AD_____

Award Number: W81XWH-09-1-0429

TITLE: Screening for Inhibitors of Essential Leishmania Glucose Transporters

PRINCIPAL INVESTIGATOR: Scott M. Landfear, Ph.D.

CONTRACTING ORGANIZATION: Oregon Health & Science University
Portland, OR 97239

REPORT DATE: July 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-07-2011	Annual	1 JUL 2010 - 30 JUN 2011

4. TITLE AND SUBTITLE
Screening for Inhibitors of Essential Leishmania Glucose Transporters

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0429

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Scott M. Landfear, Ph.D.

E-Mail: landfear@ohsu.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Oregon Health & Science University
Portland, OR 97239

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The major objective of this project is to identify compounds that function as selective inhibitors of the essential glucose transporters of the parasite Leishmania mexicana. Toward this end, a cell growth assay has been developed that can be employed in a high throughput screen (HTS) for such inhibitors. The dye SYBR green has been identified as the optimal reagent for following cell growth by fluorescence. The assay has been optimized initially for a 96-well plate assay and subsequently for a higher density 384-well plate assay. Statistical criteria have been employed to demonstrate that the assay functions with high accuracy and reproducibility under control conditions, with a Z'-factor of approximately 0.9. This assay was subsequently employed in a 'scaling screen' of the 2000 compound MicroSource Discovery Spectrum Collection to yield a Z-factor of 0.84 in the screen of a real library. The high quality of the assay indicates that it is ready to proceed to an HTS of a much larger library. The assay has been transferred to the HTS facility at St. Jude Children's Research Hospital in Memphis, TN to initiate full high throughput screens.

15. SUBJECT TERMS
Leishmaniasis, drug development, selective inhibitors of essential parasite glucose transporters, high throughput screen, fluorescence cell growth assay, compound libraries

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	18	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Introduction:

Leishmania are parasitic protozoa that cause devastating diseases throughout much of the tropical and subtropical world (4), and infections of military personnel in the Middle East have become major medical problems for U.S. troops stationed in that region (see reports in DoD – GEISWeb: www.geis.fhp.osd.mil/GEIS/IDTopics/Leishmaniasis/LeishNavyPolicy.asp, entitled 'Leishmaniasis in Military Personnel Returning From Iraq' and the Militarycom web site: www.military.com/NewsContent/0,13319,FL_sick_032004,00.html, entitled 'Troops Being Treated For Leishmaniasis'). Drugs for treatment of leishmaniasis are generally expensive, toxic, and suffer from increasing occurrence of resistant parasites (6). Hence, identification of parasite-specific targets that could be exploited for development of novel drugs is of crucial importance. Our laboratory has demonstrated that the glucose transporters of *Leishmania mexicana* are essential for survival of *Leishmania* amastigotes inside mammalian host macrophages (2), suggesting that these important permeases could serve as critical targets for drug development. Furthermore, our laboratory has developed a cell-based assay (3) that can be employed in a high-throughput format to screen for compounds that selectively inhibit *Leishmania* but not human glucose transporters. The objective of this proposal is to first optimize this assay for use in a high-throughput screen (HTS) and to then employ the assay to screen large libraries of compounds for those that selectively inhibit the parasite glucose transporters. 'Hit' compounds that emerge from such screens will be further tested in glucose uptake assays to ensure that they are selective inhibitors of the parasite permeases. They will also be examined to determine their efficacy for growth inhibition of intracellular *Leishmania* parasites (amastigotes) and their 'therapeutic index', that is their relative toxicity for *Leishmania* parasites versus human cells. These latter screens will identify compounds able to inhibit parasite growth at concentrations that are not toxic to mammalian cells. Overall, the objective is to identify selective inhibitors of *Leishmania* glucose transporters that could serve as 'leads' for development of novel anti-leishmanial drugs.

Body:

Foundation of the cell-based assay for use in the HTS. The principle of the cell-based assay is that *Leishmania* parasites that express a functional glucose transporter will grow in medium that contains glucose as a central carbon source but that does not contain an alternate carbon source, proline. Thus *L. mexicana* promastigotes (insect stage parasites that can be easily cultured in vitro) in which the glucose transporter genes have been deleted, the Δ*lmgt* null mutants (2), are not able to grow in glucose-replete/proline-deficient (glucose +/proline -) medium (3). However, if these null mutants are complemented with the major glucose transporter gene from *L. mexicana*, *LmGT2*, they will grow in glucose +/proline – medium. (*L. mexicana* encode three glucose transporter genes within a single 14 kb locus: *LmGT1, LmGT2, and LmGT3* (1). The *LmGT2* gene is the most highly expressed of these 3 genes in *L. mexicana* promastigotes. All three of these linked genes are deleted in the Δ*lmgt* null mutant.) Similarly, if the Δ*lmgt* null mutant is complemented with the human glucose transporter gene, GLUT1, the parasites will also grow in glucose +/proline – medium. However, the growth of these complemented strains depends upon the function of the complementing glucose transporter. Thus, any compound that inhibits the complementing glucose transporter will strongly inhibit growth of the parasite line (3).

The HTS assay will screen for compounds that selectively inhibit growth of the null mutants expressing the LmGT2 protein but that do not significantly inhibit growth of null mutants expressing the human GLUT1 protein. Such compounds will be selective inhibitors of LmGT2 but not of GLUT1 and will thus selectively target the parasite glucose transporter. The screen will be performed first by identifying compounds in a library that inhibit growth of the LmGT2-expressing null mutants. This subset of compounds will subsequently be rescreened against the null mutant that is expressing GLUT1 to

identify those chemicals that do not inhibit this human transporter. Those compounds that are positive (inhibit parasite growth) in the first screen but negative (do not inhibit parasite growth) in the second screen will be candidates for selective inhibitors of *Leishmania* glucose transporters. These compounds will be further investigated, as detailed in the original proposal, to identify those that inhibit uptake of [^{3}H]glucose by LmGT2 but not by GLUT1 (i.e. hit compounds).

Optimization of the fluorescence method employed for the cell-based assay. In developing any HTS, it is necessary to expend considerable effort optimizing the assay so that it can be used effectively in an automated high-throughput format (Assay Guidance Manual, http://www.ncgc.nih.gov/guidance/manual_toc.html). For initial optimization of the cell growth assay, we have examined two fluorescence assays for utility in the HTS format: i) the alamarBlue assay that monitors growth by the ability of live cells to reduce the dye alamarBlue, resulting in a change in the fluorescence spectrum; ii) the SYBR green assay that quantifies the amount of parasite DNA present by forming a fluorescent complex between the dye and DNA. To summarize, we have now determined that the SYBR green assay is superior to the alamarBlue method for several reasons. SYBR green gives a stronger fluorescence signal (excitation 485 nm, emission 528 nm) on a per cell basis than alamarBlue, SYBR green is cheaper on a per well basis than alamarBlue, the SYBR green method requires fewer steps in the assay, since fluorescence is read immediately after the dye-stop solution is added, and the SYBR green assay is an 'end point' assay in which cells are lysed at the time of dye addition. The advantage of an end point assay is that different plates can be read at different times after dye addition without adding the complication of further differential cell growth between plates that would affect the absolute values of the fluorescence signal. In contrast, this latter complication is a potential deficiency of the alamarBlue method in which cell growth is not stopped by addition of dye. For these reasons, we have chosen the SYBR green method for all our subsequent assay development studies. Thus the first accomplishment of the research program was to identify the optimal fluorescence assay to be employed for the HTS.

Test of assay quality employing control samples representing high (High), medium (Med), and low (Low) cell growth. In the first stage of development of an HTS, the assay method must be monitored for assay quality employing control samples. The details of recommended quality control tests are provided by the online Assay Guidance Manual published by Eli Lilly and company and the NIH Chemical Genomics Center (http://www.ncgc.nih.gov/guidance/manual_toc.html) that constitutes the 'gold standard' for development of an HTS. We have subsequently evaluated the SYBR green assay using first 96-well plates (data not shown but similar to that reported here) followed by miniaturization of the assay for 384-well plates that could be employed in a genuine HTS (data reported here). These control experiments have been performed on a robotics station (Synergy 4, Biotek, Winooski, VT) that performs all pipeting and fluorescence measurements in a fully automated format. In a critical evaluation of the method, a 'uniformity assay' was performed in which samples representing high cell growth (High, no added growth inhibitor), medium cell growth (Med, employing ~IC_{50} concentration of the drug phleomycin, 1.4 μM), and low cell growth (Low, employing a maximally inhibiting concentration of phleomycin, 1 mM) were arrayed in three 384-well plates in an interleaved pattern such that each well in the 384-well array received a High, Med, and Low sample among the three plates. These plates were read and the fluorescence values for all wells were measured. The purpose was to determine whether identical samples gave sufficiently reproducible readings across all wells in all three plates and whether there were any significant edge or position effects that create systematic errors in specific regions of the plates.

The results of this uniformity assay are presented in Table 1. This table summarizes the statistical criteria (Z'-factor, a statistical value that monitors assay quality (7); CV, coefficient of variation for each of the High, Med, and Low sets of samples; SD, standard deviation) and demonstrates that the

experimental values for this assay are well within the range of the 'acceptance criteria', as defined in the Assay Guidance Manual.

