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14. ABSTRACT

The objective of this project was to identify compounds that selectively inhibit the essential Leishmania glucose transporters and could hence serve as starting points for development of novel anti-leishmanial drugs. A primary high throughput assay and several secondary assays were developed and optimized for this purpose. From the primary assay, 2804 compounds were identified as hits, compounds that strongly inhibited growth of Leishmania mexicana promastigotes (insect form parasites grown in culture) at ~10 μM concentration. From the secondary assay that monitored uptake of radiolabeled glucose through the L. mexicana glucose transporter LmxGT2, 162 secondary hits were identified among the 2804 primary hits that strongly inhibited uptake. From a further use of the secondary assay in a dose-response mode, 34 tertiary hits were identified among the 162 secondary hits that inhibited uptake of radiolabeled glucose through LmxGT2 with IC50 values <1 μM. These tertiary hits are now being evaluated for those that are selective for LmxGT2 compared to the human glucose transporter GLUT1 and could thus serve as leads from continued drug development. In addition, another secondary assay has been developed that allows screening of the 2804 primary hits for those that potently inhibit growth of intracellular amastigotes (by unknown mechanisms of action), the stage of the Leishmania life cycle that invades mammalian macrophages and causes disease. A 'proof of concept' screen of a 69 compound subset of the primary hits revealed 4 compounds (~6%) that are non-toxic to host macrophages at 1 μM but potently inhibit growth of intracellular amastigotes at that concentration. These results suggest that ~170 (6% of 2804) such amastigote-active compounds may exist among the 2804 primary hits. These results make possible a facile 'phenotypic' screen that can identify multiple compounds with high potency against the disease-causing stage of the Leishmania life cycle, are non-toxic to the human host, and that have properties compatible with development of novel orally bioavailable anti-leishmanial drugs.

15. SUBJECT TERMS
high-throughput screen, large chemical library, differential inhibition of parasite glucose transport, phenotypic screen, development of new anti-leishmanial drugs

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Final Progress Report
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Introduction:

Leishmania are parasitic protozoa that cause devastating diseases throughout much of the tropical and subtropical world (6), 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 (8). 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 (3), suggesting that these important permeases could serve as critical targets for drug development. Furthermore, our laboratory has developed a cell-based assay (5) 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:

To provide a comprehensive report of work to date, this report covers work done over the past ~4 years of the project. The material toward the end of the 'Body' summarizes work done over the past year in which the validated high-throughput screen (HTS) was applied to a large chemical library of ~600,000 compounds and hits from primary and secondary screens were evaluated. In addition, the report summarizes the development of additional secondary screens that had to be developed to identify useful hit compounds.

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*lmxgt1-3* null mutants (3), are not able to grow in glucose-replete/proline-deficient (glucose +/proline -) medium (5). However, if these null mutants are complemented with the major glucose transporter gene from *L. mexicana*, *LmxGT2*, they will grow in glucose +/proline – medium. (*L. mexicana* encode three glucose transporter genes within a single 14 kb locus: *LmxGT1, LmxGT2, and LmxGT3* (2). The *LmxGT2* gene is the most highly expressed of these 3 genes in *L. mexicana* promastigotes. All 3 of these linked genes are deleted in the Δ*lmgt1-3* null mutant.) Similarly, if the Δ*lmxgt* 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 (5).

For the purpose of this summary, Δ*lmxgt1-3* null mutants expressing the LmxGT2 transporter from an *LmxGT2* transgene will be referred to simply as **LmxGT2 expressing parasites**, since LmxGT2 is the only glucose

transporter they express. Similarly, Δ*lmxgt1-3* null mutants that express the human GLUT1 transporter from a *GLUT1* transgene will be referred to as **GLUT1 expressing parasites**, since GLUT1 is the only glucose transporter they express.

