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A yeast-based high-throughput screen identifies inhibitors of trypanosomatid HRG heme transporters with potent leishmanicidal and trypanocidal activity

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ABSTRACT

Objectives: New drugs are required to treat neglected diseases caused by trypanosomatid parasites such as *Leishmania, Trypanosoma brucei* and *Trypanosoma cruzi*. An Achilles' heel of these parasites is their heme auxotrophy; they have an absolute dependence on scavenging this molecule from the host, and trypanosomatid HRG heme transporters (TrypHRG) play an important role in this process. As these proteins are essential for the parasites and have low similarity with their human orthologue, they have been proposed as attractive therapeutic targets. Here, we have developed two yeast-based assays that allow an inexpensive high-throughput screening of TrypHRG inhibitors within a cellular context.
Methods: We first assessed that *Leishmania major, Leishmania donovani* and *T. brucei* HRG proteins were heterologously expressed in the digestive vacuole membrane of a mutant heme auxotrophic yeast strain. Here, TrypHRG imports hemoglobinderived heme into the cytosol, allowing mutant yeast to grow in the presence of low hemoglobin concentrations and promoting the activity of hemeproteins such as catalase, which was used as a reporter of cytosolic heme levels.
Results: In the presence of a TrypHRG inhibitor, both catalase activity (test 1) and yeast growth (test 2) were diminished, being easily monitored. The assays were then tested on a pilot scale for HTS purposes using a collection of repurposing drugs and food antioxidants. Some of the TrypHRG inhibitors identified in yeast presented strong trypanocidal and leishmanicidal activity in the submicromolar range, proving the potential of this approach.
Conclusions: Cumulatively, it was shown that the inhibition bioassays developed were robust and applicable to large-scale HTS.

1. Introduction

The trypanosomatid parasites *Trypanosoma brucei, Trypanosoma cruzi* and *Leishmania* spp. are pathogenic for humans causing, respectively, African human trypanosomiasis or sleeping sickness, American trypanosomiasis or Chagas disease and leishmaniasis (in its different clinical manifestations: visceral, cutaneous and mucocutaneous) [1]. These protozoa have digenetic life cycles, alternating between two hosts, the insect vector and the mammalian host. In the insect, procyclic (*T. brucei*), epimastigote (*T. cruzi*) and promastigote (*Leishmania*) forms are extracellular, while in the mammalian host, the amastigote forms of *Leishmania* spp. and *T. cruzi* develop intracellularly, whereas the bloodstream forms (BSF) of *T. brucei* are extracellular. Control of these diseases is based on a very toxic, obsolete, and ineffective pharmacological arsenal against which clinical resistance has been developed [2]. In addition, access to expensive drugs is not available in many developing countries where the greatest numbers of affected people are con-

Abbreviations: Hb, haemoglobin; BSF, bloodstream forms; HTS, high-throughput screening; ALA, δ-aminolevulinic acid; IS, selectivity indexes.

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centrated. Thus, it is urgent to develop new drugs and strategies to prevent and/or treat these diseases.

An attractive approach toward this goal is to take advantage of biochemical differences between these pathogenic organisms and their human host. Heme group metabolism, an essential metabolite in almost all aerobic organisms, constitutes one of these differences as trypanosomatid parasites are auxotrophs for heme and need to scavenge it from their host [3]. Therefore, proteins involved in the uptake and intracellular trafficking of porphyrins could be potential targets for drug development [4]. One example is the members of the HRG family of heme transporters described in *Leishmania* spp. [5,6], *T. brucei* [6,7] and *T. cruzi* [8]. These proteins (TrypHRG) are responsible for heme uptake in *Leishmania* (*L*HR1) [5] and *T. cruzi* (*Tc*HTE) [8]. In addition, they import heme released from haemoglobin (Hb) digestion from the endolysosome into the cytosol in *T. brucei* (*Tb*HRG) and *Leishmania* [6]. These TrypHRG proteins are essential for the parasites [5,6,9–11]. In addition, these proteins present a low degree of identity with their human homologue (14–18%) [6] and, therefore, TrypHRG proteins represent promising targets for controlling the neglected diseases caused by these parasites.

A powerful tool for identifying new drugs is the massive screening of libraries of compounds by automatic high-throughput screening [12,13]. Classically, there are two HTS approaches, biochemical and phenotypical. The former, usually based on purified targets, are expensive and do not provide information regarding the ability of a selected compound to enter the cell or its intracellular stability. This problem is solved in phenotypic screenings, based on whole cells, but they do not provide information on the mechanism of action of the selected compound, and hits are difficult to optimize [14]. In response to these problems, target-based HTS screenings have been proposed within a cellular context, with the advantages of the previous approaches and enabling the detection of hits that interact with a known target in a cellular environment. An example is screenings made in yeast, which has been proposed as an efficient potential vehicle for neglected disease drug discovery [14,15]. In these assays, a mutant yeast with a gene deficiency is supplemented with a heterologous gene encoding the target of interest [14–16].

Interestingly, the expression of TrypHRG proteins in a mutant strain of *Saccharomyces cerevisiae* auxotrophic for heme (Δ*hem1*) enables it to grow in the presence of low concentrations of heme [5,6,8] or Hb [6]. In this yeast, TrypHRG proteins mediate heme uptake from the outside via the plasma membrane [5,6] or the digestive vacuole membrane, where they import the heme derived from endocytosed Hb into the cytosol [6]. In addition, as yeast catalases are haemoproteins regulated by heme, catalase activity can be used as an endogenous marker to measure cytosolic heme accumulated via TrypHRG proteins [6]. This catalase activity can easily be monitored without cell lysis by observing the foam generated in the heme-containing yeast after the addition of hydrogen peroxide and detergent [6,17].

