)

Melarsoprol (in STIB 900): average IC50 = 0.004

Drug preparation:

Compounds are dissolved in DMSO at 10mg/ml (SOP 9.1.). The DMSO stocks are kept at -20°C. For the assays fresh dilutions in medium are prepared each time. (Since DMSO is toxic, care has to be taken not to exceed a final concentration of 1% DMSO in the assay).

Procedure:

1. Into the wells of row H, add 75μl of medium that contains two times the highest drug concentration desired. Per plate 4 drugs can be tested (drug 1 column 1-3, drug 2 column 4-6, etc.)

2. Add 50 μl of medium at room temperature to rows A to G of a 96-well plate (row H has the drug).

3. Serial drug dilutions are prepared by using a 12-well multi-pipette. First, remove 25 μl from wells of row H and put it into row G and mix well. Next, 25 μl are taken out of row G and put into row F and so on until row B. The last 25 μl of row B are discarded. A serial dilution factor of 1:3 is thus obtained. Row A wells serve as controls without drugs.

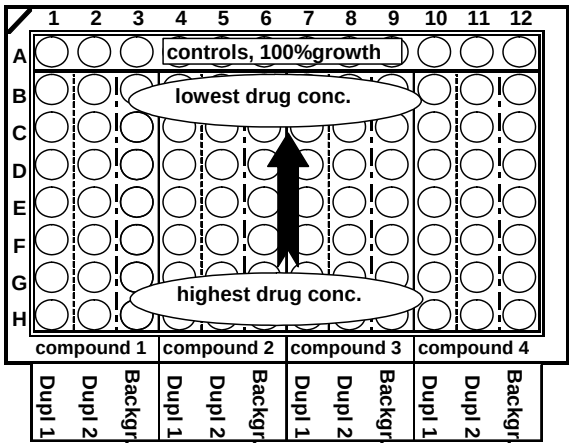
4. 50 μl of medium without trypanosomes are added to columns 3, 6, 9 and 12; these columns serve as background controls (that may be caused by the drug).

5. Dilute the trypanosomes to 3 x 10^4 tryps/ml. The trypanosome density is adjusted with a Cell Analysis System (CASY, Schärfe System, Germany) or by counting in a haemocytometer. (The trypanosome density used should be adjusted depending on the current growth characteristics of the corresponding cultures)

   Per plate, allow for the use of 3.5ml of the trypanosome stock.

6. Into the remaining wells, add 50 µl of trypanosome suspension.

7. The plates are then incubated for 69h (= 72h – time incubated with Resazurin)* at 37°C / 5% CO_2.



Evaluation

1. The plates are inspected under an inverted microscope to ensure that growth is normal. Additional information may be recorded, such as drug insolubility or contamination, etc.

2. Add 10 µl of the fluorescent dye resazurin to each well and incubate for an additional 3 hours (until a subtle color change is observed, but maximum 5 hours)*.

3. To determine an IC50 value, the plate is read at excitation wavelength 536 nm and emission wavelength 588 nm in a fluorescence reader (e.g. SPECTRAmax Gemini XS, Molecular Devices). Make sure that the values in each well are approximately 10 times the background values.

4. Data are transferred into a graphic program (Excel) and are evaluated to determine the IC50 or analyzed using the fluorescent plate reader software (e.g. SoftMax Pro).

* These are recommended times for STIB 900. When working with other strains, adjust incubation times so that an appropriate color change is achieved, while maintaining the total assay duration at 72 hours.

Drug dilutions:

90µg/ml:	4.5µl of a 10mg/ml stock + 250µl medium
30µg/ml:	1.5µl of a 10mg/ml stock + 250µl medium
10µg/ml:	5µl of a 1000µg/ml stock + 250µl medium
3µg/ml:	1.5µl of a 1000µg/ml stock + 250µl medium
1µg/ml:	5µl of a 100µg/ml stock + 250µl medium

0.1μg/ml: 5μl of a 10μg/ml stock + 250μl medium

0.01μg/ml: 5μl of a 1μg/ml stock + 250μl medium

9.3. SOP *In vitro* sensitivity assays: Axenic *Leishmania donovani amastigotes*

Standard parasite strains:

L. donovani MHOM-ET-67/HU3, axenic amastigotes

Standard drug:

Miltefosine

Standard conditions:

Medium:	SM, pH 5.4 plus 10% heat inactivated FCS
Plates:	Costar™ 96-well microtitre plates
Incubation:	37°C, 5% CO_2, 72 hours

Definition of test score:

no activity (no repeat):	IC50 > 5 μg/ml
low activity (repeat):	0.5 < IC50 < 5 μg/ml
high activity (repeat):	IC50 < 0.5 μg/ml

Miltefosine: average IC_{50} = 0.131 μg/ml

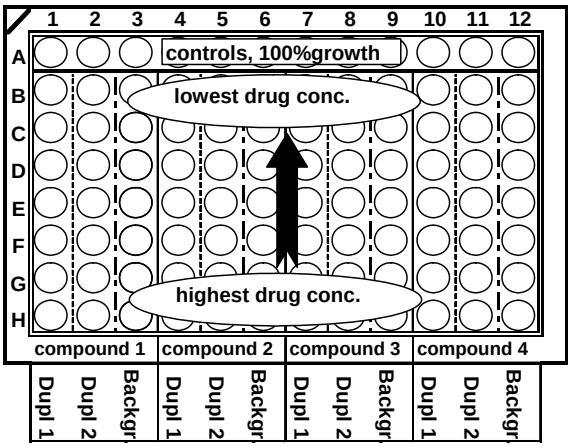
Drug preparation:

Compounds are dissolved in DMSO (SOP Nr. 9.1.). The stock solution is 10 mg/ml and stored at -20°C. Stocks are kept for 3 years. (Since DMSO is toxic, care has to be taken not to exceed a final concentration of 1% DMSO in the assay).

Procedure:

1. 75 μl of medium, containing two times the highest drug concentration, are added to the wells of row H. Four drugs can be tested this way on each plate (drug 1 columns 1-3, drug 2 columns 4-6, etc.). For each assay Miltefosin is tested as the standard with 3 μg/ml as highest concentration.
2. 50 μl of medium warmed to room temperature are added to each well of a 96-well microtiter plate in rows A to G, row H is left empty. Row A serves as control.
3. Serial drug dilutions are prepared by using a 12-well multi-pipette. First, remove 25μl from wells of row H and put it into row G and mix well. Next, 25 μl are taken out of row G and put into row F and so on until row B. The last 25 μl of row B are discarded. A serial dilution factor of 1:3 is thus obtained. Row A wells serve as controls without drugs.
4. 50 μl of medium without parasites are added to columns 3, 6, 9 and 12 which serve as controls to provide the background signal in the fluorescence scanner.

5. 50 µl of a suspension containing $2x10^6$ axenically grown amastigotes from a healthy culture in log phase are added to all the remaining wells leading to an initial parasite density of $1x10^6$/ml. (See "Preparation of amastigotes" for procedure)
6. The plates are incubated for 70 hours (= 72h – time incubated with Resazurin) at 37°C / 5% CO_2.



Evaluation

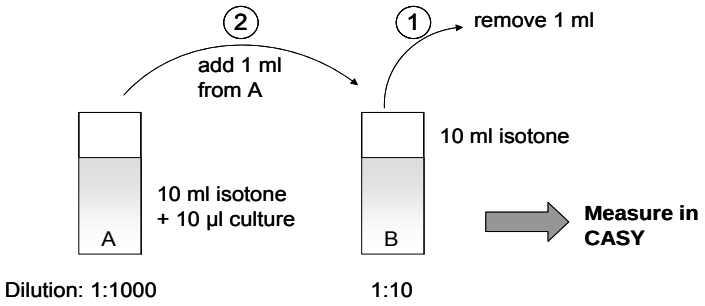
1. The plates are inspected under an inverted microscope to ensure that growth is normal. Additional information may be recorded, such as drug insolubility or contamination, etc.
2. Add 10 µl of the fluorescent dye Resazurin to each well and incubate for an additional 2 hours (until a subtle color change is observed).
3. To determine an IC50 value, the plate is read in a fluorescence scanner (e.g. SPECTRAmax GEMINI XS from Molecular Devices) at excitation wavelength 536 nm and emission wavelength 588 nm. Make sure that the values in each well are approximately 10 times the background values.
4. Data are transferred into a graphic program (Excel) and are evaluated to determine the IC50 or analyzed using the fluorescent plate reader software (e.g. SoftMax Pro).