Table 1. Summary of statistical variables calculated for the uniformity assay done with Δ*lmgt* cells expressing the LmGT2 transporter. Cells were inoculated into 384-well plates at the indicated cell density and volume. Cell growth was terminated after 72 hr by addition of 5 µl SYBR green stop solution, and fluorescence (excitation 485 nm, emission 528 nm) was read.

PLATE	Initial cell density (ml^{-1})	Final vol/well	Inc. Time	Z'	CV Min %	CV mid %	CV Max %	Mean mid % inh	SD % mid inh
1	2.5×10^6	50 ul	72 hr	0.91	2.29	3.45	2.31	45.24	4.89
2	2.5×10^6	50 ul	72 hr	0.90	2.30	3.38	2.67	43.40	5.34
3	2.5×10^6	50 ul	72 hr	0.89	2.43	3.18	2.85	44.38	4.77
Acceptance criteria				**>0.40**	**< 20%**	**< 20%**	**< 20%**	**30-70%**	**< 20%**

An illustration of the Z'-factor is shown in Fig. 1, taken from reference (7). The Z'-factor (the Z-factor for an experiment performed only with High, Med, and Low control samples but with no library samples) is defined as the Separation Band/Dynamic Range and measures the signal compared to the variability of the data from well to well for both the High and Low control samples. The formula for calculating the Z'-factor is: $Z' = 1 - [(3SD_H + 3SD_L/(Av_H - Av_L)]$ where SD_H and SD_L represent the standard deviations of measurements for High and Low control samples and Av_H and Av_L represent the means of such measurements, respectively. A perfect assay would have a Z' value of 1.0, i.e. there would be no data variability, and the Separation Band would be equal to the Dynamic Range.

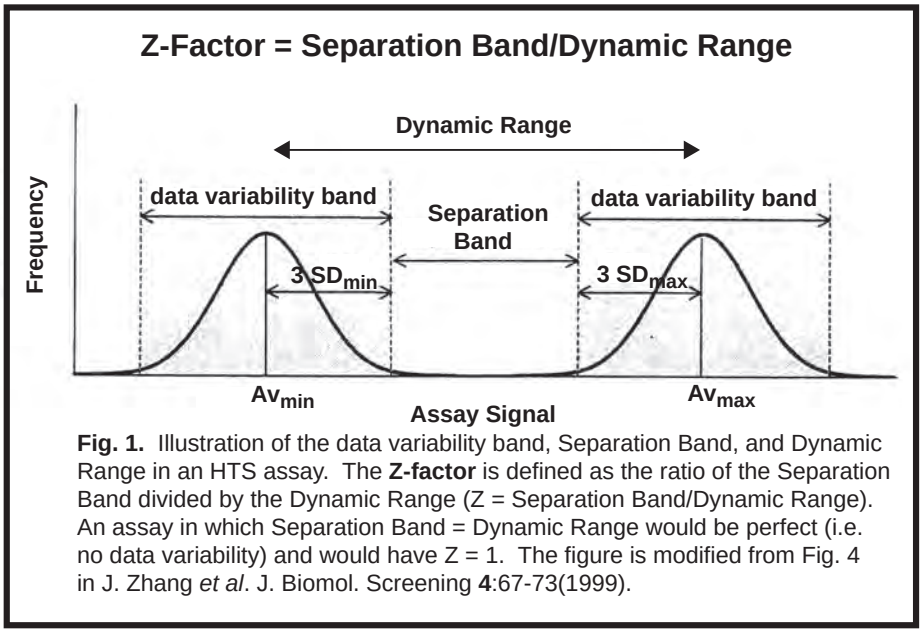

Fig. 1. Illustration of the data variability band, Separation Band, and Dynamic Range in an HTS assay. The **Z-factor** is defined as the ratio of the Separation Band divided by the Dynamic Range (Z = Separation Band/Dynamic Range). An assay in which Separation Band = Dynamic Range would be perfect (i.e. no data variability) and would have Z = 1. The figure is modified from Fig. 4 in J. Zhang *et al*. J. Biomol. Screening **4**:67-73(1999).

In practice, all assays exhibit some degree of data variability. The Z'-factor allows a quantitative measure of variability compared to signal strength and thus is a measure of assay quality. The Z' value

must be >0.40 for an acceptable assay method (Assay Guidance Manual). Table 1 indicates that our assay generates Z' values of almost 0.90 for each of the three plates tested, revealing an extremely robust assay.

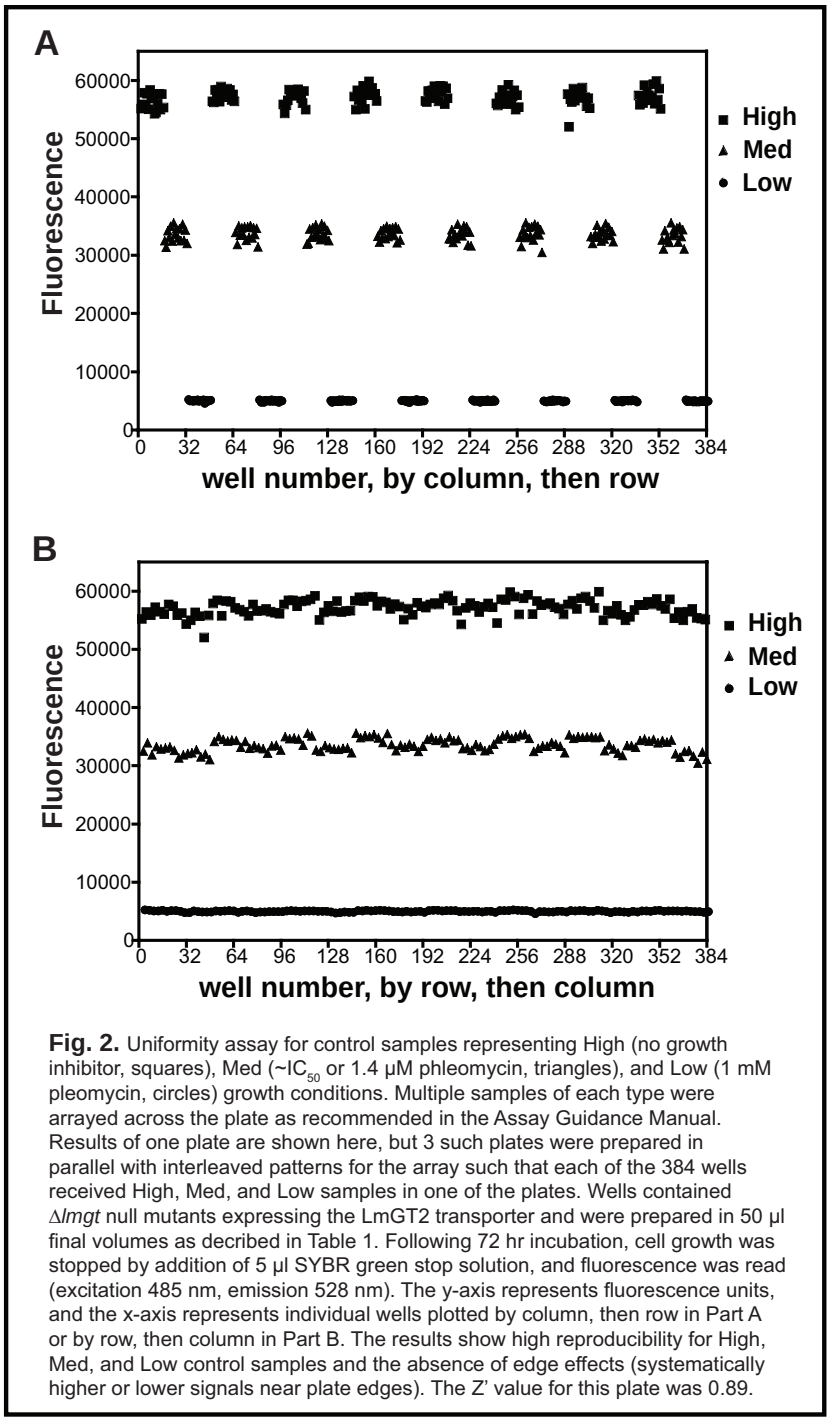

Fig. 2. Uniformity assay for control samples representing High (no growth inhibitor, squares), Med (~IC_{50} or 1.4 µM phleomycin, triangles), and Low (1 mM pleomycin, circles) growth conditions. Multiple samples of each type were arrayed across the plate as recommended in the Assay Guidance Manual. Results of one plate are shown here, but 3 such plates were prepared in parallel with interleaved patterns for the array such that each of the 384 wells received High, Med, and Low samples in one of the plates. Wells contained Δ*lmgt* null mutants expressing the LmGT2 transporter and were prepared in 50 µl final volumes as decribed in Table 1. Following 72 hr incubation, cell growth was stopped by addition of 5 µl SYBR green stop solution, and fluorescence was read (excitation 485 nm, emission 528 nm). The y-axis represents fluorescence units, and the x-axis represents individual wells plotted by column, then row in Part A or by row, then column in Part B. The results show high reproducibility for High, Med, and Low control samples and the absence of edge effects (systematically higher or lower signals near plate edges). The Z' value for this plate was 0.89.

