SOP
(abbreviated)

Antwerp University
Laboratory of Microbiology, Parasitology and Hygiene

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Standard procedures
(abbreviated SOP's)

used for the DNDi *in vitro* screening

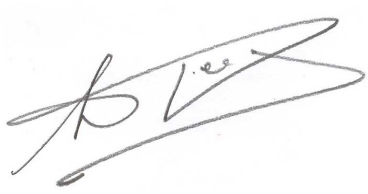
against

Sleeping sickness

Chagas disease

Leishmaniasis

Cytotoxicity



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Interpretation of results

- Activity is expressed as IC_{50}-values in µM concentrations. For compounds without exact molecular weight, IC_{50}-values are expressed in µg/ml.
- Based on the level of the IC_{50}, semi-quantitative activity scores are given (normalized across the different assays to allow comparison)
- Compounds with score ≥3 may require confirmation testing and further follow-up **if** the observed activity is selective (absence of obvious cytotoxicity, high selectivity index).

In vitro activity of reference compounds

UA	Compund code	Generic name	MW	Screen	IC_{50} (µM)	
R120	UA/ K020	TAMOXIFEN CITRATE SALT	564	MRC-5 SV2	10.2	1.44
R139	TDR/ 42268/1	NICLOSAMIDE	327		7.11	3.71
R126		MILTEFOSIN	408	L.inf	10.07	4.23
R116	UA/ K016	AMPHOTERICIN B	924		1.16	1.25
R126		MILTEFOSIN	408	L.don	6.88	3.64
R116	UA/ K016	AMPHOTERICIN B	924		0.99	0.28
R125	TDR/ 10164/1	BENZNIDAZOL	260	T.cruz	2.89	0.56
R132	TDR/ 10739/1	NIFURTIMOX	287		0.91	0.41
R131	TDR/ 10738/1	SURAMIN	1297	T.b.bruc	0.03	0.01
R152	TDR/ 9957/1	MELARSOPROL	398		0.02	0.004
R131	TDR/ 10738/1	SURAMIN	1297	T.b.rhod	0.04	0.01
R152	TDR/ 9957/1	MELARSOPROL	398		0.005	0.002

IC_{50}-values of reference compounds are based on historical control values.

Only one reference drug is included per assay.

Toxicity	***In vitro* cytotoxicity evaluation on human fibroblasts (MRC-5 cell line)**

Parasite and cell cultures

MRC-5_{SV2}[1] cells are cultured in MEM + Earl's salts-medium, supplemented with L-glutamine, $NaHC0_3$ and 5% inactivated fetal calf serum. All cultures and assays are conducted at 37°C under an atmosphere of 5% $C0_2$. Other cell types (J774, L6, Vero, Hela, *e.a.*) can also be used for determination of cytotoxicity/selectivity.

Compound solutions/dilutions

Compound stock solutions are prepared in 100% DMSO at 20 mM or mg/ml[2]. The compounds are serially pre-diluted (2-fold or 4-fold) in DMSO followed by a further (intermediate) dilution in demineralized water to assure a final in-test DMSO concentration of <1%.

Drug sensitivity assays

Assays are performed in sterile 96-well microtiter plates, each well containing 10 µl of the watery compound dilutions together with 190 µl of MRC-5_{SV2} inoculum ($1.5x10^5$ cells/ml). Cell growth is compared to untreated-control wells (100% cell growth) and medium-control wells (0% cell growth). After 3 days incubation, cell viability is assessed fluorimetrically after addition of 50µl resazurin per well[3]. After 4 hours at 37°C, fluorescence is measured (λ_{ex} 550 nm, λ_{em} 590 nm). The results are expressed as % reduction in cell growth/viability compared to control wells and an IC_{50} and an IC_{90} (50% and 90% inhibitory concentrations) are determined.

Primary screen

The MRC-5_{SV2} cell-line is used. The compounds are tested at 5 or 10 concentrations depending upon the number of compounds (4-fold compound dilutions) (64 - 16 - 4 – 1 – 0.25 – 0.0625 – 0.015625 – 0.0039 – 0.000975 and 0.00024 µM or µg/ml). The compound is classified non-toxic when the IC_{50} is higher than 30 µM. Between 30 and 10 µM, the compound is regarded as moderately toxic. When the IC_{50} is lower than 10 µM, the compound is classified as highly toxic. Cytotoxic reference compounds include vinblastine or paclitaxel (IC_{50} <0.01µM), but these are rarely included because of health hazards for laboratory personnel. Other compounds are therefore used: tamoxifen or niclosamide.

Secondary screen

The IC_{50} is determined using an extended dose range (2-fold compound dilutions) still with a highest concentration of 64µM. Other cell lines and primary cells can be included: L6, J774, Hela, Vero and PMM (primary mouse macrophages). Parallel cytotoxicity evaluation is required to assess selective action of test compounds in parasite screens.

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[1] MRC-5_{SV2} cells are diploid human embryonic lung fibroblasts. A SV-40 transformed cell line is now available to obtain unlimited sub-cultivation characteristics.