In this work, we have developed two independents but related HTS-compatible assays to identify TrypHRG inhibitors in heme auxotrophic yeast expressing these parasite proteins. As proof of concept, we screened a small library consisting of repositioning drugs and food antioxidants. The evaluation of drugs that are used for other therapeutic indications (drug repurposing) is one of the more successful approaches for the development of new treatments for neglected diseases [18], as their toxicity, pharmacodynamics and pharmacokinetics are known. On the other hand, the antioxidants used are molecules (natural or synthetic) that are already present in the human diet or additives used as preservatives in the food industry, so their low toxicity is also well known. The results show that some of the TrypHRG inhibitors found with the yeast-HTS assay were able to kill the parasites at submicromolar concentrations.

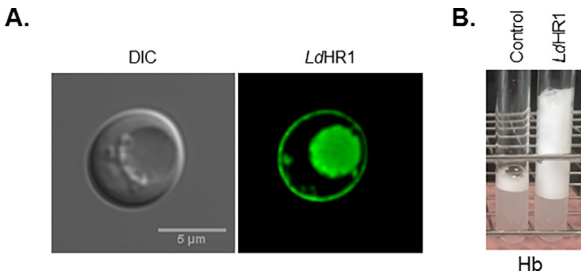


Figure 1. *Ld*HR1 mediates Hb-derived heme transport from the yeast digestive vacuole to the cytosol. (A) *Ld*HR1 shows a double localization at the plasma membrane and the digestive vacuole of yeast. *Ld*HR1 was heterologous expressed in a heme auxotrophic *S. cerevisiae* strain (*hem1*Δ) as a protein fused with fluorescent GFP (*Ld*HR1-GFP). The picture shows a representative cell expressing *Ld*HR1-GFP (green) with a fluorescence pattern similar to that of the total cell population. A Nomarski image (left) is also shown. Scale bar: 5 µm. (B) *Ld*HR1 increases yeast cytosolic heme levels after Hb endocytosis. Catalase activity, observed as foam formation as described in Materials and Methods, of *hem1*Δ yeast transformed with empty (control) or *LdHR1* containing plasmid (*Ld*HR1) and incubated with 0.5 µM Hb. Picture representative of three independent experiments.

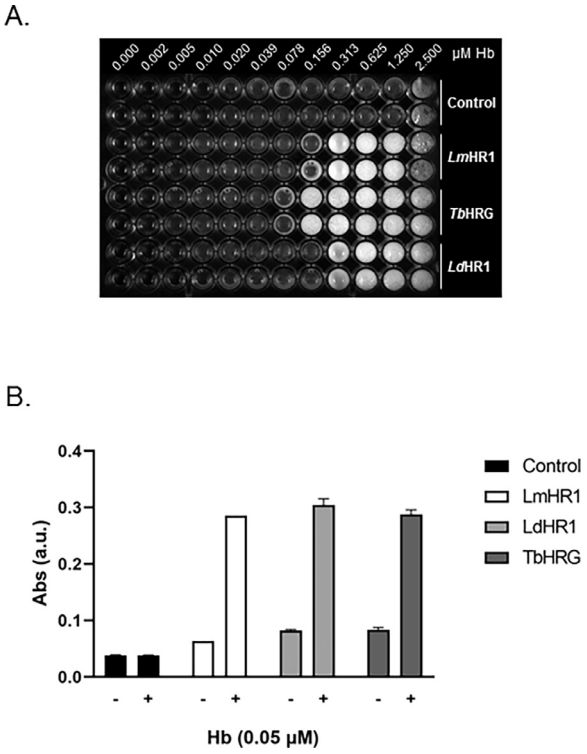


Figure 2. Development of HTS-compatible mini tests allowing the screening of inhibitors of TrypHRG proteins expressed in yeast. (A) Test based on the inhibition of an intracellular heme reporter. Δ*hem1* yeast transformed with empty plasmid (control) or containing the indicated HRG genes were cultured in 96-well plates in the presence of increasing Hb concentrations. After 24 h at 30 ºC, a mix of Triton X-100 and H_2O_2 was added to each well and the heme-dependent foam generated as a result of catalase activity was photographed 5 min later. A representative image of three assays performed independently is shown. (B) Test based on the inhibition of Hb-dependent growth. The same yeast strains were grown in the presence (+) or absence (-) of 0.05 µM Hb for 24 h at 30 ºC, and the $OD6_{00}$ of each well was then measured. Differences were significant ($P < 0.0001$) in the three yeast strains expressing TrypHRG.