The HTS assay screens for compounds that selectively inhibit growth of the null mutants expressing the LmxGT2 protein but that do not significantly inhibit growth of null mutants expressing the human GLUT1 protein. Such compounds will be selective inhibitors of LmxGT2 but not of GLUT1 and will thus selectively target the parasite glucose transporter. The screen was performed first by identifying compounds in a library that inhibit growth of the LmGT2-expressing null mutants (primary screen). This subset of compounds was subsequently rescreened (secondary screen) 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 are candidates for selective inhibitors of *Leishmania* glucose transporters. As described below, it was subsequently necessary to replace the original secondary screen with an alternative method that directly measures uptake of [^{3}H]D-glucose by LmxGT2-expressing parasites. The hit compounds that have emerged from this new secondary screen will be further investigated, as detailed in the original proposal, to identify those that inhibit uptake of [^{3}H]D-glucose by LmxGT2 but not by GLUT1.

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 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 (11); 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 LmxGT2 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%**

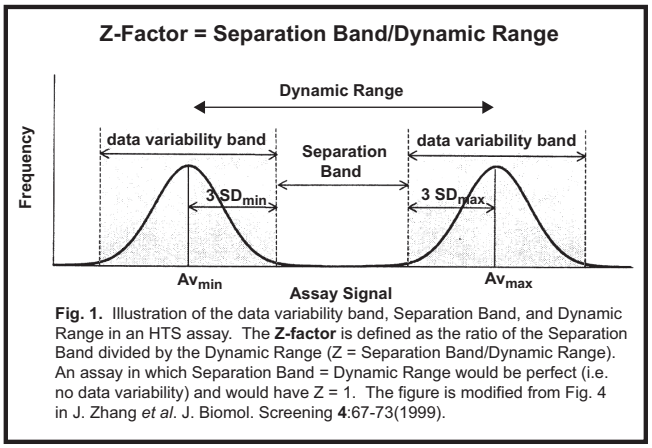

Fig. 1. Illustration of the data variability band, Separation Band, and Dynamic Range in an HTS assay. The **Z-factor** is defined as the ratio of the Separation Band divided by the Dynamic Range (Z = Separation Band/Dynamic Range). An assay in which Separation Band = Dynamic Range would be perfect (i.e. 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).

An illustration of the Z'-factor is shown in Fig. 1, taken from reference (11). 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.

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 >0.50 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.

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 passed the first set of criteria for a high quality screening method and was then employed 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 (11), 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.

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*lmxgt* parasites, complemented with either LmxGT2 or GLUT1, suspended in DME-L medium (7) at an initial cell density of 5.6 x 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.

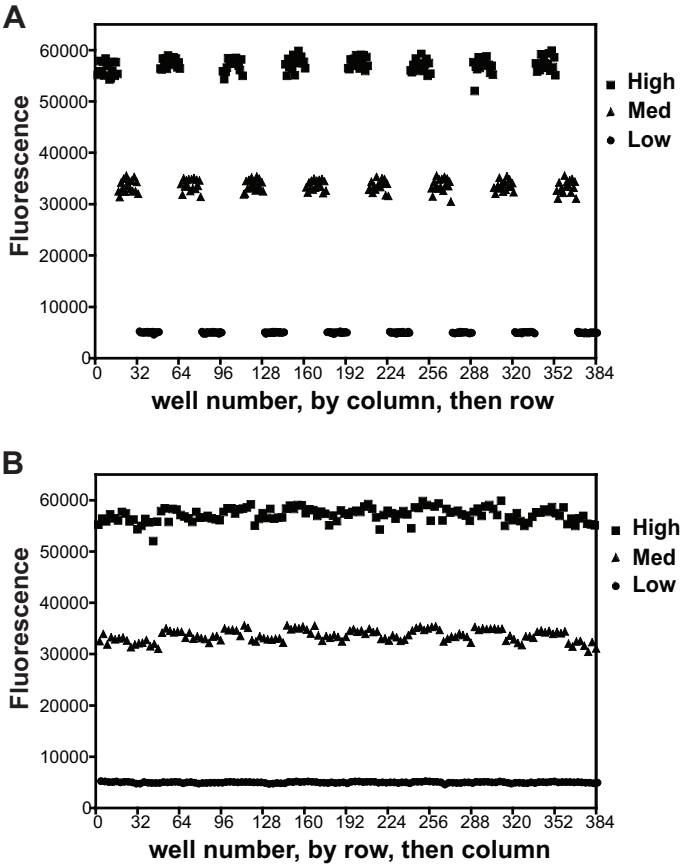

Fig. 2. Uniformity assay for control samples representing High (no growth inhibitor, squares), Med (~IC_{50} or 1.4 µM phleomycin, triangles), and Low (1 mM pleomycin, circles) growth conditions. Multiple samples of each type were arrayed across the plate as recommended in the Assay Guidance Manual. 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.