Preparation of amastigotes and cell count (CASY):

Axenic amastigotes tend to clump in culture and must be separated for counting and equal distribution in the assay.

1. Draw medium with clumped amastigotes through a 1.2 x 40mm 18G (pink) needle into a syringe. Replace the needle with a 0.5 x 16mm 25G (yellow) needle and gently expel the liquid into a fresh Bijoux bottle. Repeat this process two more times, using fresh needles, syringe and Bijoux bottle every time.

2. Fill two CASY tubes with 10 ml isotone solution. Into the first tube (A), add 10 µl culture. From the second tube (B), remove 1 ml isotone solution, and replace with 1 ml solution from tube A. Measure tube B in CASY.



3. Record counts/ml, but increase the power of 10 by 1 (CASY assumes that the dilution is 1:1000 but actually it's 1:10,000).
4. Calculate the volume needed to obtain an amastigote concentration of $2x10^6$cells/ml:

$$\frac{(\text{volume needed} = 3.5\ \text{ml/plate}) \cdot (\text{concentration desired} = 2 \cdot 10^6\ \text{cells/ml})}{(\text{cell concentration determined})} = \text{x ml}$$

Drug dilutions:

90µg/ml:	4.5µl of a 10mg/ml stock + 250µl medium
30µg/ml:	1.5µl of a 10mg/ml stock + 250µl medium
10µg/ml:	5µl of a 1000µg/ml stock + 250µl medium
3µg/ml:	1.5µl of a 1000µg/ml stock + 250µl medium
1µg/ml:	5µl of a 100µg/ml stock + 250µl medium
0.1µg/ml:	5µl of a 10µg/ml stock + 250µl medium
0.01µg/ml:	5µl of a 1µg/ml stock + 250µl medium

Media: Preparation of SM medium

Description:
Culture medium for maintenance of tsetse tissues and growth of trypanosomatids, *Leishmania* promastigotes and axenic amastigotes.

Material:

	g (for 1 Liter)	g (for 2 Liters)	g (for 4 Liters)
Inorganic salts			
MgCl2 .6H2O	3.04	6.08	12.16
MgSO4.7H2O	3.7	7.4	14.8
KCl (anh.)	2.98	5.96	11.92
CaCl2 .2H2O	0.15	0.3	0.6
NaH2PO4 .H2O	0.61	1.22	2.44
Sugars			
D(+)-Glucose	0.7	1.4	2.8
D(-)-Fructose	0.4	0.8	1.6
Sucrose (=Saccharose)	0.4	0.8	1.6
Organic acids			
L-malic acid	0.67	1.34	2.68
alpha-ketoglutaric acid (=2-Oxoglutarsäure)	0.37	0.74	1.48
Fumaric acid-Na2	0.077	0.154	0.308
Succinin acid Na2 6H2O	0.1375	0.275	0.55
Amino Acids			
β-Alanine	2	4	8
DL-alanine	1.09	2.18	4.36
L-arginine	0.44	0.88	1.76
L-asparagine. H2O	0.24	0.48	0.96
L-aspartic acid	0.11	0.22	0.44
L-cysteine HCl	0.08	0.16	0.32
L-glutamic acid	0.25	0.5	1
L-glutamine	1.64	3.28	6.56
glycine	0.12	0.24	0.48
L-histidine	0.16	0.32	0.64
DL-isoleucine	0.09	0.18	0.36
L-leucine	0.09	0.18	0.36
L-lysine	0.15	0.3	0.6
DL-methionine	0.2	0.4	0.8
L-phenylalanine	0.2	0.4	0.8
L-proline	6.9	13.8	27.6
DL-serine	0.2	0.4	0.8
Taurine	0.27	0.54	1.08
DL-threonine	0.1	0.2	0.4
L-tryptophane	0.1	0.2	0.4
L-tyrosine	0.2	0.4	0.8
DL-valine	0.21	0.42	0.84
L-cystine	0.03	0.06	0.12
Vitamin/anitibiotic/indicator mixture			
BME Vitamins(100X)	2ml	4ml	8ml
Phenol red	0.02	0.04	0.08
Add last			
MES (50mM) (only Leish axenic amastigotes medium)	12	24	48
HEPES (50mM)	12	24	48

Procedure:

1. Dissolve the compounds in the given order in about 500 ml water.
2. Adjust pH to 5.40 for axenic *Leishmania* amastigotes with 4N HCl or to pH 7.40 with 4N NaOH.
3. Fill to final volume and sterilize by filtration.