[2] Concentrations are standard expressed in molar concentrations, except for natural products, drug mixtures and if the molecular weight is not known.

[3] Resazurin stock solution in phosphate buffer: 50µg/ml. Alamar Blue™ can be used as alternative: 5µl of a 1/10 Alamar Blue™ solution is added to each well

Leishmania	***In vitro* drug screening model against *Leishmania donovani* and *Leishmania infantum***

Parasite and cell cultures

Two *Leishmania species* (*L.infantum* MHOM/MA(BE)/67 and *L.donovani* MHOM/ET/67/L82) are used. The strains are maintained in the Golden Hamster (*Mesocricetus auratus*). Amastigotes are collected from the spleen of an infected donor hamster using three centrifugation purification steps (300 rpm, keeping the supernatans, 2200 rpm, keeping the supernatans and 3500 rpm, keeping the pellet) and spleen parasite burdens are assessed using the Stauber technique[4]. Primary peritoneal mouse macrophages are used as host cell and are collected 2 days after peritoneal stimulation with a 2% potato starch suspension. All cultures and assays are conducted at 37°C under an atmosphere of 5% $C0_2$.

Compound solutions/dilutions

Compound stock solutions are prepared in 100% DMSO at 20 mM or mg/ml[5]. The compounds are serially pre-diluted (2-fold or 4-fold) in DMSO followed by a further (intermediate) dilution in demineralized water to assure a final in-test DMSO concentration of <1%.

Drug sensitivity assays

Assays are performed in 96-well microtiter plates, each well containing 10 µl of the compound dilutions together with 190 µl of macrophage/parasite inoculum (3.10^4 cells + $4{,}5.10^5$ parasites/well). The inoculum is prepared in RPMI-1640 medium, supplemented with 200 mM L-glutamine, 16.5 mM $NaHC0_3$, and 5% inactivated fetal calf serum. The macrophages are infected after 48 hours. The compounds are added after 2 hours of infection. Parasite multiplication is compared to untreated-infected controls (100% growth) and uninfected controls (0% growth). After 5 days incubation, parasite burdens (mean number of amastigotes/macrophage) are microscopically assessed after staining the cells with a 10% Giemsa solution. The results are expressed as % reduction in parasite burden compared to untreated control wells and an IC_{50} and an IC_{90} (50% and 90% inhibitory concentrations) are calculated.

Primary screen

Leishmania infantum MHOM/MA(BE)/67 strain is used. The compounds are tested at 5 or 10 concentrations depending upon the number of compounds (4-fold compound dilutions) (64 – 16 – 4 – 1 – 0.25 – 0.0625 – 0.015625 – 0.0039 – 0.000975 and 0.00024 µM or µg/ml). Amphotericin B and miltefosin are included as the reference drugs. A test compound is classified as inactive when the IC_{50} is higher than 30 µM. When IC_{50} lies between 30 and 10 µM, the compound is regarded as moderately active. If the IC_{50} is lower than 10 µM, the compound is classified as highly active on the condition that it also demonstrates selective action (absence of cytotoxicity against primary peritoneal macrophages). A final recommendation for activity is given after confirmatory evaluation in a secondary screening.

Secondary screen

Leishmania infantum MHOM/MA(BE)/67 and *L.donovani* MHOM/ET/67/L82 strains are used and the IC_{50}-values are determined using an extended dose range (2-fold compound dilutions). Amphotericin B and miltefosin are included as reference drugs.

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[4] Stauber LA. (1966): Characterization of strains of Leishmania donovani. Exp Parasitol. 18: 1-11.

[5] Concentrations are standard expressed in molar concentrations, except for natural products, drug mixtures and if the molecular weight is not known.

Chagas	***In vitro* drug screening model against *Trypanosoma cruzi***

Parasite and cell cultures

Trypanosoma cruzi, Tulahuen CL2, β galactosidase strain (nifurtimox-sensitive) is used[6]. The strain is maintained on MRC-5_{SV2} (human lung fibroblast) cells[7] in MEM medium, supplemented with 200 mM L-glutamine, 16.5 mM $NaHC0_3$, and 5% inactivated fetal calf serum. All cultures and assays are conducted at 37°C under an atmosphere of 5% $C0_2$.

Compound solutions/dilutions

Compound stock solutions are prepared in 100% DMSO at 20 mM or mg/ml[8]. The compounds are serially pre-diluted (2-fold or 4-fold) in DMSO followed by a further (intermediate) dilution in demineralized water to assure a final in-test DMSO concentration of <1%.