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 LmxGT2. 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 was next employed 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 LmxGT2 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 LmxGT2. Subsequently, the fluorescence signal for parasites exposed to each of these 140 compounds was determined for the parasites expressing LmxGT2 (y-axis of Fig. 4) versus those expressing GLUT1 (x-axis of Fig 4). In this plot, compounds that inhibit LmxGT2 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 LmxGT2 expressing parasites significantly more than they inhibited growth of GLUT1 expressing parasites. Hence, this screen of a small library did not detect any compounds that preferentially inhibited the parasite glucose transporter. Screens of considerably larger libraries constituted the next step in this program and attempted to detect selective LmxGT2 inhibitors among a much larger group of compounds.

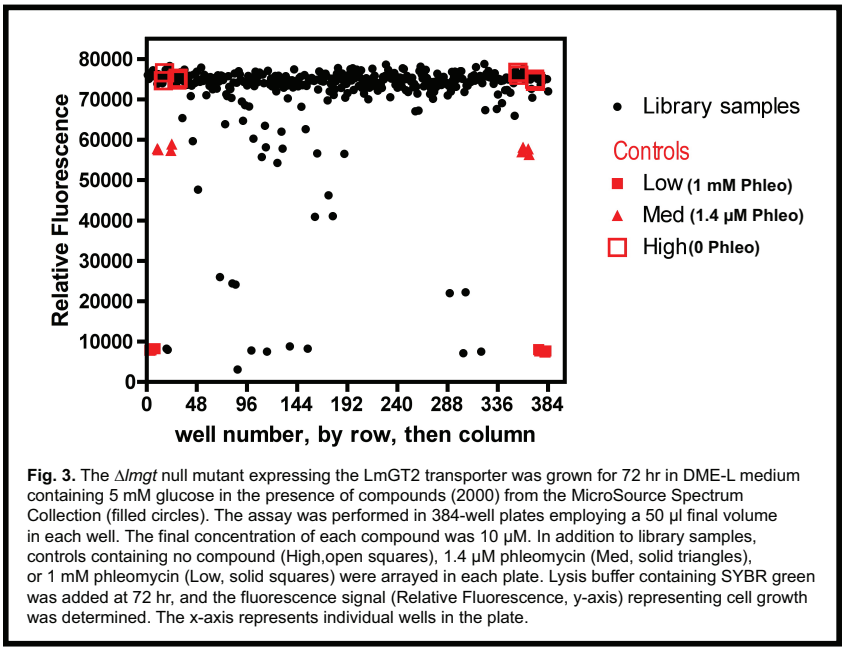

Fig. 3. The Δ*lmgt* null mutant expressing the LmGT2 transporter was grown for 72 hr in DME-L medium containing 5 mM glucose in the presence of compounds (2000) from the MicroSource Spectrum Collection (filled circles). The assay was performed in 384-well plates employing a 50 µl final volume in each 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.

Screening of the Chemical Biology and Therapeutics (CBT) Library at St. Jude Children's Research Hospital. We next performed a very large scale screen of the ~600,000 compound CBT library in collaboration with our colleagues in Dr. Kip Guy's laboratory at St. Jude Children's Research Hospital. This screen was performed using *L. mexicana* parasites expressing the LmxGT2 transporter. Primary hits (2804) form this screen were identified as those that inhibited growth of the LmxGT2-expressing line by >65%. This cutoff value was determined using Receiver Operator Characteristics (4), a statistical method that optimizes identification of the maximal number of true inhibitors while excluding the largest number of false positives (10).