Storage:

Store at +4°C

9.4. SOP *In vitro* sensitivity assays: *Leishmania donovani* infected macrophage assay

Standard parasite strains:

L. donovani MHOM-ET-67/L82, axenic amastigotes

Standard host cells:

Freshly harvested macrophages from NMRI, BalbC or CD1 mice

Standard drug:

Miltefosine: average IC50 = 0.66

Standard conditions:

Medium:	**Mouse macrophages, dilution of parasites and drugs:** RPMI 1640 plus 10% heat inactivated FCS **Cultivation of axenic *Leishmania*** SM, pH 5.4 plus 10% heat inactivated FCS
Antibiotics:	1% MäserMix
Slides:	Lab-tek™ 16-chamber slides
Incubation:	37°C, 5% CO_2, 72 hours
Fixation/Staining:	Methanol/Giemsa

Drug preparation:

Compounds are dissolved in DMSO at 10mg/ml (SOP 9.1.). The DMSO stocks are kept at -20°C. For the assays fresh dilutions in medium are prepared each time. (Since DMSO is toxic, care has to be taken not to exceed a final concentration of 1% DMSO in the assay).

Highest drug starting concentration: 10μg/ml

Procedure:

day 1 – Mouse inoculation

- One or two mice are inoculated i.p. with 2ml each of a 2% starch solution in distilled water (0.2g/10ml).

day 2 – Cell- harvesting

- Per Mouse prepare 10ml RPMI without FCS+1% MäserMix (100µl in 10ml) and put it on ice till usage.

Isolation of mouse macrophages: Euthanize mice using CO_2. Sterilise belly with 70% ethanol. Incise skin with a small cut and pull skin away from entire belly area. Wash again with ethanol. Lift skin over the sternum with forceps and inject 10ml of the prepared RPMI into the belly cavity. Grab the mouse by all four feet and swing gently in order to suspend the macrophages into the media. Lay the mouse on its back. Wash again with ethanol. Insert a 26 gauge needle on a syringe through the skin of the abdominal cavity. Enter low on the side to allow the fluid to pool. Extract medium slowly. Transfer into a tube and put it on ice.

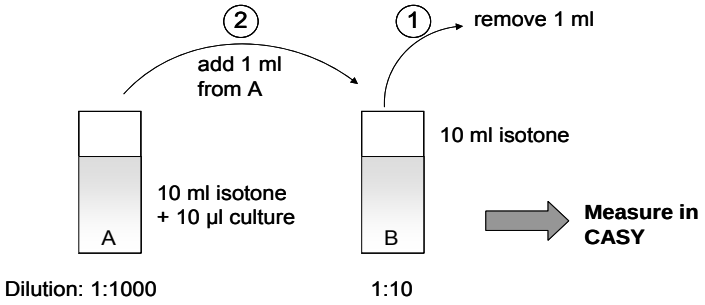
- Centrifuge macrophages for 10 min at 1500rpm. Re-suspend pellet in 5 ml RPMI Medium.
- Determine cell concentration by haemocytometer. Dilute 1:10 in Trypanblue.
- Adjust cell concentration using RPMI +10%FCS +1% MäserMix to $4x10^5$ cells/ml ($4x10^4$/well)
- 100µl of the $4x10^5$/ml macrophage suspension are dispensed into each well of the 16-chamber slides (1.6ml needed per slide)
- Incubate for 24 hrs at 37°C in 5% CO_2

day 3 – infection

- The macrophages are infected at a ratio of 1:3 (macrophages to amastigotes) with an axenic amastigote culture.
- Adjust concentration by diluting in RPMI +10%FCS to $1.2x10^6$ /ml.
- Ad 100µl of the *L. donovani* suspension to each well.(1.6ml needed per slide)
- Mix contents of the wells carefully through pipetting.
- Incubate for 24hrs at 37°C in 5% CO_2

Preparation of amastigotes and cell count (CASY):