In addition, Figure 2 shows two plots, in different formats, of the data from one plate of this uniformity assay. These plots reveal that the variation is very low for fluorescence values of replicate High, Medium, and Low samples arrayed in different wells of the plate, and there are no significant edge or position effects within the plates that would contribute to false positive or false negative effects. Hence, the assay has passed the first set of criteria for a high quality screening method and is ready to go into

production for screening of a small library. This so-called 'scaling screen' tests the assay in a screen of a real library, is an essential step in assay development (Assay Guidance Manual), and is a major objective of Specific Aim 1 of the original proposal.

Test of assay quality employing a 'scaling screen' of a small library of compounds. Following optimization of the SYBR green assay using control High, Med, and Low samples, it is necessary to test the assay against a small library to ensure that it is functioning robustly enough to employ in a genuine HTS. The principal criterion of acceptance employed at this stage is the Z-factor (7), a statistical value that is calculated similarly to the Z'-factor mentioned above but for the screen of the chemical library rather than for the measurements performed on control samples only. The formula for the Z-factor of a library screen is the same as that for the Z'-factor, except that SD_H and Av_H are replaced by SD_S and Av_S, the standard deviations and means for values determined for the library samples rather than the High control samples. As above, a Z-factor of >0.4 is considered to represent a screen of sufficient quality for advancement to the HTS stage.

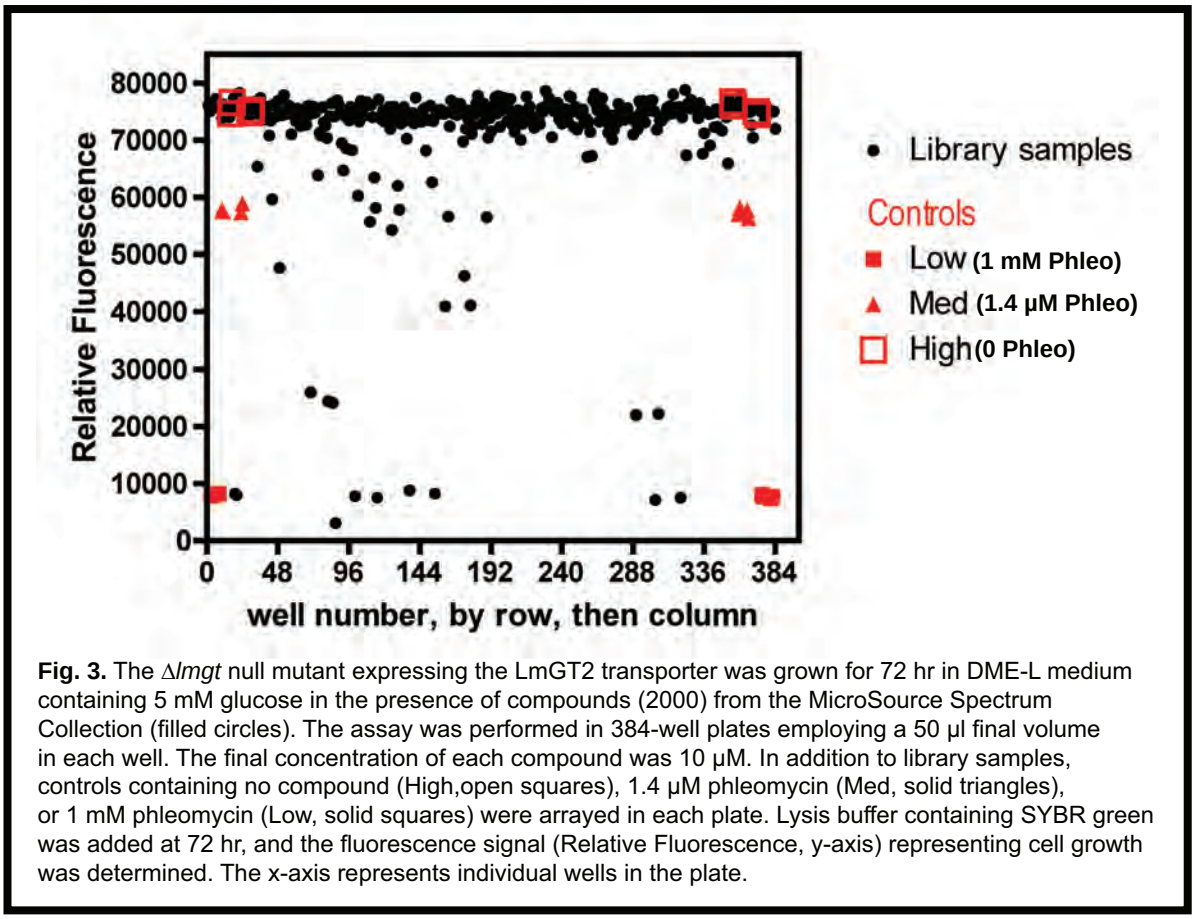

Fig. 3. The Δ*lmgt* null mutant expressing the LmGT2 transporter was grown for 72 hr in DME-L medium containing 5 mM glucose in the presence of compounds (2000) from the MicroSource Spectrum Collection (filled circles). The assay was performed in 384-well plates employing a 50 µl final volume in each well. The final concentration of each compound was 10 µM. In addition to library samples, controls containing no compound (High,open squares), 1.4 µM phleomycin (Med, solid triangles), or 1 mM phleomycin (Low, solid squares) were arrayed in each plate. Lysis buffer containing SYBR green was added at 72 hr, and the fluorescence signal (Relative Fluorescence, y-axis) representing cell growth was determined. The x-axis represents individual wells in the plate.

The library employed in the scaling screen was the MicroSource Discovery Spectrum Collection, a library consisting of ~2000 compounds of which ~1000 are approved drug components, ~600 are natural products, and ~400 are other bioactive components. The library was constructed to have a wide range of biological activities and structural diversity. The library was screened in duplicate employing 13 384-well plates. High, Med, and Low control samples were also arrayed within each plate. Each well contained 20 µl of Δ*lmgt* parasites, complemented with either LmGT2 or GLUT1, suspended in DME-L medium (5) at an initial cell density of 5.6×10^6 cells/ml, and 25 µl of each compound, as a solution of

1% DMSO, to provide a final concentration of 10 μM compound. Parasites were grown for 72 hr at 26°C, after which 5 μl of stop solution containing a 100-fold dilution of commercial stock SYBR green (Sigma, St. Louis, MO) in 10% Triton X-100 was added to terminate cell growth and generate a fluorescence signal that was proportional to the cell density in each well. Fluorescence was read using the robotic platform, and the results were exported as an Excel file for data analysis.

Fig. 3 shows the results of the read from one of the 384-well plates. This figure demonstrates that the compounds from the library (data represented by solid circles) gave a range of inhibition of growth of parasites expressing LmGT2. Most of the compounds resulted in little or no inhibition of growth, similar to data from parasites incubated without any compound, and a limited number of compounds gave a high level of growth inhibition close to that of the Low control samples. Employing data from all 13 plates, the Z-factor was 0.84 for the library samples, and a Z'-factor calculated from the High and Low control samples was 0.92. These results indicate that the assay performs extremely robustly both when employing controls and when assaying a library of compounds. Hence, the assay is now ready to proceed in screening of larger compound libraries.

In addition to the primary function of establishing the quality of the assay method, the scaling screen might identify compounds that differently inhibit LmGT2 compared to GLUT1. To determine whether any such compounds were detected in the screen, a subset of 140 compounds was identified that inhibited by 50% or more growth of parasites expressing LmGT2. Subsequently, the fluorescence signal for parasites exposed to each of these 140 compounds was determined for the parasites expressing LmGT2 (y-axis of Fig. 4) versus those expressing GLUT1 (x-axis of Fig 4). In this plot,

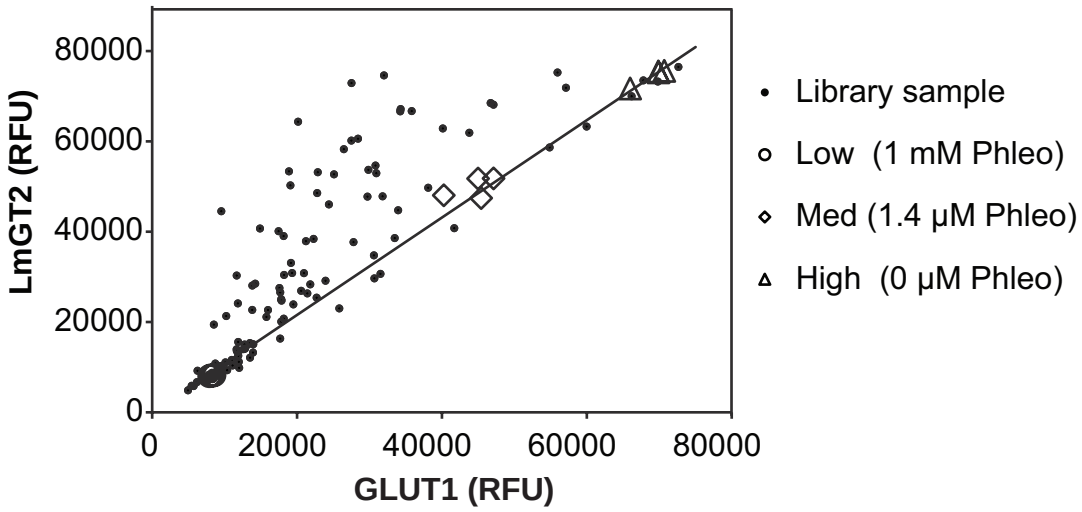

Fig. 4. The relative inhibition of growth of Δ*lmgt* null mutants expressing either LmGT2 or human GLUT1 was compared. The 140 compounds tested (filled circles; Library sample) were those from the MicroSource Discovery Collection that inhibited growth of LmGT2 expressing parasites by >50%. These compounds were retested for their ability to inhibit growth of parasites expressing GLUT1. The Relative Flurorescence Units (RFU), representing cell growth, were plotted for LmGT2 expressing parasites on the y-axis and for GLUT1 expressing parasites on the x-axis. The straight line was fitted to the data for phleomycin (Phleo; the Low, Med, and High data), a compound that inhibits growth of LmGT2 and GLUT1 expressing parasites equally. Compounds that selectively inhibit growth of LmGT2 expressing parasites over GLUT1 expressing parasites would fall below the straight line.