Drug sensitivity assays

Assays are performed in sterile 96-well microtiter plates, each well containing 10 µl of the watery compound dilutions together with 190 µl of MRC-5 cell/parasite inoculum (4.10^3 cells/well + 4.10^4 parasites/well). Parasite growth is compared to untreated-infected controls (100% growth) and non-infected controls (0% growth) after 7 days incubation at 37°C and 5% $C0_2$. Parasite burdens are assessed after adding the substrate CPRG (chlorophenolred ß-D-galactopyranoside): 50µl/well of a stock solution containing 15.2 mg CPRG + 250 µl Nonidet in 100 ml PBS. The change in color is measured spectrophotometrically at 540 nm after 4 hours incubation at 37 °C. The results are expressed as % reduction in parasite burdens compared to control wells and an IC_{50} and IC_{90} (50% and 90% inhibitory concentrations) are calculated.

Primary screen

Trypanosoma cruzi β galactosidase strain is used. Compounds are tested at 5 or 10 concentrations depending upon the number of compounds (4-fold compound dilutions) (64 – 16 – 4 – 1 – 0.25 – 0.0625 – 0.015625 – 0.0039 – 0.000975 and 0.00024 µM or µg/ml). Nifurtimox or benznidazole can be included as the reference drugs. The test compound is classified as inactive when the IC_{50} is higher than 30 µM. When IC_{50} lies between 30 and 5 µM, the compound is regarded as being moderate active. When the IC_{50} is lower than 5 µM, the compound is classified as highly active on the condition that it also demonstrates selective action (absence of cytotoxicity). A final recommendation for activity is given after confirmatory evaluation in a secondary screening.

Secondary screen

Trypanosoma cruzi β galactosidase strain is used and IC_{50}-values are determined using an extended dose range (2-fold compound dilutions). Nifurtimox or benznidazole is included as reference drugs.

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[6] Buckner FS, Verlinde CL, La Flamme AC, Van Voorhis WC. (1996): Efficient technique for screening drugs for activity against Trypanosoma cruzi using parasites expressing beta-galactosidase. Antimicrob Agents Chemother. 40: 2592-2597.

[7] MRC-5_{SV2} cells are diploid human embryonic lung fibroblasts. A SV-40 transformed cell line is now available to obtain unlimited sub-cultivation characteristics.

[8] Concentrations are standard expressed in molar concentrations, except for natural products, drug mixtures and if the molecular weight is not known.

Sleeping sickness	***In vitro* drug screening model against *Trypanosoma brucei***

Parasite and cell cultures

The *Trypanosoma brucei brucei* Squib 427 strain (suramin-sensitive) or *Trypanosoma brucei rhodesiense* (strain STIB-900) are used for screening. The strains are maintained in Hirumi (HMI-9) medium, supplemented with 10% inactivated fetal calf serum. All cultures and assays are conducted at 37°C under an atmosphere of 5% $C0_2$.

Compound solutions/dilutions

Compound stock solutions are prepared in 100% DMSO at 20 mM or mg/ml[9]. The compounds are serially pre-diluted (2-fold or 4-fold) in DMSO followed by a further (intermediate) dilution in demineralized water to assure a final in-test DMSO concentration of <1%.

Drug sensitivity assays

Assays are performed in sterile 96-well microtiter plates, each well containing 10 µl of the compound dilutions together with 190 µl of the parasite suspension ($1.5x10^4$ parasites/well - *T.brucei* or $4x10^3$ parasites/well - *T.rhodesiense*). Parasite growth is compared to untreated-infected (100% parasite growth) and uninfected controls (0% growth). After 3 days incubation, parasite growth is assessed fluorimetrically after addition of 50µl resazurin per well[10]. After 6 hours (*T.rhodesiense*) or 24 hours (*T.brucei*) at 37°C, fluorescence is measured (λ_{ex} 550 nm, λ_{em} 590 nm). The results are expressed as % reduction in parasite growth/viability compared to control wells and an IC_{50} and IC_{90} (50% and 90% inhibitory concentrations) are calculated.

Primary screen

Trypanosoma brucei brucei Squib 427 strain and *Trypanosoma brucei rhodesiense* STIB-900 strain are used. Compounds are tested at 5 or 10 concentrations depending upon the number of compounds (4-fold compound dilutions) (64 – 16 – 4 – 1 – 0.25 – 0.0625 – 0.015625 – 0.0039 – 0.000975 and 0.00024 µM or µg/ml). Suramin or Melarsoprol are included as the reference drugs. The compound is classified as inactive when the IC_{50} is higher than 5 µM. When IC_{50} lies between 5 and 1 µM, the compound is regarded as being moderate active. When the IC_{50} is lower than 1 µM, the compound is classified as highly active on the condition that it also demonstrates selective action (absence of cytotoxicity). A final recommendation for activity is given after confirmatory evaluation in a secondary screening.

Secondary screen

Trypanosoma brucei brucei and *T.b.rhodesiense* are both included used and IC_{50}-values are determined using an extended dose range (2-fold compound dilutions). Suramin and melarsoprol are included as reference drugs.

[9] Concentrations are standard expressed in molar concentrations, except for natural products, drug mixtures and if the molecular weight is not known.

[10] Resazurin stock solution in phosphate buffer: 50µg/ml. // Alamar Blue™ can be used as alternative: 5µl of a 1/10 Alamar Blue™ solution is added to each well