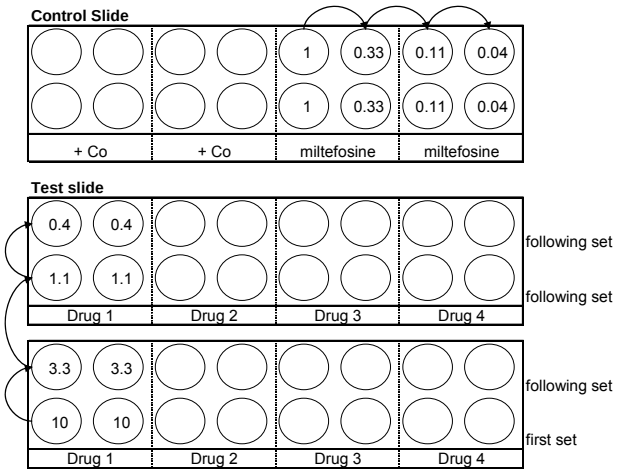
1. Draw medium with clumped amastigotes through a 1.2 x 40mm 18G (pink) needle into a syringe. Replace the needle with a 0.5 x 16mm 25G (yellow) needle and gently expel the liquid into a fresh Bijoux bottle. Repeat this process two more times, using fresh needles, syringe and Bijoux bottle every time.
2. Fill two CASY tubes with 10 ml isotone solution. Into the first tube (A), add 10 µl culture. From the second tube (B), remove 1 ml isotone solution, and replace with 1 ml solution from tube A. Measure tube B in CASY.



3. Record counts/ml, but increase the power of 10 by 1 (CASY assumes that the dilution is 1:1000 but actually it's 1:10,000).

day 4 – Drug treatment

- The degree of infection is checked by fixing (100% methanol, 5 min) and staining (10% Giemsa stain, 10 min) one slide for microscopic evaluation. Or, fix 1 well by adding 100µl methanol for 2-5 min, remove the methanol and add 20µl of Giemsa**.**
- For the other slides, the medium is removed and replaced by fresh RPMI 1640+10%FCS medium
- Mix medium, remove again and add 200µl of fresh medium.
- In a 48 Well Plate add 300µl RPMI 1640+10%FCS medium per well and 9µl of a 1mg/ml drug-stock-solution.(=3xhighest drug concentration)
- Add 100µl of pre-diluted drug from the 48 well-plate to the first set of wells in test slides.
- Mix and transfer 100µl into the following set of wells. Repeat this step for all wells, continuing onto the second test slide (1:3 serial drug dilution)
- Discard the last 100µl so that all wells contain 200µl.
- For the control slide, the first 8 wells are left without drugs. In the remaining 8 wells, standard drug (miltefosine) is diluted longwise along the control slide (start conc. 1 µg/ml)
- Incubate for 96 hours at 37°C in 5% CO_2



day 8 – Staining

- The medium and then the chambers are removed, the slides are fixed in 100% methanol for 5 min and stained with Giemsa (10%) for 10 min.
- The slides are then examined microscopically: the ratio of infected to uninfected macrophages is determined and the IC_{50} calculated by linear regression analysis.

9.5. SOP *In vitro* sensitivity assays: Cytotoxicity

Standard cell lines:

L-6 (rat skeletal myoblast cells)
or HT-29 (human bladder carcinoma cells)

Standard drug:

Podophyllotoxin (PPT); starting concentration: 0.1μg/ml
average IC50 = 0.006μg/ml

Standard conditions:

Medium:	RPMI 1640 + 10% FCS + 1.7μM L-Glutamine (850μl 200mM for 100ml)
Culture vessel:	Costar™ 96-well microtiter plates
Incubation:	37°C, 5% CO_2

Drug preparation:

Compounds are dissolved in DMSO (SOP 9.1). The stock solution is 10 mg/ml and stored at -20°C. Stocks are kept for 3 years. (Since DMSO is toxic, care has to be taken not to exceed a final concentration of 1% DMSO in the assay).

Procedure:

1. Add 100μl of medium to wells of columns 3, 6, 9 and 12 of a microtiter plate. These wells serve as controls.
2. 100 μl of a cell suspension of $4x10^4$ cells/ml is added into the remaining columns (1 and 2, 4 and 5, 7 and 8, and 10 and 11). Cells are allowed to attach over night.

Per plate, allow for 6.5ml of cell suspension to be used.