compounds that inhibit LmGT2 better than GLUT1 would be represented by spots below the line in the

graph. (The line was determined by a least squares fit from control High, Medium, and Low samples only and represents data for a compound, phleomycin, that inhibits growth of both parasites lines equally.) It is clear from Fig. 4 that none of the 140 compounds inhibited growth of LmGT2 expressing parasites significantly more than they inhibited growth of GLUT1 expressing parasites. Hence, this screen of a small library has not detected any compounds that preferentially inhibit the parasite glucose transporter. Screens of considerably larger libraries will constitute the next step in this program and will attempt to detect selective LmGT2 inhibitors among a much larger group of compounds (up to 500,000).

Transfer of the HTS Assay to Automated Screening Facility at St. Jude Children's Research Hospital. As described in our original proposal, the high throughput screens will be performed at the cutting edge fully automated screening facility of our collaborator, Dr. Kip Guy, Chair of the Department of Therapeutics and Chemical Biology at St. Jude Children's Research Hospital. We believe this facility is likely the best one in the world in which to perform these studies, given Dr. Guy's outstanding reputation in development and execution of high throughput screens and his longstanding interest in molecular parasitology. During the course of the previous year, St. Jude has initiated a stringent review process that must be carried out for all such collaborative projects. For this reason, we were unexpectedly required to schedule and present a detailed description of our assay to their review committee. Unfortunately, this process proved to be quite time consuming. The committee did not schedule a meeting for several months, and it subsequently took them several more months to render a decision. However approval was obtained, and we were able to schedule a first visit to St. Jude in late May of this year. During this visit, two of our personnel (Diana Ortiz and Carolyn Elya) established cultures of the appropriate *Leishmania* strains at St. Jude and carried out uniformity assays. A scaling screen of a St. Jude library consisting of ~4000 compounds is currently being performed. We are also obtaining support from Dr. Armand Guiguemde, a senior research associate in Dr. Guy's group, to carry out this screening project.

The unexpected administrative obstacles at St. Jude, which were completely beyond our control, have delayed the schedule for screening, but the authorization to proceed has been obtained and the experiments are now in progress.

Key Research Accomplishments:

- Determined that SYBR green represents a superior assay method, compared to alamarBlue, to monitor cell growth for a HTS. This method provides high fluorescence signal at low cost and has the advantage that it is an 'end point' assay.

- Established, using control samples representing High, Medium, and Low cell growth, that the cell growth assay performed both in 96-well and 384-well plates meets robust statistical criteria for use in a HTS, as outlined in the Assay Guidance Manual. In particular, a Z'-factor of close to 0.9 was obtained from these control experiments, far above the acceptable level of 0.4.

- Also established using 'uniformity assays' that the assay method does not suffer from position effects within plates or from unacceptable variation from plate to plate or from day to day when separate assays are performed.

- Performed in duplicate a 'scaling screen' of the MicroSource Discovery Spectrum Collection library of ~2000 compounds to further validate the assay in a screen of a small library. This screen generated a Z-factor of 0.84, indicating that the automated assay method is extremely robust.

- Searched for compounds in the MicroSource library that might differentially inhibit the parasite LmGT2 transporter compared to the human GLUT1 glucose transporter. No such compounds were detected in this small-scale screen, indicating that screens of considerably larger libraries are warranted.

- Assay has been transferred to St. Jude Children's Hospital HTS facility operated by our collaborator Dr. Kip Guy. This transfer entailed visits to St. Jude by two of our personnel and optimization of the uniformity assays on the St. Jude fully automated screening decks. A scaling screen of a St. Jude library (~4000 compounds) is currently in progress. Following optimization of this scaling screen, full deck screens of a large library of compounds will be carried out.

Reportable Outcomes:

1. Seminar and research discussion, Department of Biochemistry, University of Iowa, January 2010.
2. Presentation of research results to the Portland Area Malaria Research Group in May 2010.
3. Research discussion, School of Pharmacy, University of Georgia, June 2010.
4. Research presentation to review committee, St Jude Children's Research Hospital, December 14, 2010.
5. Research presentation to Advinus Pharmaceuticals, January 6, 2011.
6. Research presentation to Merck, June 2, 2011.

Conclusion:

The importance of the research accomplished during the first two years of this grant is that an assay for detection of compounds that selectively inhibit *Leishmania* glucose transporters has been developed. This assay has been optimized and shown to function robustly, employing various statistical and reproducibility criteria. The assay is now fully validated for use in HTS of large chemical libraries, and the assay has been transferred to the HTS facility at St. Jude. We will be engaging in such HTS experiments in the coming year of the project.

'So what section'. The importance of this assay development is that it can be employed to identify 'hit' compounds that can be further explored for development of anti-leishmanial therapeutics. Initial hit compounds, those that selectively inhibit growth of parasites expressing LmGT2 compared to GLUT1, will be further examined for their ability to inhibit uptake of [^{3}H]glucose by LmGT2 compared to GLUT1 and for their ability to inhibit growth of *L. mexicana* amastigotes within mammalian macrophages. Compounds that inhibit growth of *Leishmania* parasites without significant toxicity to mammalian cells can be further pursued as potential leads for anti-leishmanial drugs.

References:

1. **Burchmore, R. J. S., and S. M. Landfear.** 1998. Differential regulation of multiple glucose transporter genes in the parasitic protozoan *Leishmania mexicana*. J. Biol. Chem. **273:**29118-29126.
2. **Burchmore, R. J. S., D. Rodriguez-Contreras, K. McBride, P. Merkel, M. P. Barrett, G. Modi, D. L. Sacks, and S. M. Landfear.** 2003. Genetic characterization of glucose transporter function in *Leishmania mexicana*. Proc. Natl. Acad. Sci. U S A **100:**3901-3906.
3. **Feistel, T., C. A. Hodson, D. H. Peyton, and S. M. Landfear.** 2008. An expression system to screen for inhibitors of parasite glucose transporters. Mol Biochem Parasitol **162:**71-6.
4. **Herwaldt, B. L.** 1999. Leishmaniasis. Lancet **354:**1191-1199.
5. **Iovannisci, D. M., and B. Ullman.** 1983. High efficiency plating method for *Leishmania* promastigotes in semidefined or completely-defined medium. J. Parasitol. **69:**633-636.

6. **Mishra, J., A. Saxena, and S. Singh.** 2007. Chemotherapy of leishmaniasis: past, present and future. Curr Med Chem **14:**1153-69.
7. **Zhang, J. H., T. D. Chung, and K. R. Oldenburg.** 1999. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen **4:**67-73.

Molecular & Biochemical Parasitology 162 (2008) 71–76

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

An expression system to screen for inhibitors of parasite glucose transporters

Torben Feistel[a], Cheryl A. Hodson[b], David H. Peyton[b], Scott M. Landfear[a,*]

[a] Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, OR 97239, USA
[b] Department of Chemistry, Portland State University, Portland, OR 97207, USA

ARTICLE INFO

Article history:
Received 14 May 2008
Received in revised form 16 July 2008
Accepted 17 July 2008
Available online 30 July 2008

Keywords:
Leishmania mexicana
Plasmodium falciparum
Null mutant
Glucose transporter
High-throughput screening

ABSTRACT

Chemotherapy of parasitic protists is limited by general toxicity, high expense and emergence of resistance to currently available drugs. Thus methods to identify new leads for further drug development are increasingly important. Previously, glucose transporters have been validated as new drug targets for protozoan parasites including *Plasmodium falciparum*, *Leishmania mexicana* and *Trypanosoma brucei*. A recently derived glucose transporter null mutant (Δ*lmgt*) of *L. mexicana* was used to functionally express various heterologous glucose transporters including those from *T. brucei* THT1, *P. falciparum* PfHT and human GLUT1—resulting in recovery of growth of the Δ*lmgt* null mutant in glucose replete medium. This heterologous expression system can be employed to screen for compounds that retard growth by inhibiting the expressed glucose transporter. The ability of this expression system to identify specific glucose transporter inhibitors was demonstrated using 3-*O*-undec-10-enyl-D-glucose, a previously described specific inhibitor of PfHT.

