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Stable expression of *Escherichia coli* β-glucuronidase A (GusA) in *Giardia lamblia*: application to high-throughput drug susceptibility testing

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Objectives: In order to create a suitable model for high-throughput drug screening, a *Giardia lamblia* WB C6 strain expressing *Escherichia coli* glucuronidase A (GusA) was created and tested with respect to susceptibility to the anti-giardial drugs nitazoxanide and metronidazole.

Methods: GusA, a well-established reporter gene in other systems, was cloned into the vector pPacVInteg allowing stable expression in *G. lamblia* under control of the promoter from the glutamate dehydrogenase (*gdh*) gene. The resulting transgenic strain was compared with the wild-type strain in a vitality assay, characterized with respect to susceptibility to nitazoxanide, metronidazole and—as assessed in a 96-well plate format—to a panel of 15 other compounds to be tested for anti-giardial activity.

Results: GusA was stably expressed in *G. lamblia*. Using a simple glucuronidase assay protocol, drug efficacy tests yielded results similar to those from cell counting.

Conclusions: *G. lamblia* WB C6 GusA is a suitable tool for high-throughput anti-giardial drug screening.

Keywords: high throughput drug screening, reporter gene, stable expression, transfection

Introduction

During recent decades, novel anti-infective drugs have regained importance because of increasing resistance to well-established antibiotics and due to the emergence of new infectious diseases.[1] *Giardia lamblia*, for instance, responsible for several million cases of persistent diarrhoea worldwide, is currently treated with metronidazole, representing the main drug so far to be available on a global scale.[2] The thiazolide nitazoxanide is a good alternative.[3,4] In case of resistance formation, albendazole[5,6] or isoflavones[7] are other options.

In order to find novel drug candidates, a high-throughput screening system is required. Present screening methods include counting of trophozoites,[8,9] incorporation of [³H]thymidine[10] or a vitality assay based on the reduction of resazurin (Alamar Blue) quantified by fluorimetry.[11] These methods are time-consuming or need expensive equipment. A more suitable method would be the use of a reporter strain as shown for instance for *Toxoplasma gondii* expressing β-galactosidase.[9,12] Here, we present a short study describing the construction of a

transgenic *Giardia* cell line expressing *Escherichia coli* glucuronidase A (GusA), and first drug efficacy tests using a protocol adapted to a 96-well format.

Materials and methods

Tissue culture media, biochemicals and drugs

If not otherwise stated, the biochemical reagents were from Sigma (St Louis, MO, USA). All compounds tested were kept as 100 mM stock solutions in DMSO at −20°C.

Culture of Giardia trophozoites and drug treatment assays

Trophozoites from *G. lamblia* WB clone C6 were grown under anaerobic conditions in 10 mL culture tubes (Nunc, Roskilde, Denmark) or in 96-well-plates containing modified TYI-S-33 medium as described previously.[7,8] For drug treatment assays, cultures with confluent trophozoite layers were incubated on ice for

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15 min. Suspended motile trophozoites were counted (Neubauer chamber, ×200) and 5×10^3 trophozoites/mL were inoculated into 24-well plates (1 mL per well) or 96-well plates (0.2 mL per well) in the presence of compound or a solvent control (DMSO). The plates were incubated in an anaerobic chamber for 72 h, and attached trophozoites were counted by light microscopy[7] or processed further for a glucuronidase assay or for a resazurin (Alamar Blue) vitality assay as described previously.[11]

Cloning of GusA

In a first round, GusA was cloned into the His tag expression vector pET151 directional TOPO (Invitrogen, Carlsbad, CA, USA); the primers pGusF and pGusR (Table 1) were created for the amplification of a 1811 bp product encoding the GusA polypeptide with four additional bases at the 5′ end allowing directional cloning as previously described.[13] For amplification by PCR, genomic DNA was extracted from 10^8 *E. coli* TOP 10 cells using the DNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The resulting DNA was diluted 10× in ultrapure water. The PCR was performed using *pfu* DNA polymerase (Promega, Madison, WI, USA) as described. The resulting 1811 bp product was inserted into the pET151 vector using the respective cloning kit according to the manufacturer, and the vector was transformed into *E. coli* TOP 10 cells (Invitrogen). Positive clones were expressed in *E. coli* BL21 as described.