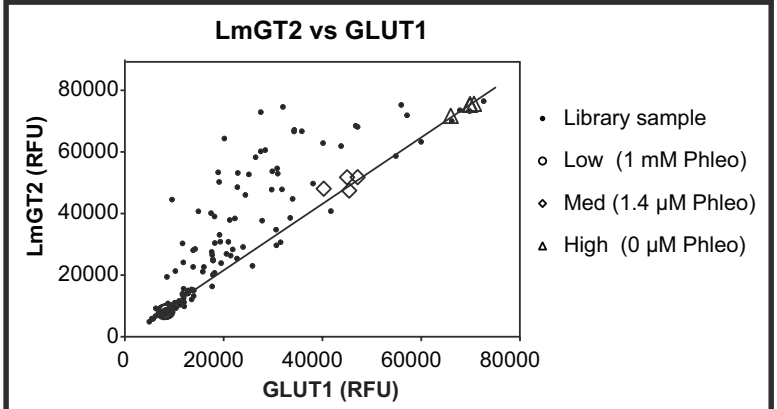

Fig. 4. The relative inhibition of growth of Δ*lmgt* null mutants expressing either LmGT2 or human GLUT1 was compared. The 140 compounds tested (filled circles; Library sample) were those from the MicroSource Discovery Collection that inhibited growth of LmGT2 expressing parasites by >50%. These compounds were retested for their ability to inhibit growth of parasites expressing GLUT1. The Relative Fluroresence 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.

These primary hits were subsequently subjected to a secondary screen to identify compounds likely to be selective inhibitors of LmxGT2 compared to GLUT1. This screen involved measuring dose-response (DR) curves for inhibition of growth of LmxGT2- and GLUT1-expressing Δ*lmxgt1-3* parasites. Compounds were designated hits if they exhibited: 1) an IC_{50} for growth inhibition of LmxGT2-expressing parasites that was <1 µM, and 2) a differential IC_{50} for growth of LmxGT2- versus GLUT1-expressing parasites that was >2-fold. From this secondary screen, 14 compounds emerged that fell within these cutoff values; differential inhibition ranged from 2- to 13-fold lower IC_{50} for LmxGT2- versus GLUT1-expressing parasites. The 14 secondary hits were subsequently assayed for their ability to inhibit uptake of [^{3}H]D-glucose by LmxGT2 compared to GLUT1. However, none of these initial hits proved to be selective inhibitors of [^{3}H]D-glucose uptake by LmxGT2.

Development of an <u>Alternative</u> Secondary Screen - Direct Measurement of Uptake of [^{3}H]D-glucose. One potential limitation of the strategy described above is that the secondary screen relied upon an 'indirect' measurement of differential inhibition: the ability to inhibit growth of LmxGT2- versus GLUT1-expressing parasites. It is possible that a more direct secondary screen that measures uptake of glucose directly would identify more accurately selective glucose transporter inhibitors. For this reason, within the past year we developed an alternative secondary assay that measures uptake of radiolabeled glucose into ∆*lmxgt1-3* null mutant parasites expressing the LmxGT2 transporter. This assay was based upon the 'low throughput' cell filtration assay that has been employed in the past to measure glucose uptake in small numbers of samples of *Leishmania* parasites (9), but the assay was adapted for a 96-well format (Millepore MultiScreen glass fiber filter plates) to allow measurement of glucose uptake in up to several thousand cell samples (medium throughput). First compounds are added to each well of the plate in 100 µl Phosphagte Buffered Saline (PBS). Then LmxGT2 expressing parasites ($1x10^7$) in 90 µl PBS are added to each well and preincubated with compound for 5 min, and the uptake reaction is initiated by addition of 10 µl of [^{3}H]D-glucose to give a final concentration of 200 µM and 2.5 µCi per well. Parasites, compound, and labeled glucose are incubated for 5 min, and the uptake reaction is rapidly stopped by addition of 50 µl of 4% formaldehyde in PBS. The labeled glucose is removed by vacuum filtration of the plate (Millepore MultiScreen Vacuum Manifold), the wells are washed 2x with 250 µl of PBS and dried. To each well 100 µl of scitillation fluid is added and the radiolabel remaining inside the parasites is quantified by liquid scintillation counting. While this radiolabel uptake assay generated more scatter in plate uniformity assays (Fig. 5, Z'=0.61) than the SYBR Green fluorescence assay used to monitor parasite growth in the primary screen (Fig. 2, Z'=0.89), it still met all the acceptance criteria for an acceptable HTS.