1. Introduction

Parasitic protozoa such as *Leishmania* species, *Trypanosoma brucei*, and *Plasmodium falciparum*, the causative agents of leishmaniasis, African sleeping sickness, and malaria, respectively, are responsible for onerous diseases that afflict millions of people across the globe. While drug therapies exist for each disease, they typically suffer from high expense, toxicity, and development of drug resistance, so that there is an urgent need for novel therapies [1–4]. One strategy to identify new drugs that could supplement or improve the current armamentarium is to target essential parasite pathways or biological processes in a search for selective inhibitors that can serve as leads for development of new anti-parasitic drugs.

Glucose is an important nutrient for many organisms, and uptake of sugars through glucose transporters has been demonstrated to be essential for viability of the infectious stages of *Leishmania mexicana* [5,6], *T. brucei* [7–10] and *P. falciparum* [11]. Hence, parasite glucose transporters may provide valid targets for identification of novel chemotherapies. Indeed previous studies by other groups have demonstrated that selective inhibitors of the *T. brucei* [12] or *P. falciparum* [11] glucose transporters are cytotoxic to those parasites and are able to kill the parasite in both culture and in animal models of infection. However, to explore potential inhibitors of parasite glucose permeases, it is essential to design an assay that would enable medium or high-throughput screens of chemical libraries for compounds that selectively inhibit these carriers. In this report, we describe the use of a glucose transporter null mutant of *L. mexicana*, designated Δ*lmgt* [5], to functionally express heterologous glucose transporters from several parasites and from humans. This null mutant was developed in the promastigote or insect stage of the parasite life cycle and, unlike the amastigote form that lives inside mammalian macrophages, is viable provided that an alternative energy source such as proline is present in the culture medium. Furthermore, Δ*lmgt* null mutants expressing heterologous glucose permeases are dependent upon both the permease and glucose for growth in medium replete in glucose but deficient in proline. Hence, these transgenic parasites can be employed in a cell growth assay to monitor for compounds that selectively inhibit each parasite glucose transporter but do not inhibit human glucose transporters such as GLUT1 [13–15]. We demonstrate here that such a cell growth assay, based upon complemented Δ*lmgt* mutants, can be used to monitor for selective inhibitors of the *P. falciparum* glucose transporter PfHT and hence represents a valid approach to screen

Abbreviations: BF, bloodstream form; DME-L, Dulbecco's modified Eagle medium adapted for *Leishmania*; iFBS, heat-inactivated fetal bovine serum; ORF, open reading frame; RFU, relative fluorescence unit.

* Corresponding author at: Department of Molecular Microbiology and Immunology, Oregon Health & Science University, 3181 S.W. Sam Jackson Park Road, Portland, OR 97239, USA. Tel.: +1 503 494 2426; fax: +1 503 494 6862.
E-mail address: landfear@ohsu.edu (S.M. Landfear).

0166-6851/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2008.07.005

small molecule libraries for inhibitors of parasite glucose transporters.

2. Materials and methods

2.1. Generation of complemented Δ*lmgt* cell lines and cell culture

The Δ*lmgt* null mutant was complemented individually with the *GLUT1* (NM006516), *PfHT* (GeneBank: AJ131457), *THT1* (GeneDB: Tb10.6k15.2040) or the *TgGT1* (GeneBank: AF518411) ORF. The region of each gene containing the ORF was subcloned into the *Leishmania* expression vector pX63NEO [16] transfected [5] into the Δ*lmgt* line, and selected in G418 (Cellgrow, Canada) containing medium to generate the Δ*lmgt*[*pGLUT1*], Δ*lmgt*[*pPfHT*], Δ*lmgt*[*pTgGT1*] and Δ*lmgt*[*pTHT1*] lines. Promastigotes of complemented Δ*lmgt* null mutant lines were cultured in RPMI 1640 medium (Gibco, USA), pH 7.2, supplemented with 10% heat-inactivated fetal bovine serum (iFBS) (HyClone, USA), 0.1 mM xanthine (Sigma, USA), and 5 μg/ml hemin (Sigma, USA), and 100 μg/ml G418. Continuous cultures were maintained by periodic dilution of logarithmic phase parasites, and new parasite cultures were initiated frequently from frozen stocks.

2.2. Uptake assays

Assays for uptake of [6-^{3}H(*N*)] D-glucose (Perkin-Elmer Life Sciences, USA) in promastigotes of wild type *L. mexicana*, Δ*lmgt*, and Δ*lmgt* complemented with each glucose transporter gene were performed as reported [17]. Wild type and Δ*lmgt* promastigotes in middle-late logarithmic phase of growth were assayed for sugar uptake at several substrate concentrations between 100 μM and 4 mM. Uptake assays were performed between 0 and 120 s and the data were fitted to a straight line by linear regression. Dose–response curves for compound 3361 were fitted by non-linear regression to a one-site competition model using Graph Pad Prism version 4.0b software (Graph Pad, USA).

2.3. alamarBlue™ assays

Cells were cultured to early log phase at 26 °C in RPMI 1640 medium (Gibco, USA), pH 7.2, supplemented with 10% iFBS, 0.1 mM xanthine and 5 μg/ml hemin containing 100 μg/ml G418. Cells were washed twice with Dulbecco's modified Eagle's medium adapted for *Leishmania* [18] (DME-L) (Gibco, USA) supplemented with 10% iFBS, 5 mM glucose (Sigma, USA), 0.1 mM xanthine and 5 μg/ml hemin at room temperature. Parasites in 50 μl DME-L were seeded in black bottom plates (Greiner, Germany) and mixed with 50 μl DME-L containing 2% DMSO (Mallinckrodt, USA) and twice the indicated concentration of each drug. Following an incubation time of 3 days in a humid chamber at 26 °C, 10 μl alamarBlue™ (Biosource, USA) were added and the incubation was continued for another 24 h. Relative fluorescence units were read using a Spektra Max Gemini XS plate reader (Molecular Devices, USA). Means and standard deviations were calculated in Microsoft Excel 2000 software. Dose–response curves were fitted as described above using Graph Pad Prism version 4.0b software.

2.4. Synthesis of 3-*O*-undec-10-enyl-D-glucose

3-*O*-Undec-10-enyl-D-glucose (compound 3361) was synthesized as described [19]. 1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose (2.6 g) was dissolved in 15 ml anhydrous DMSO and treated with 15 ml of 1.4 M solution of NaH in anhydrous DMSO (15 ml) dropwise while the solution was stirred and maintained at room temperature, followed by the dropwise addition of 11-bromo-1-undecene (4.29 g). The solution was stirred for 3 h, then quenched by adding 40 ml of ice water. The resulting solution was extracted with diethyl ether, concentrated under reduced pressure and subjected to flash chromatography on silica with $CHCl_3$ to isolated 3-*O*-undec-10-enyl-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose which was then converted to 3-*O*-undec-10-enyl-D-glucose (Compound 3361) by refluxing with Amberlite CG-120 (H+ type, 4.35 g) in water (100 ml) for 36 h. The reaction mixture was filtered and extracted with ether. The product was isolated from solvent under reduced pressure, subjected to flash chromatography on silica with $CHCl_3$/MeOH (10:1), recrystallized from ethanol and confirmed by NMR. M.P. 133.3–135.8 °C.

3. Results

3.1. Heterologous expression of glucose transporter homologs in Δ*lmgt* null mutants

Previous results demonstrated that the *L. mexicana* glucose transporter knock out cell line Δ*lmgt* is unable to take up glucose and exhibits reduced growth in the promastigote stage in media such as RPMI that contains proline, but do not grow in proline deficient medium such as DME-L [5]. Glucose uptake can be restored in the null mutant by expression of any of the three *L. mexicana* glucose transporters LmGT1, LmGT2, or LmGT3 [6]. To determine whether the endogenous glucose transporters can be substituted by transporter homologs, the ORFs of the *P. falciparum PfHT*, *T. brucei THT1* and human *GLUT1* were subcloned into the *Leishmania* expression vector pX63NEO [16] and transfected into the Δ*lmgt* cell line. Uptake of 100 μM 6-[^{3}H]D-glucose was measured over a time course of 120 s for each transfected cell line (Fig. 1A). Null mutants complemented with each of the glucose transporter homologs showed robust restoration of uptake, whereas no uptake was measured in the null mutants transfected with empty vector.

To determine whether expression of a heterologous glucose transporter is sufficient to restore the ability to grow, cell densities for all cell lines were monitored over an 8-day time course. To ensure that growth would be dependent upon glucose uptake, cells were cultured in DME-L [18], a medium containing glucose but no alternative source of metabolic energy such as proline [20]. Whereas Δ*lmgt* cells complemented with empty vector showed no increase in cell density, robust growth comparable to that of wild type cells was observed for Δ*lmgt* cells complemented with each of the glucose transporter homologs (Fig. 1B). These results indicate that the Δ*lmgt* cell line is a potent system for expression of exogenous glucose transporters, restoring glucose uptake and enabling growth under conditions where glucose is an essential nutrient.

3.2. Heterologous glucose transporters retain native function when expressed in the Δ*lmgt* null mutant

To investigate if the expression of the exogenous glucose transporters in Δ*lmgt* influences their fundamental biochemical properties, the uptake kinetics for PfHT and GLUT1 were examined in greater detail. Measurement of substrate saturation curves for D-glucose revealed K_m values of 0.3 and 1.2 mM for PfHT and GLUT1, respectively (data not shown). These K_m values are very close to those determined for PfHT expressed in *Xenopus* oocytes (0.48 mM), another heterologous expression system [21], and for GLUT1 in human red blood cells (1–2 mM) [22]. This concordance of kinetic constants suggests appropriate folding and tertiary structure of the heterologous permeases in the *L. mexicana* plasma membrane.