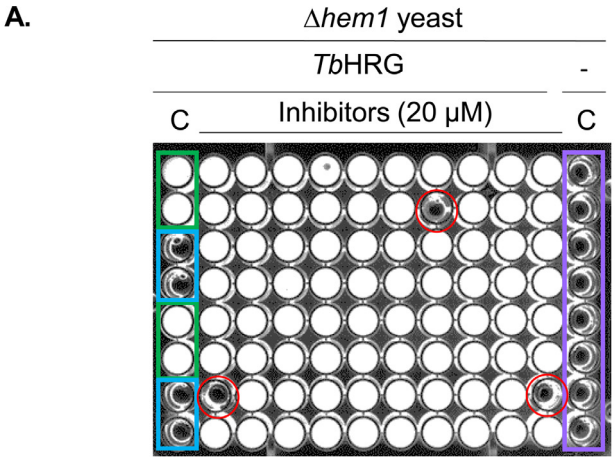
2. Results and discussion

2.1. Development of HTS-compatible tests for screening for inhibitors of TrypHRG proteins expressed in yeast

We decided to use a yeast-based HTS screening strategy to search for TrypHRG inhibitors. The use of an assay based on yeast expressing parasite targets can overcome some of the obstacles of phenotypic and biochemical tests, as it offers an inexpensive platform for high-throughput target-directed screening within a eukaryotic cellular context. In fact, this type of yeast-based test has recently been proposed as a good alternative to search for drugs against neglected diseases [14–16]. We took advantage of the *S. cerevisiae* Δ*hem1* mutant strain with a deletion in the gene *hem1*, which encodes the δ-aminolaevulinic acid (ALA) synthase enzyme, the first enzyme of the heme biosynthesis pathway, and, therefore, auxotrophic for heme. Many HRG transporters expressed in this mutant strain functionally rescue its growth defect in the presence of low concentrations of hemin or Hb [5,6,8,19]. As discussed above, TrypHRGs present different localizations in yeast, plasma membrane and/or digestive vacuole, depending on the species [5,6,8]; this allows mutant yeast to use not only hemin [20] but also Hb, as a heme source [6]. In addition, catalase activity can be used as an endogenous marker to measure cytosolic heme accumulated via TrypHRG proteins [6].

Since *Leishmania donovani*, which causes visceral leishmaniasis, is one of the *Leishmania* spp. that causes more deaths, we started by confirming that, as in *Lm*HR1 and *Tb*HRG [6], *L. donovani* HR1 (*Ld*HR1) located at the digestive vacuole membrane (Fig. 1A). Subsequently, we analysed the effect of *Ld*HR1 expression on yeast cytosolic heme levels, measuring the activity of the heme-dependent enzyme catalase [6,21]. For its assessment, we added a mix of hydrogen peroxide and detergent (Triton X-100) to yeast previously cultured in the presence of Hb, expressing *Ld*HR1 or not. Then, we observed the foam produced when oxygen bubbles, generated during the catalase-mediated decomposition of hydrogen peroxide, were trapped by the detergent [17]. As shown in Fig. 1B, the foam developed in the tubes indicated that Δ*hem1* yeast expressing *Ld*HR1 and incubated in the presence of Hb had a significantly higher catalase activity than control yeast.

To adapt this assay to a HTS format, we miniaturized it to a 96-well plates (which could be extrapolated to the 384-well format), developing two related but independent tests that measure the availability of cytosolic heme: a) by analysing catalase activity, as described above; and b) by evaluating yeast growth by measuring the OD_{600}. The first test allows us to qualitatively measure an inhibitor-mediated decrease in intracellular heme levels, observing the absence of foam formed by the catalase activity, used as a haemoprotein reporter, allowing a very simple visual screening assay. Several parameters, such as the starting inoculum, incubation time and Hb concentration, which determine the growth of yeast expressing HRG proteins, were optimized. An example is shown in Fig. 2A, where control and TrypHRG-expressing *hem1*Δ yeasts were incubated at a final OD_{600} = 0.1 in SC-medium supplemented with increasing concentrations of Hb (0 to 2.5 μM). After incubation for 24 h at 30 ºC, hydrogen peroxide and detergent



B.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.512	0.539	0.523	0.537	0.443	0.562	0.513	0.538	0.568	0.556	0.557	0.079
B	0.524	0.503	0.495	0.457	0.466	0.513	0.490	**0.155**	0.478	0.515	0.523	0.082
C	0.053	0.499	0.553	0.594	0.490	0.502	0.534	0.525	0.551	0.574	0.605	0.054
D	0.056	0.499	0.506	0.528	0.468	0.507	0.499	0.538	0.561	0.551	0.549	0.095
E	0.572	0.555	0.551	0.551	0.506	0.641	0.598	0.571	0.526	0.537	0.620	0.072
F	0.542	0.596	0.618	0.599	0.548	0.560	0.558	0.370	0.574	0.534	0.588	0.084
G	0.054	**0.044**	0.598	0.595	0.613	0.599	0.623	0.590	0.622	0.610	**0.045**	0.079
H	0.056	0.287	0.313	0.265	0.454	0.377	0.497	0.559	0.525	0.553	0.513	0.067

Figure 3. HTS screening of TbHRG inhibitors. (A) HTS screening based on inhibition of an intracellular heme reporter. Representative 96-well plate showing the foam (white wells) generated as a result of catalase activity of Δ*hem1* yeast transformed with the *Tb*HRG protein and grown in the presence of 0.5 μM Hb and 20 μM of each compound. Potential hits preventing foam development (black wells) are highlighted in red. Positive controls (*Tb*HRG yeast incubated with Hb but without drug) are marked in green, whereas negative controls are marked in blue (*Tb*HRG yeast incubated without Hb) and purple (control Δ*hem1* yeast transformed with an empty plasmid, incubated with Hb). (B) HTS screening based on inhibition of Hb-dependent growth. Equivalent 96-well plate to that of A, showing OD values measured at 600 nm of yeast transformed with *Tb*HRG protein and grown in the presence of 0.05 μM Hb and 20 μM of each compound. The wells containing the assay compounds are shown in gray, highlighting in red those wells where yeast growth was inhibited by more than 75%. Green wells are positive controls (*Tb*HRG yeast incubated with Hb, but without drug) and blue wells are negative controls (*Tb*HRG yeast incubated without Hb). Purple wells are also negative controls using control Δ*hem1* yeast transformed with an empty plasmid, cultured in the presence of 0.05 μM Hb.

