African trypanosomiasis: in vitro screening model

Parasite cultures

Three strains of *Trypanosoma brucei* spp. are used in this study: (a) *T. b. rhodesiense* STIB 900 (a clone of a population isolated in 1982 from a patient in Tanzania), which is known to be susceptible to all currently used drugs; (b) *T. b. gambiense* STIB 930 (a derivative of strain TH1/78E (031), isolated in 1978 from a patient in Ivory Coast), which is known to be sensitive to all drugs used; and (c) *T. b. brucei* STIB 950 (a clone of a population isolated in 1985 from a bovine in Somalia), which shows drug resistance to diminazene, isometamidium and quinapyramine. Bloodstream-form trypomastigotes of the strains (a) and (c) are maintained in Minimal Essential Medium (MEM) with Earle’s salts supplemented according to Baltz et al. (EMBO J. 4, 1273-1277, 1985) with 25 mM N-2-hydroxyethylpiperazine-N’-2-ethane-sulphonic acid (HEPES), 1 g/l additional glucose, 1% MEM non-essential amino acids (100x), 0.2 mM 2-mercaptoethanol, 2 mM sodium pyruvate, 0.1 mM hypoxanthine and 15% heat-inactivated horse serum. Bloodstream-form trypomastigotes of strain (b) are maintained in MEM with Earle’s salts supplemented with 25 mM HEPES, 1 g/l additional glucose, 1% MEM non-essential amino acids (100x), 0.2 mM 2-mercaptoethanol, 2 mM sodium pyruvate, 0.1 mM hypoxanthine, 0.05 mM bathocuproine disulphonic acid, 0.15 mM L-cysteine and 15% heat-inactivated pooled human serum. All cultures and assays are conducted at 37°C under an atmosphere of 5% CO₂ 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 does not exceed 1%.

Assays are performed in 96-well microtiter plates, each well containing 100 µl of culture medium with 8 x 10³ bloodstream forms with or without a serial drug dilution. The highest concentration for the test compounds is 90 µg/ml. Seven 3-fold drug dilutions are used, covering a range from 90 µg/ml to 0.123 µg/ml. Each drug is tested in duplicate. Active compounds are tested twice for confirmation. The final result is the mean of the four individual IC₅₀ values. After 72 hrs of incubation, the plates are inspected under an inverted microscope to assure growth of the controls and sterile conditions. Ten µl of Alamar Blue (12.5 mg resazurin dissolved in 100 ml distilled water) are then added to each well and the plates are incubated for another 2 hours. The plates are read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wave length of 536 nm and an emission wave length of 588 nm. IC₅₀ values are determined using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA).
**Primary screen**

The preliminary screen uses the *T. b. rhodesiense* strain. The compounds are tested at seven concentrations (drug concentrations ranging from 90 µg/ml to 0.123 µg/ml in 3-fold dilutions).

- If the IC₅₀ is > 3µg/ml, the compound is classified as inactive.
- If the IC₅₀ is between 0.2 and 3 µg/ml, the compound is classified as moderately active.
- If the IC₅₀ is < 0.2 µg/ml, the compound is classified as active.

The standard drug is melarsoprol, which is run in the same assay; the IC₅₀ for melarsoprol is 2.1 ng/ml (range 1.3 - 4.0 ng/ml; n = 20).

**Secondary screen**

Active compounds (IC₅₀ < 0.2 µg/ml) are tested against *T. b. gambiense* STIB 930 and the drug-resistant *T. b. brucei* STIB 950 strains, following the protocol described above. The standard drug is melarsoprol which is run in the same assay; the IC₅₀ for melarsoprol is 2.05 ng/ml (range 1.08 - 3.82 ng/ml; n = 34) with STIB 930 and 6.2 ng/ml with STIB 950.