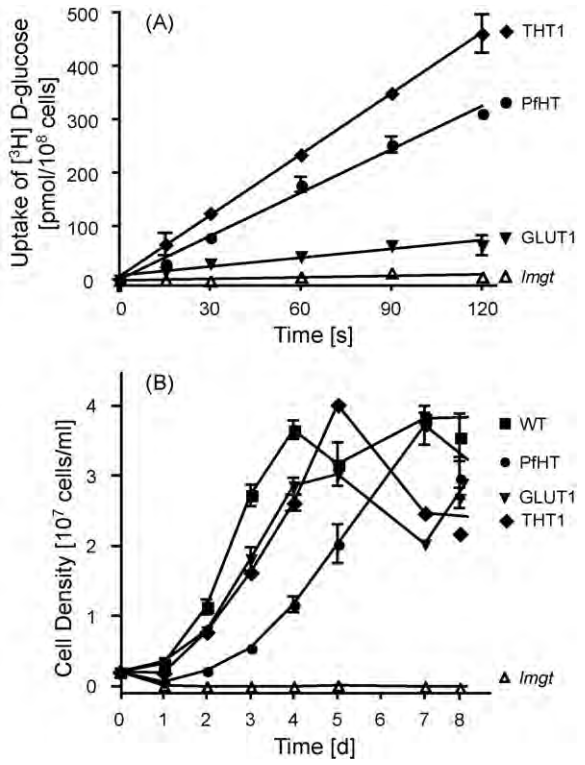

Fig. 1. Restoration of uptake and growth of complemented Δ*lmgt* cells lines. (A) Uptake of [^{3}H]D-glucose by uncomplemented null mutants (Δ*lmgt*) or Δ*lmgt* complemented with the *THT1*, *PfHT* or *GLUT1* genes on an episomal expression vector. (B) Wild type (WT) *L. mexicana* promastigotes or Δ*lmgt* either not complemented (open triangles) or complemented with the *PfHT*, *GLUT1*, or *THT1* genes on an episomal expression vector were grown in DME-L. Cell density was monitored by counting on a hemacytometer. Each data point represents the average and standard deviation (error bars) of three measurements.

3.3. Differential inhibition of glucose uptake by 3-*O*-undec-10-enyl-D-glucose for PfHT and GLUT1

Previous studies revealed that 3-*O*-undec-10-enyl-D-glucose (compound 3361), a glucose derivate with an $-(CH_2)_9-CH{=}CH_2$ substitution of the C^3 hydroxyl group [11], selectively inhibits glucose uptake mediated by PfHT but not GLUT1 [11,21]. This compound kills *P. falciparum* in vitro with an IC_{50} value of 36.7 μM at 4 mM D-glucose and causes a reduction of 40% of the parasitemia in mice infected with *P. berghei* [11]. The availability of such an inhibitor allowed us to investigate the distinct inhibition properties of the heterologously expressed PfHT and GLUT1 glucose transporter proteins in the *L. mexicana* expression system. The uptake of [^{3}H]D-glucose was examined in the complemented null mutant lines Δ*lmgt*[*pPfHT*] and Δ*lmgt*[*pGLUT1*] in the presence of various concentrations of compound 3361 (Fig. 2A and B). Compound 3361 inhibited uptake by PfHT with an IC_{50} of 5.7 μM in 100 μM glucose, resulting in a calculated K_i [23] value of 1.3 μM, which is even lower than the K_i value of 53 μM previously reported for inhibition of PfHT expressed in *Xenopus* oocytes [11]. In contrast, compound 3361 was a very poor inhibitor of GLUT1 expressed either in Δ*lmgt* null mutant (Fig. 2A) or in *Xenopus* oocytes [11]. These results confirm that the Δ*lmgt* expression system can be employed to detect selective inhibitors of parasite glucose transporters such as PfHT.

To determine the mode of glucose uptake inhibition by compound 3361, uptake assays of [^{3}H]D-glucose using Δ*lmgt* expressing PfHT were performed with glucose concentrations up to 4 mM and several concentrations of compound 3361. Lineweaver–Burk plots (Fig. 2B) exhibit increasing slopes but identical *y*-intercepts for increasing concentrations of compound 3361, revealing the expected competitive inhibition by this glucose analog.

To determine whether compound 3361 is also able to inhibit other parasitic glucose transporters, we analyzed glucose uptake in the previously derived Δ*lmgt* lines separately expressing *L. mexicana* glucose transporter LmGT1, LmGT2 and LmGT3 [5], as well as the more recently developed Δ*lmgt* lines expressing the *T. brucei* transporter THT1 and the *Toxoplasma gondii* transporter TgGT1 [24] (Fig. 2C and D). While the *Leishmania* glucose transporters showed little if any inhibition by compound 3361 (Fig. 2C), THT1 and TgGT1 exhibited weak inhibition with estimated IC_{50} values of 100 μM and 50 μM, respectively but with incomplete inhibition even at 10^{-4} M 3361 (Fig. 2D). Again, comparable results for TgGT1 in *L. mexicana* and *Xenopus* oocytes [11] indicate similar biochemical properties of the expressed proteins in both systems.

3.4. Development of the Δlmgt expression system to screen for inhibitors of parasite glucose transporters

Since the growth in DME-L of Δ*lmgt* parasites complemented with heterologous glucose transporters is dependent upon glucose uptake, selective inhibition of each transporter should inhibit growth of these transgenic parasites. Hence a convenient assay for parasite growth could be employed to screen libraries of small compounds for those that inhibit growth of the null mutant expressing a parasite transporter, but not of the null mutant expressing human GLUT1. One such growth assay that is amenable to a high-throughput format relies upon reduction of the cell-permeable dye alamarBlue™ by viable *Leishmania* parasites [25]. The reduced dye emits a strong fluorescence signal at 590 nm when illuminated at 544 nm, and measurement of the fluorescence signal can be used to quantitate parasite growth.

A first step in the development of a screening assay is to determine the range of linearity of the signal as a function of cell number. Various numbers of Δ*lmgt*[*pPfHT*] or Δ*lmgt*[*pGLUT1*] cells ranging from 1.6×10^4 to 1×10^6 were seeded into 100 μl DME-L medium containing 5 mM glucose. Following incubation for 3 days, 10 μl alamarBlue™ were added and incubation was continued for another 24 h, after which the relative fluorescence unit (RFU) were measured at 590 nm following excitation at 544 nm (Fig. 3A). For both cell lines, the resulting curve showed saturation for high numbers of cells, but a linear correlation between the number of initially seeded cells and the RFU at cell densities up to 2.5×10^5 cells/100 μl (Fig. 3A, inset) was observed.

Since many of the compounds in chemical libraries are relatively water insoluble and therefore dissolved in DMSO, the tolerance of *L. mexicana* promastigotes towards DMSO was determined. 2×10^5 Δ*lmgt*[*pPfHT*] and Δ*lmgt*[*pGLUT1*] cells were cultured in increasing concentrations of DMSO for 3 days and cell-growth was determined by the alamarBlue™ assay (Fig. 3B). No decrease of the RFU was observed with cells in medium containing 1% DMSO. In 2% DMSO, the RFU values were reduced by ~30–40%, whereas above 2% DMSO, the RFU values were reduced by >80%. Hence cell growth assays can be performed reliably in a final 1% concentration of DMSO.

3.5. Compound 3361 inhibits growth of Δlmgt[pPfHT] but not Δlmgt[pGLUT1]

To establish 'proof of principle' that transgenic Δ*lmgt* parasites can be used to screen for selective inhibitors of parasite glucose transporters, we examined the ability of compound 3361 to selectively inhibit growth of Δ*lmgt*[*pPfHT*] but not Δ*lmgt*[*pGLUT1*] parasites. However, to first establish the efficacy of the growth