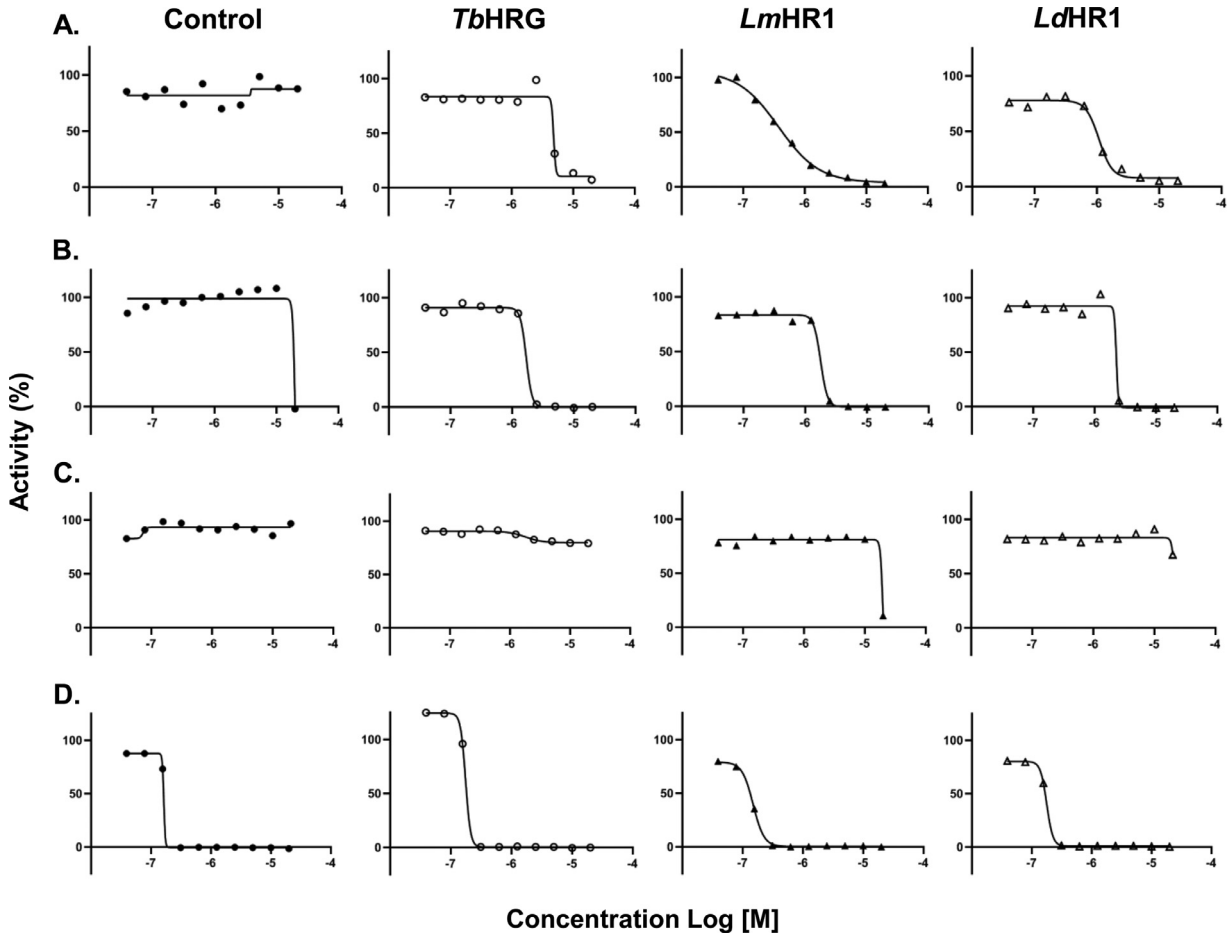


Figure 4. Dose-response curves of representative selected and nonselected compounds based on the yeast cytotoxicity assay. Examples of dose-response curves obtained for the compounds tested in control Δ*hem1* yeast and Δ*hem1* yeast transformed with the indicated TrypHRG transporters. (A) Menadione, (B) Chlorhexidine, (C) Meclocycline sulfosalicylate and (D) Thimerosal.

were added and foam formation was observed in the wells. Fig. 2A shows that submicromolar Hb concentrations produced catalase-dependent foam (white wells) only when the yeast expressed the trypanosomatid transporters, *Tb*HRG being the most efficient (foam at 0.156 µM Hb). A concentration of 0.5 µM of Hb was selected for the performance of this test to allow foam development in all cases.

The second test measured the OD_{600} of the well plates to monitor the yeast growth inhibition produced by putative TrypHRG inhibitors. In this case, we incubated control yeast and yeast transfected with the three TrypHRG genes with 0.05 µM Hb (according to [6]) for 24 h and determined growth by measuring the OD_{600}. Fig. 2B shows that, at this Hb concentration, yeast was only able to grow when expressing one of the three genes. The quality and reproducibility of this whole-cell-based assay was determined by calculating the Z factor, where a score of 0.5 and 1.0 indicates an excellent assay [22]. In our case, the values obtained were greater than 0.8 for all three transformed yeast lines (0.87 for LmHR1 and LdHR1 and 0.84 for TbHRG), indicating that the assays were appropriate for the HTS format.