3. The next day, the medium is removed from row H (do this for half of the plates and go to step 4 and return to step 3 for the second half, so the cells don't dry out).
4. 150 μl of medium containing the highest drug concentration is added to the wells of row H. Four drugs can be tested on one plate (drug 1 column 1-3, drug 2 column 4-6, etc.).
5. Serial drug dilutions are prepared by using a 12-channel multi-pipette. First, remove 50 μl from wells of row H and put into row G and mix well. Next, 50 μl are taken out of row G and put into row F and so on until row B. The last 50 μl of row B are discarded. A serial dilution factor of 1:3 is thus obtained. Wells in row A serve as control wells without drugs.
6. The plates are then incubated for 70 hrs at 37ºC / 5% CO_2.

Evaluation:

1. The plates are inspected under an inverted microscope to ensure that growth is normal. Additional information may be recorded, such as drug insolubility or contamination, etc.
2. Add 10 μl of the fluorescent dye Resazurin to each well and incubate the plates for another 2 hours (until a color change is observed, but maximum 3 hours).
3. To determine an IC50 value, the plate is read at excitation wavelength 536 nm and emission wavelength 588 nm. Make sure that the values in each well are approximately 10 times the background values.
4. Data are transferred into a graphic program (Excel) and are evaluated to determine the IC50 or analyzed using the fluorescent plate reader software (e.g. SoftMax Pro).

Cell count:

1. Remove the medium from the flask of cells.
2. Add 2ml of EBSS, swirl for a few seconds and then remove.
3. Add 2ml of 0.25% Trypsin and swirl gently; then remove.
4. If required, place the flask in the incubator.
5. When the cells are visibly starting to come off the flask, use 5ml of medium to fully remove the cells from the bottom and into a homogeneous solution.
6. Mix 10μl of the cells with 90μl of Trypan Blue.
7. Add 10μl of this solution to the haemocytometer and count the cells in all 4 quadrants.
8. Divide the total number by 4 and multiply by 10^5.

$$\frac{(\text{volume needed} = 6.5\ \text{ml/plate}) \cdot (\text{concentration desired} = 4 \cdot 10^4\ \text{cells/ml})}{(\text{cell concentration determined})} = \text{x ml}$$

Drug dilutions

90μg/ml: 4.5μl of a 10mg/ml stock + 500μl medium
30μg/ml: 1.5μl of a 10mg/ml stock + 500μl medium

10μg/ml: 5μl of a 1000μg/ml stock + 500μl medium

1μg/ml: 5μl of a 100μg/ml stock + 500μl medium

9.6. SOP: *Preliminary* acute toxicity test *in vivo* model to assess highest tolerated dose (HTD)

Standard parasite strain:	None
Standard drugs:	None
Standard drugs preparation:	Distilled H_2O or DMSO + H_2O (max. 10% of DMSO) or Tween80 + Ethanol (70/30)
Standard conditions:	The drugs are dissolved in distilled H_2O. When not soluble in H_2O, they are first dissolved in 100 % DMSO followed by the addition of dissolved in distilled H_2O to a final maximal concentration of 10 % DMSO
Mice:	NMRI mice, females, 25 g
Cages:	Standard macrolon cages type III
Maintenance:	Standard condition with 22°C and 60 – 70 % relative humidity, special pellets

Test procedure:

day 0	One mouse is injected i.p. or p.o., when the route is i.p., the mouse is injected every 2 hours with an increasing dose, starting at 20 mg/kg, then rising to 30 mg/kg, 50 mg/kg and 50 mg/kg. The cumulative dose is the sum of the injected doses, and raises from 20 to 50 mg/kg, then to 100 mg/kg and finally to 150 mg/kg. The aim is to determine roughly the highest tolerated i.p. dose (HTD). When the route is p.o., the mouse receives an application every 2 hours with an increasing dose, starting at 20 mg/kg, then rising to 30 mg/kg, 50 mg/kg and 100 mg/kg. The cumulative dose corresponds to the sum of the injected doses, and raises from 20 to 50 mg/kg, then to 100 mg/kg and finally to 200 mg/kg. The aim is to determine roughly the highest tolerated p.o. dose (HTD).

day 1 - 2	The mice are inspected several times per day for toxic and subtoxic symptoms. 48 hours after injection the HTD is determined. This information will be used to decide on the dose used for the acute model test