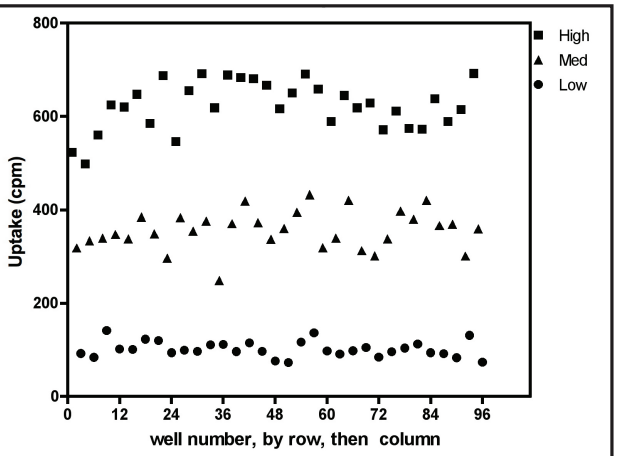

Fig. 5. Plate uniformity graph for uptake of [^{3}H]D-glucose by LmxGT2 expressing parasites using 96-well filter plate assay. Samples designated 'High' have no inhibitor of glucose uptake, those desginated 'Med' included 1 mM D-fructose, an alternate substrate and thus inhibitor for LmxGT2, and those designated 'Low' have 15 mM D-fructose as inhibitor.

Secondary Screen of the 2804 Primary Hits Using the [^{3}H]D-glucose Uptake Assay as Secondary Screen. The 2804 hits of the primary screen were subsequently screened in the [^{3}H]D-glucose uptake assay and 162 compounds were identified that inhibited by >90% uptake of 100 µM glucose by LmxGT2 when they were applied at a concentration of 20-30 µM. These 162 secondary hits were then screened again in the glucose uptake assay, but his time the assay was performed in a dose-response format employing 10 different concentrations of each compound from 0.01-100 µM. From this screen, 34 compounds were identified that inhibited uptake of [^{3}H]D-glucose by LmxGT2 with an EC_{50} value of <1 µM, and these compounds are designated 'tertiary hits'. Of these 34 tertiary hits, 15 corresponded to scaffolds with especially desirable chemical properties and are thus assigned first priority for further analysis.

Currently, we are ordering available compounds from the list of 34 teritiary hits. These compounds will be tested agains ∆*lmxgt1-3* null mutants that are expressing either LmxGT2 or the human glucose transporter GLUT1 to identify those that inhibit LmxGT2 with a significantly lower EC_{50} value than for GLUT1. The objective is to identify compounds that inhibit LmxGT2 in the submicromolar range without strongly inhibiting GLUT1 (and ultimately other human GLUTs). These compounds will then also be tested in a dose-response format for those that inhibit growth of *L. mexicana* amastigotes inside murine macrophages (see luciferase assay below) and which thus have potential for development as anti-leishmanial compounds.

Getting the Most out of the Primary Screen – Using the Results for a 'Phenotypic Screen'. The approach we have employed in this project is to target specific proteins of *Leishmania* parasites, the glucose transporters, for development of novel anti-leishmanial drugs – a 'target-based screen'. An alternative approach to drug development is the so-called 'phenotypic screen' in which compounds are selected solely on the basis of their ability to kill the parasite without knowledge of which target or targets they affect. Indeed, the

2804 compounds that emerged from the primary screen (growth inhibition measured by SYBR Green fluorescence) represent the first step of such a phenotypic screen.