2.2. Screening of TrypHRG inhibitors

As a proof of concept, we used these tests to screen a drug chemolibrary consisting of around 1000 drugs approved by the FDA for clinical use in pathologies other than leishmaniasis or trypanosomiasis (part of the US Drug Collection (Microsource)). The advantage of this type of drug is that many of them are orally administered and there is a great deal of information available regarding their toxicity, pharmacokinetics and pharmacodynamics. This saves time and costs if any of these compounds reach clinical trials and, in fact, this approach of using repurposed drugs against parasites responsible for neglected diseases has been previously explored [18,23–25]. In addition, we have also included several antioxidants, of natural and synthetic origin, in use in the food industry, for which there is much data available concerning their safety profile [26]. For the initial screening, compounds were used at 20 µM in 96-well plates and both their effects on catalase activity and yeast growth were analysed and compared. This relatively high concentration was chosen as the drug must cross not only the yeast cell wall but also an efficient drug efflux system formed by ABC transporters [27], barriers that limit intrayeast drug accumulation and are absent in parasite and mammalian cells. As the amount of compounds in the library was limited, we performed the initial screening against yeast transformed with *Tb*HRG, as this strain presented the highest growth and catalase activity in the presence of low Hb concentrations (Fig. 2A and [6]).

Thus, we cultured *Tb*HRG-expressing yeast in 96-well plates in the presence of 0.5 µM Hb and 20 µM of each test compound. After 24 h, we added 30 µl of 1% detergent (Triton X-100) and 30 µl of H_2O_2 (30% (w/w) in H_2O) to each well, obtaining pictures of the plates 5 min later and selecting those compounds that inhibited foam formation. Fig. 3A shows an example of selected compounds in one plate. In parallel, we cultured yeast under the same conditions but in the presence of 0.05 µM Hb. In this case, after 24 h we measured its growth (as OD_{600}), selecting those com-

pounds producing a growth inhibition of higher than 75% with respect to yeast cultured in the absence of drug (control). Fig. 3B shows the result of the equivalent plate shown in Fig. 3A. Using both methodologies, the same compounds (positions G2, B8 and G11 on the plates) were selected in both plates, and this correlation was maintained throughout the screening. Therefore, we did not include any control to determine a putative inhibition of the catalase activity produced by the selected drugs. Indeed, although we carried out HTS screening with both tests, in future larger-scale screenings, only one of the two should be chosen since the same 66 compounds were selected with each test from the 1007 compounds in the library (6.55%).

The phenotypes measured in both selection methods could be due not just to the TrypHRG inhibition that mediated the decrease in yeast growth or catalase activity, but also to a toxic effect of the drugs on the yeast. Indeed, the library included some fungicidal drugs. Therefore, we performed a drug toxicity assay using control yeast grown in medium supplemented with ALA, the product of the deleted *Hem1* protein. Thus, we obtained dose-response curves for the 66 selected compounds measuring their effect on the growth (as OD_{600}) of control yeast cultured in the presence of ALA and yeast transformed with *Tb*HRG, *Lm*HR1 or *Ld*HR1 and cultured in the presence of 0.05 µM Hb and the absence of ALA. These curves were fitted to the data using the Condoseo application of the Genedata Screener software (Genedata, Inc., Basel, Switzerland), allowing the determination of EC_{50}, the drug concentration required for half-maximal inhibition of the cellular growth rate [28] (Table 1).

Fig. 4 represents examples of the four basic types of dose-response curves obtained for compounds: a) selected compounds that inhibited the growth of yeast expressing TrypHRG proteins but not of control cells in the presence of ALA (at least until 20 µM); b) selected compounds active against yeast expressing TrypHRG proteins at concentrations at least three times lower than that required to inhibit the growth of control cells; c) nonselected compounds that were not active against yeast expressing TrypHRG proteins (not reproducing the effect observed in the initial screening) or not effective at concentrations three times lower than those necessary to inhibit the control; and d) nonselected compounds showing similar toxicity in yeast expressing TrypHRG proteins and control cells. This assay allowed us to eliminate compounds that were toxic to yeast, false positives and nonselective, and to select 25 active compounds (2.48% of the original collection) from the 66 selected above. Most of these compounds inhibited all TrypHRG transporters in this toxicity assay.

2.3. Effect of the selected compounds on T. brucei and Leishmania spp

Selected compounds were purchased and evaluated as described in the experimental section against BSF of *T. brucei* and the promastigote form of *Leishmania major*. Compounds active against *L. major* promastigotes were also assayed against intracellular amastigote forms of *L. major* and *L. donovani* (Table 2). For all parasites, the highest concentration assayed was 10 µM.

In the case of BSF of *T. brucei*, 15 of the 25 compounds assayed presented an EC_{50} below 10 µM (66%) (Table 2). Three of these compounds had an activity of between 1 and 10 µM, nine between 0.1 and 1 µM and three had EC_{50} values under 0.1 µM. The three most active compounds against *T. brucei* presented EC_{50} values of 2.3 nM (bleomycin), 60 nM (pyrvinium pamoate) and 70 nM (lauryl gallate).

For the promastigote forms of *L. major*, 11 of the 25 compounds assayed presented an EC_{50} below 10 µM (44%). Of these, three

Table 1
Activity of selected compounds on different yeast strains.

