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An expression system to screen for inhibitors of parasite glucose transporters

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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 ($\Delta lmg1$) 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 $\Delta lmg1$ 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.

Keywords

Leishmania mexicana; *Plasmodium falciparum*; Null mutant; Glucose transporter; High-throughput screening

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 Δlmg t [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, Δlmg t 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 Δlmg t mutants, can be used to monitor for selective inhibitors of the *P. falciparum* glucose transporter PfHT and hence represents a valid approach to screen small molecule libraries for inhibitors of parasite glucose transporters.

2. Materials and methods

2.1. Generation of complemented Δlmg t cell lines and cell culture

The Δlmg t 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 Δlmg t line, and selected in G418 (Cellgrow, Canada) containing medium to generate the Δlmg t[p*GLUT1*], Δlmg t[p*PfHT*], Δlmg t[p*TgGT1*] and Δlmg t[p*THT1*] lines. Promastigotes of complemented Δlmg t 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), 0.1mM xanthine (Sigma, USA), and 5 μ g/ml hemin (Sigma, USA), and 100 μ g/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 [6-³H(*N*)] D-glucose (Perkin-Elmer Life Sciences, USA) in promastigotes of wild type *L. mexicana*, Δlmg t, and Δlmg t complemented with each glucose transporter gene were performed as reported [17]. Wild type and Δlmg t promastigotes in middle-late logarithmic phase of growth were assayed for sugar uptake at several substrate concentrations between 100 μ M and 4mM. 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, 0.1mM xanthine and 5 μ g/ml hemin containing 100 μ g/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, 5mM glucose (Sigma, USA), 0.1mM xanthine and 5 μ g/ml hemin at room temperature. Parasites in 50 μ l DME-L were

seeded in black bottom plates (Greiner, Germany) and mixed with 50 μ l 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 μ l 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- β -glucose

3-O-Undec-10-enyl- β -glucose (compound 3361) was synthesized as described [19]. 1,2:5,6-Di-*O*-isopropylidene- α - β -glucofuranose (2.6 g) was dissolved in 15 ml anhydrous DMSO and treated with 15 ml of 1.4M 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 isolate 3-*O*-undec-10-enyl-1,2:5,6-di-*O*-isopropylidene- α - β -glucofuranose which was then converted to 3-*O*-undec-10-enyl- β -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 $\text{CHCl}_3/\text{MeOH}$ (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 μ M 6- ^3H -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 Δlmg t null mutant

To investigate if the expression of the exogenous glucose transporters in Δlmg t 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 1.2mM 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 (1–2mM) [22]. This concordance of kinetic constants suggests appropriate folding and tertiary structure of the heterologous permeases in the *L. mexicana* plasma membrane.

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 μ M at 4mM 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 [3H] D -glucose was examined in the complemented null mutant lines Δlmg t [*pPfHT*] and Δlmg t [*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 μ M in 100 μ M glucose, resulting in a calculated K_i [23] value of 1.3 μ M, which is even lower than the K_i value of 53 μ M 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 Δlmg t null mutant (Fig. 2A) or in *Xenopus* oocytes [11]. These results confirm that the Δlmg t 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 [3H] D -glucose using Δlmg t expressing PfHT were performed with glucose concentrations up to 4mM 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 Δlmg t lines separately expressing *L. mexicana* glucose transporter LmGT1, LmGT2 and LmGT3 [5], as well as the more recently developed Δlmg t 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 μ M and 50 μ M, 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 Δlmg t expression system to screen for inhibitors of parasite glucose transporters

Since the growth in DME-L of Δlmg t 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 590nm 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 $\Delta lmg1[pPfHT]$ or $\Delta lmg1[pGLUT1]$ cells ranging from 1.6×10^4 to 1×10^6 were seeded into 100 μ l DME-L medium containing 5 mM glucose. Following incubation for 3 days, 10 μ l alamarBlue™ were added and incubation was continued for another 24 h, after which the relative fluorescence unit (RFU) were measured at 590nm 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 μ l (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 $\Delta lmg1[pPfHT]$ and $\Delta lmg1[pGLUT1]$ 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 $\Delta lmg1[pPfHT]$ but not $\Delta lmg1[pGLUT1]$

To establish ‘proof of principle’ that transgenic $\Delta lmg1$ 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 $\Delta lmg1[pPfHT]$ but not $\Delta lmg1[pGLUT1]$ parasites. However, to first establish the efficacy of the growth inhibition assay with a well characterized cytotoxic compound, 2×10^5 $\Delta lmg1[pPfHT]$ cells/100 μ l DME-L were cultured for 3 days in the presence of various concentrations of phleomycin (Fig. 4A). Incubation with 10 nM to 10 μ M phleomycin revealed an IC₅₀ value of 0.52 μ M. 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 $\Delta lmg1[pPfHT]$ and $\Delta lmg1[pGLUT1]$ parasites were cultured for 3 days in DME-L containing compound 3361 in concentrations ranging from 100 nM to 100 μ M. Growth of $\Delta lmg1[pPfHT]$ was inhibited with an IC₅₀ value of 3.7 μ M, somewhat lower than the IC₅₀ value (53 μ M) reported for inhibition of growth of *P. falciparum* [26], whereas growth of $\Delta lmg1[pGLUT1]$ parasites was scarcely inhibited at 10⁻⁴M compound 3361 (Fig. 4B). Hence the growth assay described here can be employed to screen for selective inhibitors of essential parasite glucose transporters.

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*, Δlmg t, can be functionally complemented with parasite and human glucose transporters. When expressed in Δlmg t 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 Δlmg t 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 Δlmg t 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 Δlmg t[pPfHT] parasites would then be rescreened for their ability to inhibit growth of Δlmg t[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 Δlmg t[pPfHT] but not Δlmg t[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.

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.

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Fig. 1.

Restoration of uptake and growth of complemented $\Delta lmgf$ cells lines. (A) Uptake of [³H]_D-glucose by uncomplemented null mutants ($\Delta lmgf$) or $\Delta lmgf$ complemented with the *THT1*, *PfHT* or *GLUT1* genes on an episomal expression vector. (B) Wild type (WT) *L. mexicana* promastigotes or $\Delta lmgf$ 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.

Fig. 2.

Inhibition of glucose uptake by compound 3361. (A) Inhibition of $[^3\text{H}]\text{D-glucose}$ (100 μM) uptake in $\Delta lmg1$ 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 $[^3\text{H}]\text{D-glucose}$ by $\Delta lmg1[pPfHT]$ inhibited with 15 μM , 10 μM or no compound 3361 plotted as a Lineweaver–Burk diagram. (C) Uptake of $[^3\text{H}]\text{D-glucose}$ (100 μM) mediated by *L. mexicana* glucose transporters LmGT1, LmGT2 and LmGT3 expressed in $\Delta lmg1$ with various concentrations of compound 3361. (D) Uptake of $[^3\text{H}]\text{D-glucose}$ (100 μM) mediated by *T. gondii* (TgGT1) and *T. brucei* (THT1) glucose transporters expressed in $\Delta lmg1$ 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.

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 $\Delta lmg1[pPfHT]$ (squares and solid line) and $\Delta lmg1[pGLUT1]$ (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 $\Delta lmg1[pPfHT]$ (black bars) and $\Delta lmg1[pGLUT1]$ (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 $\Delta lmgf[pPfHT]$ parasites in the presence of phleomycin. (B) Growth of $\Delta lmgf[pPfHT]$ (squares and solid line) and $\Delta lmgf[pGLUT1]$ (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 ($n = 9$).