The next step to determine which of these 2804 primary hits could be useful for drug development is to perform a secondary screen to determine which of them inhibit growth of *Leishmania* amastigotes grown inside macrophages (the infectious stages for the vertebrate host). Toward this end, we have developed a convenient assay to monitor growth of intracellular amastigotes in a medium to high-throughput format. The *Renilla* luciferase gene (1) was integrated into the rDNA locus of *L. mexicana* to generate a transgenic line that expressed this reporter enzyme. Subsequently, J774 murine macrophages are deposited in wells of a 96-well tissue culture plate and infected with these transgenic parasites to allow development of intracellular amastigotes. Five days after infection, the infected macrophages are lysed and the luminescence signal generated by oxidation of the substrate coelenterazine by *Renilla* luciferase is monitored on a luminometer.

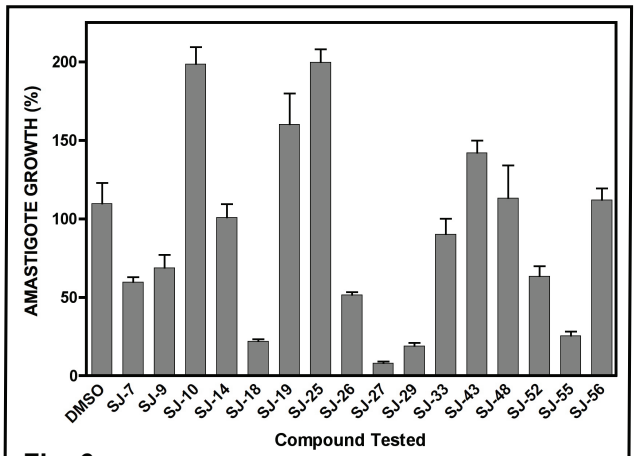

Fig. 6. **Inhibition of amastigote growth inside murine macrophages measured using luciferase assay. DMSO is the solvent control, and there are 16 SJ compounds whose inhibition of amastigote growth has been measured (n=2).**

To validate this assay, a 69 compound subset of the 2804 primary hits was assayed by this method. However, these compounds were first incubated at a concentration of 1 μM with uninfected macrophages, and those that inhibited growth of these host cells were removed as likely cytotoxic compounds. The remaining 16 hits that were not toxic to macrophages were subsequently monitored at 1 μM (using the luciferase assay) for their ability to inhibit growth of luciferase-transgenic amastigotes in J774 macrophages. The results of this secondary screen are shown in Fig. 6 and reveal that 4 of the 16 compounds (SJ-18, SJ-27, SJ-29, and SJ-55) inhibit growth of intracellular amastigotes by >75%. Hence, we designate these compounds as 'secondary hits' in a phenotypic screen, that is compounds that at a 1 μM concentration potently inhibit growth of intracellular amastigotes but are not toxic to host macrophages. Furthermore, an initial dose-response curve (Fig. 7) for inhibition of amastigote (open circles, EC_{50} ~ 0.11 μM) or macrophage (filled circles, EC_{50} ~5.2 μM) growth reveals a 'therapeutic index' of ~50 for SJ-27, confirming that his compound strongly inhibits growth of intracellular parasties at concentrations well below those that affect host macrophages. Since 4/69 (~6%) of the primary hits qualify as secondary hits by these criteria, we estimate that approximately (2804)(0.06)=~170 of the primary hits may emerge as secondary hits using this luciferase based amastigote viability assay.

Hence as one component of this project, we have performed the first step in a phenotypic screen generating 2804 primary hits, and we have developed a convenient secondary assay to monitor in high-thorughput format which of these compounds have potential for killing intracellular disease-causing amastigotes at 1 μM or lower concentration without overt toxicity to the host macrophages. Applying this secondary luciferase screen to all 2804 hits represents a promising direction for future research with the potential to detect multiple chemical 'scaffolds' with promise for development of novel anti-leishmanial drugs. We consider this development to be one of the most promising of this research program. Indeed current pharmacological treatments for leishmaniasis suffer from use of a limited number of compounds that were often selected on an empirical basis (8). The phenotypic screen described here has the potential to advance a substantial number of candidate scaffolds that can be