	Control	*Tb*HRG	*Lm*HR1	*Ld*HR1
Acriflavinium hydrochloride	>20	>20	>20	20
Acrisrocin	>20	>20	4.96	>20
Amiodarone hydrochloride	>20	12.93	8.91	7.07
Atorvastatin calcium	>20	>20	>20	>20
Benzalkonium chloride	>20	2.52	7.06	5.32
Benzbromarone	>20	6.87	2.0	3.66
Benzethonium chloride	>20	1.93	6.87	6.18
Bht. Butylated hydroxutoluene	>20	16.3	12.1	12.5
Bifonazole	3.2	2.69	2	4
Bithionate sodium	4.360	1.37	0.698	0.941
Bleomycin	3.961	0.678	0.074	0.127
Cetrimonium bromide	4.19	1.28	1.33	1.82
Cetylpyridinium chloride	7.50	1.87	2.37	3.81
Chlorhexidine	15.21	1.75	1.77	1.68
Chloroxine	7.52	6.52	4.3	6.3
Ciclopirox olamine	3.2	3.3	3.1	3.1
Clioquinol	3.1	1.8	1.5	1.7
Clomiphene citrate	>20	13.1	13.8	16,9
Clotrimazole	1.610	0.131	0.135	0.124
Cloxiquin	15.1	14.2	12.5	15.3
Cycloheximide	0.212	0.063	0.039	0.044
Dactinomycin	>20	>20	15.7	18.4
Daunorubicin	>20	>20	>20	>20
Disulfiram	13.1	15.3	6.2	11.6
Doxorubicin	>20	>20	>20	>20
Epirubicin hydrochloride	>20	>20	>20	>20
Estradiol acetate	>20	>20	>20	>20
Florfenicol	>20	3.0	4.3	6.3
Fluconazole	>20	13.6	11.7	18.33
Fluorescein	>20	>20	4.7	18.2
Fluorouracil	3.45	3.54	0.94	1.21
Gentian violet	1.57	0.74	1.01	2.02
Hexachlorophene	7.71	1.86	1.02	1.30
Iodoquinol	14.1	10.2	6.8	8.7
Ketoconazole	4.53	0.45	0.34	0.59
Lauryl gallate	>20	12.3	5.2	7.4
Lynestrenol	>20	7.2	5.0	7.1
Mafenide hydrochloride	>20	>20	>20	>20
Meclinize hydrochloride	>20	>20	12.3	>20
Meclocycline sulfosalicylate	>20	>20	>20	>20
Meclofenamate sodium	>20	>20	18.2	17.9
Menadione	>20	3.82	0.37	1.08
Merbromin	>20	7.3	3.9	4.6
Methoxamine hydrochloride	>20	>20	>20	>20
Methylbenzethonium chloride	7.3	2.4	7.8	7.4
Methylene blue	>20	>20	10.1	19.9
Mycophenolic acid	>20	18.3	2.5	15.2
Oxiconazole nitrate	<0.039	<0.039	<0.039	<0.039
Oxyquinoline hemisulfate	>20	>20	>20	>20
Pararosaniline pamoate	>20	>20	>20	>20
Penfluridol	>20	>20	17.1	>20
Phenelzine sulfate	>20	>20	16.0	18.3
Phenoxybenzamine hydrochloride	>20	>20	9.1	>20
Phenylmercuric acetate	<0.039	<0.039	<0.039	<0.039
Pyrithione zinc	>20	15.37	>20	>20
Pyrvinium pamoate	>20	13.3	10.2	15.1
Rosuvastatin calcium	>20	>20	>20	>20
Sirolimus	<0.039	<0.039	<0.039	<0.039
Sulconazole nitrate	0.469	0.377	0.041	0.127
Suloctidil	>20	>20	>20	>20
Tamoxifen citrate	15.2	7.8	11.0	15.3
Thimerosal	0.23	0.21	0.15	0.20
Tioconazole	0.092	<0.039	<0.039	<0.039
Toremiphene citrate	>20	>20	>20	>20
Triclosan	16.1	13.6	10.2	10.3
Undecylenic acid	>20	>20	8.4	>20
Zoxazolamine	>20	>20	>20	>20

Half-maximal effective concentration (EC_{50}) values obtained from the dose-response curves performed with the compounds selected from the HTS screening. Selected compounds are highlighted in bold.

Table 2
Parasiticidal effect of selected TrypHRG inhibitors.

	T. brucei	*L. major*		*L. donovani*	THP-1 (Toxicity)
	Bloodstream forms	Promastigotes	Amastigotes	Amastigotes	
Acrisorcin (4-hexylresorcinol)	>10	>10	n.d.	n.d.	>10
Acrisorcin (9-aminoacridine)	0.917 ± 0.045	1.628 ± 0.222	4.076 ± 0.410	4.055 ± 0.254	7.421 ± 0.101
Amiodarone hydrochloride	2.19 ± 0.49	1.701 ± 0.59	2.425 ± 0.202	7.156 ± 0.638	>10
Benzalkonium chloride	0.122 ± 0.011	0.231 ± 0.096	0.129 ± 0.021	1.364 ± 0.361	4.382 ± 0.026
Benzbromarone	>10	>10	n.d.	n.d.	n.d.
Benzethonium chloride	0.241 ± 0.028	0.261 ± 0.037	0.250 ± 0.064	>10	>10
Bithionate sodium	>10	>10	n.d.	n.d.	n.d
Bleomycin	0.0023 ± 0.001	0.605 ± 0.111	>10	2.913 ± 0.713	>10
Cetrimonium bromide	2.489 ± 0.436	1.276 ± 0.070	2.486 ± 0.295	1.155 ± 0.108	6.083 ± 0.780
Cetylpyridinium chloride	0.185 ± 0.022	0.189 ± 0.084	0.486 ± 0.150	0.382 ± 0.167	6.033 ± 0.569
Clorhexidine	0.247 ± 0.024	0.299 ± 0.093	0.458 ± 0.101	1.006 ± 0.024	>10
Clotrimazole	7.764 ± 0.112	>10	n.d.	n.d.	n.d.
Cycloheximide	0.146 ± 0.014	0.165 ± 0.016	0.321 ± 0.039	1.180 ± 0.256	8.505 ± 3.039
Florfenicol	>10	>10	n.d.	n.d.	n.d.
Fluorescein	>10	>10	n.d.	n.d.	n.d.
Fluorouracil	>10	>10	n.d.	n.d.	n.d.
Hexachlorophene	>10	>10	n.d.	n.d.	n.d.
Ketoconazole	>10	>10	n.d.	n.d.	n.d.
Lauryl gallate	0.070 ± 0.015	>10	n.d.	n.d.	n.d.
Lynestrenol	>10	>10	n.d.	n.d.	n.d.
Menadione	0.998 ± 0.332	0.300 ± 0.061	1.279 ± 0.385	3.305 ± 0.343	7.08 ± 0.852
Merbromin	0.152 ± 0.086	>10	n.d.	n.d.	n.d.
Mycophenolic acid	0.287 ± 0.024	>10	n.d.	n.d.	n.d.
Pyrvinium pamoate	0.060 ± 0.011	0.024 ± 0.007	0.024 ± 0.006	0.449 ± 0.168	8.284 ± 1.464
Sulconazole nitrate	>10	>10	n.d.	n.d.	n.d.