9.7. SOP: *Trypanosoma brucei brucei* acute *in vivo* model

Standard parasite strains: *Trypanosoma brucei brucei*; STIB 795

Drugs:

Standard drugs:	Melarsoprol (Arsobal^R, Sanofi-Aventis, France) Diminazene aceturate (Berenil^R, Hoechst, Germany)
Stock solutions:	The drugs are first dissolved in 100 % DMSO followed by the addition of distilled H_2O to a final concentration of 10 % DMSO

Standard conditions:

Mice:	NMRI mice, females, 20 - 25 g
Cages:	Standard Macrolon cages type III
Maintenance:	Standard conditions with 22°C and 60-70% relative humidity, commercial pellets and water *ad libitum*

Test procedure

day 0	For each group (control as well as experimental) 4 mice are infected i.p. with $1x10^4$ bloodstream forms from a stock of cryopreserved stabilates containing 10% glycerol. The stabilate is suspended in PSG 6:4 to obtain a trypanosome concentration of $4x10^4$/ml. Each mouse is injected with 0.25 ml.
day 3 to 6	Animals are treated on 4 consecutive days with the drug by i.p. or p.o. route with a tolerated dose which was determined earlier (see SOP 9.6 *Preliminary* acute toxicity test *in vivo* model to assess highest tolerated dose (HTD)). The drug concentration is such that 0.1 ml/ 10 gr. can be injected.
day 7	Parasitaemia of all mice is checked by tail blood examination and recorded.
day 10	Parasitaemia of all mice is checked by tail blood examination and recorded. Thereafter parasitaemia is monitored twice per week until day 30. Mice with parasitaemia relapses are euthanized and the day of parasitaemia relapse is recorded.
day 30	Surviving and aparasitaemic mice are considered cured. They are euthanized.

9.8. SOP: *Trypanosoma brucei rhodesiense* acute *in vivo* model

Standard parasite strains: *Trypanosoma brucei rhodesiense*; STIB 900

Drugs:

Standard drugs: Melarsoprol (Arsobal^R, Sanofi-Aventis, France)
Diminazene aceturate (Berenil^R, Hoechst, Germany)

Stock solutions: The drugs are dissolved in distilled H_2O.
When not soluble in H_2O, they are first dissolved in 100 % DMSO followed by addition of distilled H_2O to a final concentration of 10 % DMSO

Standard Conditions:

Mice: NMRI mice, females, 20 - 25 g

Cages: Standard Macrolon cages type III

Maintenance: Standard conditions with 22°C and 60-70% relative humidity, commercial pellets and water *ad libitum*

Test procedure

day 0	For each group (control as well as experimental) 4 mice are infected i.p. with $2x10^4$ bloodstream forms from a stock of cryopreserved stabilates containing 10% glycerol. The stabilate is suspended in PSG 6:4 to obtain a trypanosome concentration of $8x10^4$/ml. Each mouse is injected with 0.25 ml.
day 3 to 6	Animals are treated on 4 consecutive days with the drug by i..p. or p.o. route with a tolerated dose which was determined earlier The drug concentration is such that 0.1 ml/ 10 gr. can be injected.
day 7	Parasitaemia of all mice is checked by tail blood examination and recorded.
day10	Parasitaemia of all mice is checked by tail blood examination and recorded. Thereafter parasitaemia is monitored twice per week until day 60. Mice with parasitaemia relapses are euthanized and the day of parasitaemia relapse is recorded.
day 60	Aparasitaemic mice are considered cured. They are euthanized.

9.9. SOP: *Trypanosoma brucei brucei* CNS chronic *in vivo* model (GVR35)

Standard parasite strains: *Trypanosoma brucei brucei*; GVR35

Drugs:

Standard drugs: Melarsoprol (Arsobal^R, Sanofi-Aventis, France)
Diminazene aceturate (Berenil^R, Hoechst, Germany)

Stock solutions: The drugs are first dissolved in 100 % DMSO followed by addition of distilled H_2O to a final concentration of 10 % DMSO

Standard conditions:

Mice: NMRI mice, females, 20 - 25 g

Cages: Standard Macrolon cages type III

Maintenance: Standard conditions with 22°C and 60-70% relative humidity, commercial pellets and water *ad libitum*