A clone with appropriate expression of the recombinant enzyme was selected by purification of the recombinant enzyme followed by a glucuronidase assay as described below and then used for the re-cloning of *gusA* into the giardial vector pPacVInteg using the XbaI and PacI sites for integration.[14] For this purpose, a forward primer was designed starting with the XbaI site followed by the glutamate dehydrogenase promoter, a strong promoter as previously characterized in *G. lamblia*.[15] In the reverse primer, a sequence encoding an HA tag was included before the PacI site (Table 1). PCRs were performed using the *pfu* polymerase as described above, and fragments were cloned into the Zero Blunt TOPO vector (Invitrogen) according to the manufacturer's instructions. Inserts were cut out with XbaI and PacI and ligated into pPacV thus yielding pPacV-GusA. Prior to transfection, the vector pPac-GusA was linearized by digestion with SwaI in order to allow chromosomal integration of the transgene by homologous recombination. Then, transfection of *G. lamblia* WB C6 and selection of stable transfectants (containing GusA integrated in the genome) by resistance to the antibiotic puromycin was performed as previously described.[14]

Glucuronidase assay

Glucuronidase was assayed in 50 mM NaHPO$_4$, pH 7, containing 5 mM dithiothreitol, 1 mM EDTA and 0.1% Triton X-100 (assay buffer).[16] For assays in cuvettes, trophozoites were suspended in 1.5 mL of assay buffer. The reaction was started by adding 15 μL of 100 mM *p*-nitrophenyl-glucuronide (1 mM final concentration). At various timepoints, 0.2 mL aliquots were removed and mixed with 0.8 mL of 0.2 M Na$_2$CO$_3$ (stop solution) in order to stop the reaction and enhance colour development. For assays in 96-well microtitre plates, plates with trophozoites were washed once with PBS prewarmed at 37°C in order to remove unattached trophozoites and debris. Then, cells were suspended in 50 μL of assay buffer containing the substrate and incubated at 37°C. In the control wells, the reaction was stopped immediately by adding 200 μL of stop solution. In the other wells, the reaction was stopped depending on colour formation. Absorption was then read at 410 nm and activity calculated using the extinction coefficient 18.3 M^{-1}cm^{-1}.

Table 1. Overview of primers used in this study

Primer	CDS	Length	Sequence
GusF	1785074–1785074	1811	CACCATGGTCCGTCCTGTAGAAAC
GusR			TCATTGTTTGCCTCCCTGCT
GusF_Glex	XbaI + GDH promotor + Gus	52 + 1811 + 40	GATCTAGACCACAAATAACGCCTTTAATTACAGGCGCCCCAGATTTTAAAATATGGTCCGTCCTGTAGAAAC
GusR_Glex	PacI + stop + HA tag + Gus	52 + 1811 + 40	GATTAATTAATCACGCGTAGTCTGGGACATGGTATCATTGTTTGCCTCCCTGCT

CDS, coding sequence in the genome of *E. coli* K-12.

More suitable for high-throughput screening was the following assay system based on the formation of an insoluble, blue product. The assay mixture consisted of 50 mM $NaHPO_4$ containing 1 mM EDTA, 50 μM potassium ferrocyanide, 20% methanol and 1 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronide cyclohexylammonium salt (X-glucuronide) and was applied to trophozoites grown in microtitre plates as described above (100 μL per well).[17] The colour formation was monitored visually and by reading the absorption at 625 nm at various timepoints.

Statistics

IC_{50} values were calculated after the logit-log-transformation of the relative growth (RG; control=1) according to the formula $\ln[RG/(1-RG)]=a \times \ln(\text{drug concentration})+b$ followed by regression analysis using the corresponding software tool contained in the Excel software package (Microsoft, Seattle, WA, USA).
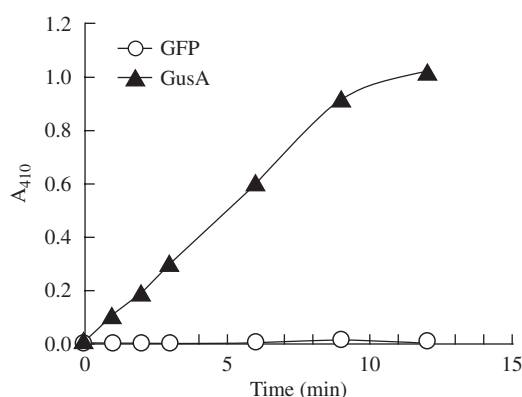