EC_{50} values (μM) of the selected compounds from Table 1 on BSF of *T. brucei*, promastigotes and intracellular amastigote forms of *L. major* and *L. donovani*. Toxicity on THP-1 cells is also shown. Acrisorcin (Table 1) is a combination of the active ingredients 9-aminoacridine and 4-hexylresorcinol, which we tested separately here. Experiments were performed three times in duplicate and data expressed as mean ± SEM. n.d.: not determined. Most interesting compounds (EC_{50} values in the parasites between 1 and 10 μM marked in blue, between 0.1 and 1 μM in green and under 0.1 μM in red) are highlighted in bold. n.d.: not determined.

were active between 1 and 10 μM, whereas seven had EC_{50} values between 0.1 and 1 μM, and one was active below 0.1 μM. This most active compound (pyrvinium pamoate) had an EC_{50} of 24 nM. These promastigote forms are clinically less interesting; however, they serve to validate the test since LmHR1 is essential at this stage [6] and they have also been used for a first screening of leishmanicidal compounds [29].

Those 11 compounds with an EC_{50} below 10 μM in the promastigote forms of *L. major* were tested against intracellular amastigote forms of *L. major* and *L. donovani* (Table 2). Beforehand, we measured their cytotoxicity against the human THP-1 macrophage cell line used as a host for the parasites. Most of the compounds tested for toxicity presented an EC_{50} greater than 5 μM (Table 2); only benzalkonium chloride was lower (EC_{50} 4.38 ± 0.03 μM) but, also, as will be seen below, far higher than that seen in the intracellular amastigote forms of *L. major* and *L. donovani*. We then analysed the effect of these compounds against intracellular amastigotes of *L. major* and *L. donovani*, calculating the selectivity indexes (IS) for each active compound. To this end, we used *L. major* [30] and *L. donovani* strains expressing the luciferase gene (generated as described in Materials and Methods) to infect the macrophage-differentiated THP-1 cells. These infected cells were incubated for 120 h with increasing concentrations of the selected compounds, and amastigote growth was determined by measuring the luminescence of *Leishmania*-infected macrophages [31]. In the case of *L. major* intracellular amastigotes, 10 of the 11 compounds assayed (90.9%) showed EC_{50} values lower than 5 μM. Of these, four were active between 1 and 5 μM, five had activities between 0.1 and 1 μM (with Selectivity Index (SI) ranging from 12.4 to >40) and one was active below 0.1 μM. This last compound was, as seen in *L. major* promastigotes, pyrvinium pamoate, presenting the same EC_{50} value (24 nM) and an SI of 345. With respect to intracellular amastigotes of *L. donovani*, 10 of the 11 compounds assayed (90.9%) were active under 5 μM. Eight had EC_{50} values between 1 and 5 μM, whereas two were active between 0.1 and 1 μM. The two most active compounds against *L. donovani* intracellular amastigotes presented EC_{50} values of 382 nM (cetylpyridinium chloride, SI 15.8) and 449 nM (pyrvinium pamoate, SI 18.4).

Many of the compounds selected against TrypHRG proteins in the yeast test presented activity against *T. brucei* (15/25) and *Leishmania* (11/25), with most (about 75%) being effective against both species. The fact that they were compounds active for *T. brucei* and *Leishmania spp.* supported that they inhibit a common protein in both species, such as HRG proteins. Indeed, in the yeast assay, most of these compounds inhibited all TrypHRG transporters. However, some compounds were active only against *T. brucei*: clotrimazole (EC_{50}: 7.76 μM), lauryl gallate (EC_{50}: 70 nM), merbromin (EC_{50}: 152 nM) and mycophenolic acid (EC_{50}: 287 nM) (Table 2). This result could be due to the compounds being internalized more effectively in one parasite compared with the other or that they effect their antiparasitic action by inhibiting a target other than the TrypHRGs.

Among these active compounds, there are some whose effect against *Leishmania* spp. and/or *T. brucei* has already been described in the literature, although in most cases their targets are unknown. Examples include pyrvinium pamoate, amiodarone hydrochloride, bleomycin, menadione (vitamin K3,) benzethonium chloride, cycloheximide and 9-aminoacridine. On the other hand, we did not find any reference to activity against these trypanosomatid parasites for the other compounds identified as active in this study. Some examples include benzalkonium chloride, cetylpyridinium chloride, merbromin, cetrimonium bromide, chlorhexidine, and lauryl gallate. See Supplementary Material for more details.

2.4. Effect of selected compounds on human HRG1 (Hs*HRG1*)

Humans also present an HRG protein (*Hs*HRG1) that plays an important role in heme recycling from senescent erythrocytes [32]. It cannot be ruled out that the inhibitors identified in Table 3 (IC_{50} at least three-fold lower in yeast expressing any of the TrypHRGs than in control cells) also affect the human transporter. Therefore,

Table 3
Effect of selected compounds on Δ*hem1* yeasts expressing human HRG1 (*Hs*HRG1).