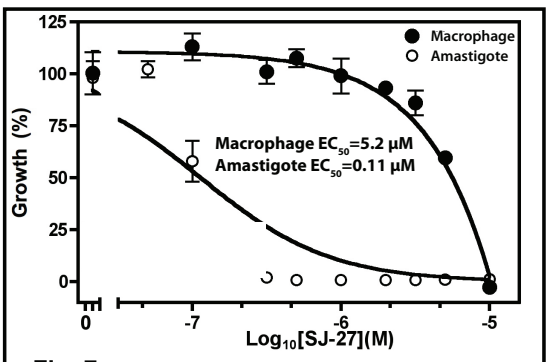

Fig. 7. **Dose-response curves for compound SJ-27 for intracellular amastigotes (open circles) and for J774 macrophages (filled circles). The EC_{50} values for each curve are indicated.**

interrogated systematically to identify those with the highest potential for development of novel and improved anti-leishmanial drugs.

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 0.5.

- 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 LmxGT2 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.

- The CBT library of ~600,000 compounds has been screened in high-throughput format, and 2804 primary hits have emerged.

- A secondary screen of the primary hits was performed using a dose-response assay. Some 14 secondary hits were identified, but none of these hits emerged as selective inhibitors of LmxGT2 compared to GLUT1.

- An improved secondary screen has been developed based upon direct measurement of inhibition of glucose uptake by each compound employing 96-well glass fiber filter plates. This assay generates an acceptable Z value of 0.61.

- The 2804 primary hits were screened using the [^{3}H]D-glucose uptake assay to generate 34 tertiary hits that are potential potent inhibitors of LmxGT2.

- These tertiary hits are being screened by conventional [^{3}H]D-glucose uptake assays to determine definitively which hits are high affinity inhibitors of LmxGT2 with poor inhibitory capacity for mammalian glucose transporters such as GLUT1.

- Another secondary assay (based upon measurement of luminescence signal from transgenic parasites expressing the *Renilla* luciferase gene) has been developed that allows the 2804 primary hits to be screened for those that potently inhibit growth of intracellular amastigotes without exhbiting toxicity toward host macrophages. When employed with a 69 compound subset of the 2804 primary hits, ~6% of the compounds met the criteria for secondary hits (at 1 μM concentration, >75% inhibition of amastigote growth but no detectable inhibition of macrophage growth). In future studies, this assay can be employed with the 2804 primary hits to complete a 'phenotypic screen' for multiple novel anti-leishmanial compounds. This work forms the foundation of several new grant applications to develop novel anti-leishmanial drugs.

- We anticipate that at least 2 publications will emerge from this work once appropriate experiments have been completed.

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.
7. Poster presentation summarizing results of screening at OHSU Research Forum, May 2012.
8. Seminar, University of Washington, Department of Pharmaceutics, School of Pharmacy, January 31, 2012
9. Seminar, Washington State University, Department of Pharmaceutical Sciences, June 13, 2013

Conclusion:

The importance of the research accomplished as a result of this project 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, and a secondary assay that directly measures uptake of radiolabeled glucose, were subsequently employed in primary and secondary screens of a large chemical library, and 34 promising tertiary hits have emerged. These hits are now being evaluated for those that are high affinity selective inhibitors of the *Leishmania* glucose transporters. In addition and perhaps of equal importance, a phenotypic screen accomplished as part of the screen for glucose transporter inhibitors has identified 2804 primary hits that inhibit growth of *Leishmania* parasites in vitro. A secondary screen has been developed that allows identification of those primary hits (~6%) that inhibit growth if the diease causing intracellular amastigote stage of the parasite without apparent toxicity to host macrophages.

'So what section'. The importance of this work is that compounds have been identified that either function i) as potential selective inhibitors of essential *Leishmania* glucose transporters or ii) that kill *Leishmania* parasites by currently unknown mechanisms. Both sets of compounds represent a valuable resource for development of novel anti-leishmanial drugs. Following further steps to identify the most promising compounds in each set, it is likely that this work will deliver multiple chemical scaffolds with signicant promise for drug development. A major advantage of identifying multiple promising scaffolds is that it maximizes the chances to identify at least one and potentially several novel anti-leishmanial drugs.

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