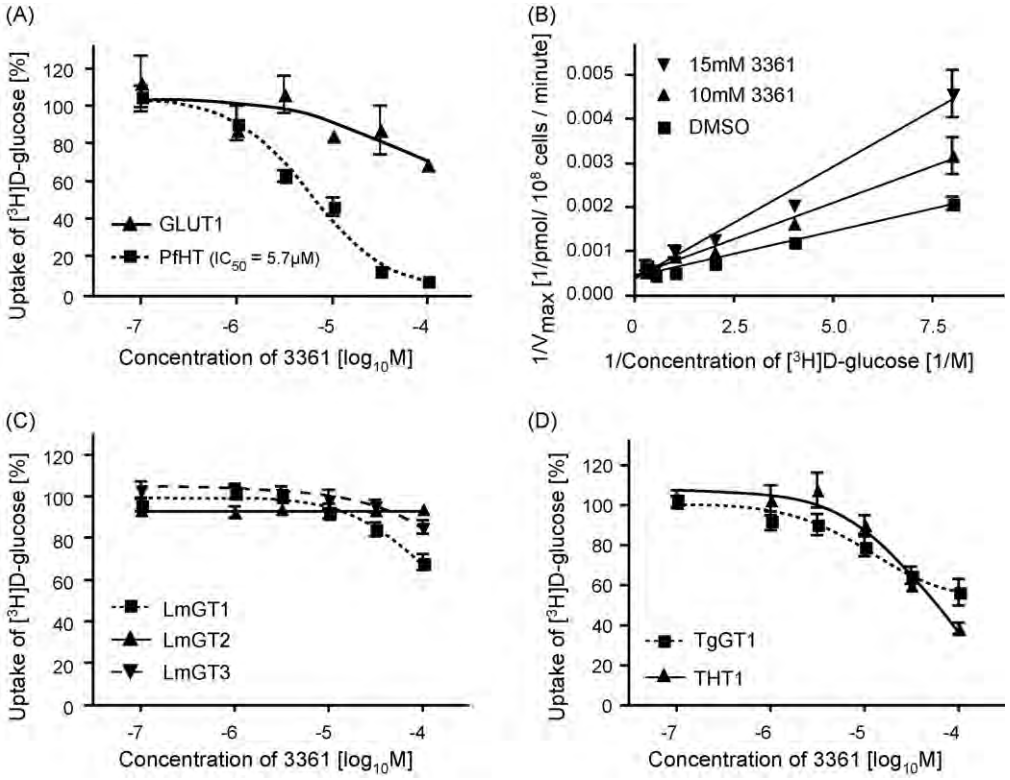

Fig. 2. Inhibition of glucose uptake by compound 3361. (A) Inhibition of [^{3}H]D-glucose (100 μM) uptake in Δ*lmgt* complemented either with *GLUT1* (solid line) or *PfHT* (dotted line) by various concentrations of compound 3361. (B) Uptake of various concentrations (100 μM–4 mM) of [^{3}H]D-glucose by Δ*lmgt*[*pPfHT*] inhibited with 15 μM, 10 μM or no compound 3361 plotted as a Lineweaver–Burk diagram. (C) Uptake of [^{3}H]D-glucose (100 μM) mediated by *L. mexicana* glucose transporters LmGT1, LmGT2 and LmGT3 expressed in Δ*lmgt* with various concentrations of compound 3361. (D) Uptake of [^{3}H]D-glucose (100 μM) mediated by *T. gondii* (TgGT1) and *T. brucei* (THT1) glucose transporters expressed in Δ*lmgt* with various concentrations of compound 3361. Inhibited uptakes are shown as percentage of the control uptake with 1% DMSO. Each data point represents the average and standard deviation of nine replicates.

inhibition assay with a well characterized cytotoxic compound, 2×10^5 Δ*lmgt*[*pPfHT*] cells/100 μl DME-L were cultured for 3 days in the presence of various concentrations of phleomycin (Fig. 4A). Incubation with 10 nM to 10 μM phleomycin revealed an IC_{50} value of 0.52 μM. Thus, the ability of a compound to inhibit growth of transgenic *Leishmania* promastigotes can be monitored reliably using this assay. To test for the capability to identify compounds that selectively inhibit parasite glucose transporters, the Δ*lmgt*[*pPfHT*] and Δ*lmgt*[*pGLUT1*] parasites were cultured for 3 days in DME-L containing compound 3361 in concentrations ranging from 100 nM to 100 μM. Growth of Δ*lmgt*[*pPfHT*] was inhibited with an IC_{50} value of 3.7 μM, somewhat lower than the IC_{50} value (53 μM) reported for inhibition of growth of *P. falciparum* [26], whereas growth of Δ*lmgt*[*pGLUT1*] parasites was scarcely inhibited at 10^{-4} M compound 3361 (Fig. 4B). Hence the growth assay described here can be employed to screen for selective inhibitors of essential parasite glucose transporters.

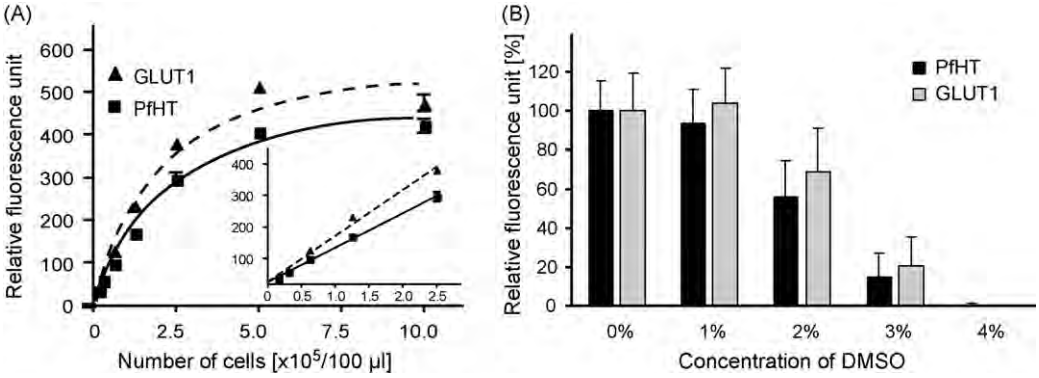

Fig. 3. Sensitivity of the growth assay to initial cell number and percent DMSO. (A) Measurements of the relative fluorescent unit (RFU) from different numbers of Δ*lmgt*[*pPfHT*] (squares and solid line) and Δ*lmgt*[*pGLUT1*] (triangles and dotted line) parasites seeded in 96 well plates after 72 h incubation in DME-L and 24 h incubation with alamarBlue™. The number of initially seeded cells and the RFU show a linear correlation in a range from 0 to 2.5×10^5 cells/100 μl. (B) Effect of DMSO on growth of Δ*lmgt*[*pPfHT*] (black bars) and Δ*lmgt*[*pGLUT1*] (grey bars). 2×10^5 cells were incubated for 3 days in DME-L containing various concentration of DMSO. RFU were measured after incubation for 24 h with alamarBlue™. Data are shown as percent RFU of the control containing no DMSO. Each data point represents the average and standard deviation of three independent experiments each with three repeats ($n = 9$).

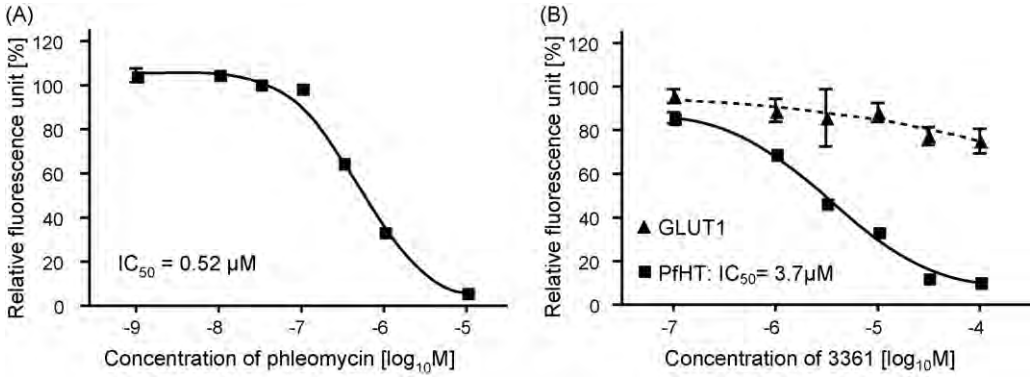

Fig. 4. Inhibition of cell growth by phleomycin and compound 3361 monitored by alamarBlue™ assay. (A) Dose–response curve for the growth of Δ*lmgt*[*pPfHT*] parasites in the presence of phleomycin. (B) Growth of Δ*lmgt*[*pPfHT*] (squares and solid line) and Δ*lmgt*[pGLUT1] (triangles and dotted line) in DME-L containing various concentration of compound 3361. 2×10^5 cells/100 μl were incubated for 3 days followed by a 24 h incubation with 10 μl alamarBlue™. Data are shown as percent of growth of the control containing 1% DMSO but no inhibitor. Each data point represents the average and standard deviation of three independent experiments with three repeats each ($n = 9$).

4. Discussion

Recent studies have indicated that glucose uptake and metabolism is vital for several medically important parasites. Thus *L. mexicana* glucose transporter null mutants were unable to survive as amastigotes inside murine macrophages or as culture form amastigotes [5]. Another kinetoplastid parasite, *T. brucei*, lives in the high glucose environment of the mammalian bloodstream and has dispensed with the Krebs Cycle and the mitochondrial respiratory chain; hence, the sole source of ATP for bloodstream trypanosomes is glycolysis [8]. Consequently, uptake of glucose and subsequent glycolysis have attracted considerable attention as potential targets for drug development [9]. Indeed, Azema et al. [12] showed that the glucose analog acetylbromo-D-glucosamine and the fructose analog acetylbromo-6-amino-D-fructose inhibited glucose uptake by bloodstream form (BF) trypanosomes and were able to kill BF trypanosomes in vitro. For the human malaria parasite *P. falciparum*, strong evidence for a crucial role of the hexose transporter PfHT (systematic name PFB0210C) among the three sugar transporter-like genes present in the parasite's 'permeome' [27] comes from work by the Krishna laboratory [11], which has identified a glucose analog, compound 3361, as a selective inhibitor of PfHT. However, no data about function, substrate or inhibition by compound 3361 of the other two *Plasmodium* putative sugar transporters (PFI0785C and PFI0955W) are currently available. Treatment with compound 3361 of intraerythrocytic *P. falciparum* cultured in human red blood cells and of mice infected with the murine malaria parasite *P. berghei* led to significant reduction of parasitemia. These results suggest that glucose transporters may be valid targets for drug development in each parasite.