Test procedure

day 0	For each group (control as well as experimental) 5 mice are infected i.p. with $2x10^4$ bloodstream forms from a stock of cryopreserved stabilates containing 10% glycerol. The stabilate is suspended in PSG 6:4 to obtain a trypanosome concentration of $8x10^4$/ml. Each mouse is injected with 0.25 ml.
day 14	Parasitaemia of all mice is checked by tail blood examination to ensure that all mice are infected. Negative mice are removed.
day 21	A control group is treated with 40mg/kg diminazene aceturate ip for one day only. The diminazene treated group should relapse between day 30 and 45 post infection. A second control group treated with 10mg/kg melarsoprol i.p. for 5 days can be added. The melarsoprol treated group should become cured.
day 21 – 25 (30)	Experimental groups are treated on 5 (10) consecutive days with the highest tolerated dose by i.p. route. The drug concentration is such that 0.1 ml/ 10 gr. can be injected.
day 28 (33)	Parasitaemia of all mice is checked by tail blood examination and thereafter twice a week until day 50. After day 50 the mice are checked only once per week until day 180. Mice with parasitaemia relapses are euthanized and the day of parasitaemia relapse is recorded.
day 180	Aparasitaemic mice are considered cured. They are euthanized.

9.10. SOP: *Leishmania donovani in vivo* primary screening model

Standard parasite strains: sodium stibogluconate sensitive *Leishmania donovani* (strain MHOM/ET/67/HU3, also known as LV9 or L82)

Drugs:

Standard drugs: Pentostam® (GSK), miltefosine (Paladium, Canada), AmBisome (Gilead, USA)

Stock solutions: the drugs are dissolved in sterile double distilled H_20. Where applicable, manufacturer's instructions are followed

Standard conditions:

Mice: Female BALB/c mice (18-20g); also Syrian hamsters - *Mesocricetus auratus*

Cages: Standard Macrolon cages

Maintenance: Standard conditions with 22°C and 60-70% relative humidity, commercial pellets and water *ad libitum*

Test procedure

day 0	All mice are weighed. For 20g mice the infecting bolus is 2.0×10^7 amastigotes via the lateral tail vein. Infected mice are randomly assorted into groups of five animals. *L. donovani* amastigotes are isolated from the spleen of a heavily infected donor animal. Parasite burden is assessed on a Giemsa stained spleen impression smear using the Stauber method [J. Protozool. 5(4), 269-273 (1958)] and by Thoma™ counting chamber. For 20g mice an inoculums of $1x10^8$ amastigotes/ml RPMI 1640 is prepared and kept on ice until use.
day 7	One mouse is sacrificed, livers smears taken, methanol fixed and Giemsa stained to assess level of infection. Amastigotes should be readily seen in the majority of fields of view (x100, oil immersion). All groups are weighed.
day 7 - 11	Infected mice are treated intraperitoneally, at 50mg/kg/day, once a day for 5 consecutive days unless previous toxicity data indicate a preferable lower dose. One group is treated with test drug vehicle only; another group is treated with 15mgSbV/kg sodium stibogluconate by the subcutaneous route.

day 14	All groups are weighed and the mice humanely killed. In this model significant weight loss is regarded as an indicator of drug toxicity rather than disease pathology. Evaluation endpoint: At necropsy the liver is dissected and weighed. Fixed and stained liver impression smears are evaluated microscopically (x100, oil immersion) to determine parasite burden. The number of amastigote/500 liver cells is counted. NB: Giemsa stains nuclear material. The burden is expressed as Leishman Donovan Units (LDU's): mean number of amastigotes per liver cell x weigh of the organ (mg) [Braley & Kirkley, Clin Exp Immunol. (1977) 30; 119-129]. The LDU of drug-treated groups is compared to that of untreated mice and the % inhibition calculated. For the average level of infection the standard SbV treatement of 15mg/kg.day x 5 should reduce the parasite load by about 50%
	Evaluation endpoint: methanol fixed, 10% Giemsa-stained liver impressions smear are used for the microscopical determination of the total parasite burden. The number of amastigotes/500 liver cells is counted microscopically (5x100, oil immersion). The results are expressed as Leishman Donovan Units (LDU): mean number of amastigotes per liver cell x mg liver [Bradley & Kirkley, Clin. For the average level of infection, the standard drug treatment of (Pentostam®, GSK) 15mg SbV/kg s.c. x 5 days should give around 50% reduction of liver parasite load.

Acknowledgments

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