	*Hs*HRG1
Acrisorcin (4-hexylresorcinol)	>20
Acrisorcin (9-aminoacridine)	>20
Amiodarone hydrochloride	16.2
Benzalkonium chloride	8.59
Benzbromarone	12.5
Benzethonium chloride	16.7
Bithionate sodium	4.91
Bleomycin	0.185
Cetrimonium bromide	8.06
Cetylpyridinium chloride	2.28
Clorhexidine	1.93
Clotrimazole	0.392
Cycloheximide	<0.02
Florfenicol	>20
Fluorescein	>20
Fluorouracil	>20
Hexachlorophene	3.68
Ketoconazole	12.2
Lauryl gallate	4.51
Lynestrenol	>20
Menadione	0.507
Merbromin	3.28
Mycophenolic acid	>20
Pyrvinium pamoate	>20
Sulconazole nitrate	0.983

EC_{50} values (μM) obtained from dose-response curves of the selected compounds from Table 1 on Δ*hem1* yeast expressing HsHRG1. Compounds showed selectivity (> 3-fold) with at least one of the TrypHRGs are highlighted in bold.

we have analysed their selectivity using Δ*hem1* yeasts expressing *Hs*HRG1. This protein increases yeast cytosolic heme levels after Hb endocytosis (Fig. S1). As shown in Table 3, almost 70% of these compounds (16 from 24) showed selectivity (> three-fold) with at least one of the TrypHRGs, probably because of the low identity of *Hs*HRGs with that of the parasites (around 15%) [6]. Furthermore, it is likely that a possible partial inhibition of the human transporter during the administration of a TrypHRG inhibitor would not have many consequences as *Hs*HRG1 knockout mice do not present any problems as long as they have sufficient iron intake in the diet; these mice avoid heme toxicity by producing inert hemozoin, just like the malaria parasite *Plasmodium* [33].

3. Conclusion

In this work, we have developed and validated two yeast-based assays that allow HTS of inhibitors of HRG transporters in trypanosomatid parasites. Many of the inhibitors identified presented activity in the submicromolar range on the BSF of *T. brucei* and promastigote and amastigote forms of *L. major* and *L. donovani*. In addition to serving as proof of concept for future screenings of collections with a greater number of compounds, some of the inhibitors identified may be of interest since they come from a collection of drugs in clinical use, therefore, their safety profiles are well established. Moreover, this type of yeast-based HTS to select HRG inhibitors could find wider use. Besides TrypHRGs, this transporter family is present in other human and animal disease-causing pathogens. For example, the nematode that produces filariasis in humans (*Brugia malayi*) and the barber's pole worm (*Haemonchus contortus*) are also auxotrophic for heme and require HRG proteins (BmHRG-1 and HcHRG-1, respectively) for heme acquisition and survival [34,35]. HRG proteins are, therefore, also target candidates for the control of nematode infection [35,36]. In addition, HRG proteins are important for disease vector organisms in humans and other animals, such as ticks [37].

4. Materials and methods

The chemical compounds, strains and culture conditions used, the strategy followed for gene deletion in *L. major* using the CRISPR-Cas9 system and the expression of *Ld*HR1 in yeast are described in Supplementary Material.

4.1. Heterologous expression of TrypHRG in yeast and generation of bioluminescent Leishmania lines

*Tb*HRG (Tb927.8.6010), *Lm*HR1 (LmjF.24.2230) and *Ld*HR1 (LDHU3_24.2870) were expressed in yeast as described in [6]. To study intracellular amastigote susceptibility to these compounds, we generated *L. major* and *L. donovani* expressing the luciferase gene as described in [30].

4.2. HTS test

For the yeast growth-based test, *Tb*HRG-expressing *hem1*Δ yeasts were incubated at a final OD_{600} = 0.1 in SC-medium supplemented with 0.05 μM of Hb and 20 μM of each compound in 96-well plates in a volume of 100 μl per well. Control yeast grown with Hb and *Tb*HRG-expressing yeast grown without Hb were used as negative controls, and *Tb*HRG grown with Hb but without drug were used as positive controls. After 24 h incubation at 30 ºC, OD_{600} was measured. The Z-factor of this test was determined for each TrypHRG as described in [22], via the following formula:

$$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{\mu_{c+} + \mu_{c-}}$$

Where σ is the typical deviation (σ_{c+} for positive controls and σ_{c-} for negative ones) and μ the mean of the values (μ_{c+} for positive controls and μ_{c-} for negative ones).

For the catalase-based test, yeast was incubated as described above but increasing the Hb concentration to 0.5 μM. After 24 h, 30 μl of 1% Triton X-100 and 30 μl of H_2O_2 (30% (w/w) in H_2O) were added to each well. Pictures of the plates were obtained after 5 min to allow the development of the foam.

4.3. Drug susceptibility analysis in parasites

The concentration of compound required to inhibit cell growth by 50% (EC_{50}) was determined as described for BSF of *T. brucei* S16, promastigotes of *Leishmania* spp THP-1 cells, or intracellular *Leishmania* amastigotes [31].

4.4. Selectivity analysis of the selected compounds with respect to human HRG (HsHRG1)

Δ*hem1* yeasts transformed with pESC-URA-HsHRG1 (NM_017842.2) were induced with 2% p/v raffinose and 0.4% p/v galactose as described in [10] and incubated as indicated above for the catalase-based test with 20 μM of each selected TrypHRG inhibitor. Those compounds that did not prevent foam formation were identified (IC_{50}>20uM) and, along with the other compounds, dose-response curves were performed as described above.

Declarations

Ethical approval: Not required.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2024.107092.

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