In this study, we have shown that the glucose transporter null mutant of *L. mexicana*, Δ*lmgt*, can be functionally complemented with parasite and human glucose transporters. When expressed in Δ*lmgt* null mutants, PfHT and GLUT1 showed no major differences in K_m values compared to the proteins expressed in either their original cell type, e.g. red blood cells for GLUT1, or in another heterologous expression system such as *Xenopus* oocytes for PfHT. These observations validate that the *L. mexicana* heterologous expression system does not alter the kinetic properties of each permease. In addition, compound 3361 selectively inhibited PfHT expressed in Δ*lmgt* null mutants with an K_i value even lower than that determined in *Xenopus* oocytes, whereas GLUT1 was very poorly inhibited by compound 3361 in both the heterologous expression system and in oocytes. These results provide 'proof of principle' that heterologous expression of PfHT in the *L. mexicana* null mutant can be employed to screen for compounds that selectively inhibit the parasite permease and thus could represent leads for development of therapeutically useful inhibitors of glucose uptake by the parasite.

In principle, there may be other heterologous expression systems that would provide convenient platforms to search for inhibitors of parasite hexose transporters. We have attempted to express *L. mexicana* glucose transporters in a strain of *Saccharomyces cerevisiae* in which 20 sugar transporter-like genes have been deleted [28] to provide a system with a low background for uptake of sugars. However, we were not able to obtain functional expression of the parasite permeases in this background and have thus focused upon the expression system described here.

Measurement of cell growth by monitoring reduction of alamarBlue™ should provide a convenient assay that can be adapted for high-throughput screening for compounds that inhibit various parasite glucose transporters. The fluorescence signal from the reduced dye is linear over a broad range of cell density (Fig. 3A), indicating that the assay can accurately monitor cell number over this range. Furthermore, cell growth as measured by this assay is relatively unaffected by up to 1% DMSO (Fig. 3B), establishing that stock compounds dissolved in this solvent can be tested for their ability to inhibit heterologous glucose transporters at a final 1% concentration of this organic solvent. Thus it should be possible to develop a microtiter plate assay that would measure inhibition of growth of Δ*lmgt* parasites expressing PfHT, another parasite glucose transporter, or the human transporter GLUT1 by individual compounds in libraries of small molecules. Presumably, most of the growth inhibitory compounds detected in such a screen would diminish growth of the *L. mexicana* parasites for reasons other than inhibition of the heterologous glucose transporter. However, the subset of compounds that inhibited growth of Δ*lmgt*[*pPfHT*] parasites would then be rescreened for their ability to inhibit growth of Δ*lmgt*[*pGLUT1*] parasites. This two-step screen would remove all chemicals that affected growth for 'off-target' reasons or that inhibited both PfHT and GLUT1. Those compounds that inhibited growth of Δ*lmgt*[*pPfHT*] but not Δ*lmgt*[*pGLUT1*] parasites would be candidates for selective inhibitors of PfHT, that could be tested for such selective inhibition in direct substrate uptake assays and might be used in further drug development strategies.

Acknowledgements

We thank Dr. Nishith Gupta (Humboldt University, Berlin) for providing the TgGT1 clone and Dr. Diana Rodriguez-Contreras for the transfection of the TgGT1 clone into *L. mexicana*. We also thank Dr. Choukri Ben Mamoun for providing genomic DNA from *P. fal-*

ciparum strain 3D7 for PCR amplification of the *PfHT* gene. This work was supported by grants AI25920 (to S.M.L.) and AI067837 (to D.H.P.) from the National Institutes of Health.

References

[1] Croft SL, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. Clin Microbiol Rev 2006;19(1):111–26.
[2] Daily JP. Antimalarial drug therapy: the role of parasite biology and drug resistance. J Clin Pharmacol 2006;46(12):1487–97.
[3] Matovu E, Seebeck T, Enyaru JC, Kaminsky R. Drug resistance in *Trypanosoma brucei* spp., the causative agents of sleeping sickness in man and nagana in cattle. Microbes Infect 2001;3(9):763–70.
[4] Leite EA, Grabe-Guimaraes A, Guimaraes HN, Machado-Coelho GL, Barratt G, Mosqueira VC. Cardiotoxicity reduction induced by halofantrine entrapped in nanocapsule devices. Life Sci 2007;80(14):1327–34.
[5] Burchmore RJ, Rodriguez-Contreras D, McBride K, et al. Genetic characterization of glucose transporter function in *Leishmania mexicana*. Proc Natl Acad Sci USA 2003;100(7):3901–6.
[6] Rodriguez-Contreras D, Feng X, Keeney KM, Bouwer HG, Landfear SM. Phenotypic characterization of a glucose transporter null mutant in *Leishmania mexicana*. Mol Biochem Parasitol 2007.
[7] Barrett MP, Tetaud E, Seyfang A, Bringaud F, Baltz T. Functional expression and characterization of the *Trypanosoma brucei* procyclic glucose transporter, THT2. Biochem J 1995;312(Pt 3):687–91.
[8] Cazzulo JJ. Aerobic fermentation of glucose by trypanosomatids. FASEB J 1992;6(13):3153–61.
[9] Verlinde CL, Hannaert V, Blonski C, et al. Glycolysis as a target for the design of new anti-trypanosome drugs. Drug Resist Update 2001;4(1):50–65.
[10] Bringaud F, Baltz T. Differential regulation of two distinct families of glucose transporter genes in *Trypanosoma brucei*. Mol Cell Biol 1993;13(2):1146–54.
[11] Joet T, Eckstein-Ludwig U, Morin C, Krishna S. Validation of the hexose transporter of *Plasmodium falciparum* as a novel drug target. Proc Natl Acad Sci USA 2003;100(13):7476–9.
[12] Azema L, Claustre S, Alric I, et al. Interaction of substituted hexose analogues with the *Trypanosoma brucei* hexose transporter. Biochem Pharmacol 2004;67(3):459–67.
[13] Mueckler M, Caruso C, Baldwin SA, et al. Sequence and structure of a human glucose transporter. Science 1985;229(4717):941–5.
[14] Kirk K, Saliba KJ. Targeting nutrient uptake mechanisms in *Plasmodium*. Curr Drug Targets 2007;8(1):75–88.
[15] Manolescu AR, Witkowska K, Kinnaird A, Cessford T, Cheeseman C. Facilitated hexose transporters: new perspectives on form and function. Physiology (Bethesda) 2007;22:234–40.
[16] LeBowitz JH, Coburn CM, Beverley SM. Simultaneous transient expression assays of the trypanosomatid parasite *Leishmania* using beta-galactosidase and beta-glucuronidase as reporter enzymes. Gene 1991;103(1):119–23.
[17] Seyfang A, Landfear SM. Four conserved cytoplasmic sequence motifs are important for transport function of the *Leishmania* inositol/H(+) symporter. J Biol Chem 2000;275(8):5687–93.
[18] Iovannisci DM, Ullman B. High efficiency plating method for *Leishmania promastigotes* in semidefined or completely-defined medium. J Parasitol 1983;69(4):633–6.
[19] Ikekawa T, Irinoda K, Saze K, et al. Studies on synthesis of 3-*O*-alkyl-D-glucose and 3-*O*-alkyl-D-allose derivatives and their biological activities. Chem Pharm Bull (Tokyo) 1987;35(7):2894–9.
[20] Ter Kuile BH, Opperdoes FR. A chemostat study on proline uptake and metabolism of *Leishmania donovani*. J Protozool 1992;39(5):555–8.
[21] Woodrow CJ, Penny JI, Krishna S. Intraerythrocytic *Plasmodium falciparum* expresses a high affinity facilitative hexose transporter. J Biol Chem 1999;274(11):7272–7.
[22] Mueckler M, Hresko RC, Sato M. Structure, function and biosynthesis of GLUT1. Biochem Soc Trans 1997;25(3):951–4.
[23] Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_1) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 1973;22(23):3099–108.
[24] Joet T, Holterman L, Stedman TT, et al. Comparative characterization of hexose transporters of *Plasmodium knowlesi*, *Plasmodium yoelii* and *Toxoplasma gondii* highlights functional differences within the apicomplexan family. Biochem J 2002;368(Pt 3):923–9.
[25] Mikus J, Steverding D. A simple colorimetric method to screen drug cytotoxicity against *Leishmania* using the dye Alamar Blue. Parasitol Int 2000;48(3):265–9.
[26] Ionita M, Krishna S, Leo PM, Morin C, Patel AP. Interaction of *O*-(undec-10-en)-yl-D-glucose derivatives with the *Plasmodium falciparum* hexose transporter (PfHT). Bioorg Med Chem Lett 2007.
[27] Martin RE, Henry RI, Abbey JL, Clements JD, Kirk K. The 'permeome' of the malaria parasite: an overview of the membrane transport proteins of *Plasmodium falciparum*. Genome Biol 2005;6(3):R26.
[28] Wieczorke R, Krampe S, Weierstall T, Freidel K, Hollenberg CP, Boles E. Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. FEBS Lett 1999;464(3):123–8.