Figure 1. Glucuronidase activity in *G. lamblia* WB C6 expressing *E. coli* GusA or GFP as a control. Confluent cultures were harvested and suspended in GusA assay buffer (10^6 trophozoites/mL). Enzyme reactions were initiated by adding *p*-nitrophenyl-glucuronidase. At various timepoints, aliquots were removed and added to stop solution. Mean values and standard errors (smaller than symbols) correspond to three independent cultures per clone.
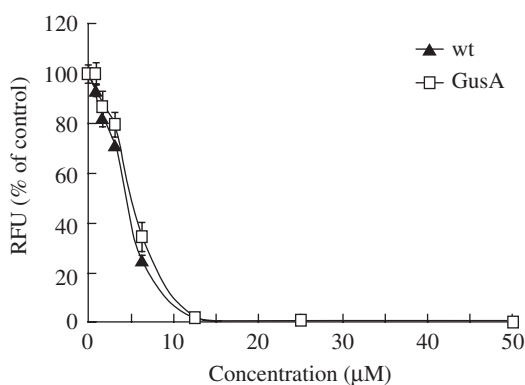

Figure 2. Comparison of *G. lamblia* WB C6 expressing *E. coli* GusA with its corresponding wild-type (wt) using a vitality assay. Plates (96-well) were inoculated with 2×10^2 trophozoites of wt or GusA trophozoites per well and grown in the presence of metronidazole at various concentrations. After 72 h, cell growth was monitored by a vitality assay based on the reduction of resazurin (Alamar Blue) to a pink product that was assayed fluorimetrically. Mean values of the RFU normalized to the control values ($\pm$SE) are given for six replicates.

Results and discussion

GusA can be expressed in *G. lamblia*

G. lamblia WB C6 trophozoites were transfected with pPacV-GusA and stable transfectants were selected by resistance to puromycin (see above). After removal of puromycin, cells were grown for >10 generations. As a control, trophozoites were transformed with pPacV–GFP (expressing green fluorescent protein) and cultivated in parallel. Three independent cultures were harvested and glucuronidase was assayed. Extracts from GFP-transformed trophozoites yielded a background activity close to zero. Extracts from GusA-transformed cells, however, had strong glucuronidase activity with a linear increase of absorption between 0 and 1 (Figure 1). Thus, GusA could be expressed in *G. lamblia* and was stable for >10 generations.
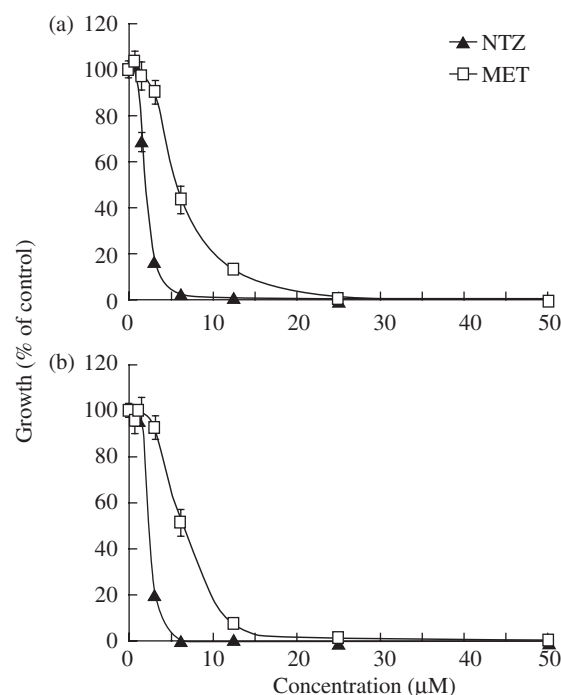

Figure 3. Suitability of *G. lamblia* WB C6 GusA for drug efficacy assays. Plates (96-well) were inoculated with 2×10^2 trophozoites per well and grown in the presence of nitazoxanide (NTZ) or metronidazole (MET) at various concentrations. After 72 h, cell growth was monitored either by counting (a) or by a glucuronidase assay based on the hydrolysis of *p*-nitrophenyl-glucuronide (b). Mean values $\pm$ SE are given for six replicates.

Table 2. IC_{50} values of nitazoxanide (NTZ) and metronidazole (MET) for transgenic *G. lamblia* WB C6 GusA

Treatment	IC_{50} (μM)
NTZ counting	2.7 (1.2)
MET counting	5.9 (1.1)
NTZ Gus	3.2 (1.2)
MET Gus	6.3 (1.1)

The values were calculated from the mean values either determined by counting or by a glucuronidase assay (Gus) as shown in Figure 2. Standard deviations are noted in parentheses.

Table 3. Application of *G. lamblia* WB C6 GusA for anti-giardial drug screening

Compound	Reference(s)	Effective against *G. lamblia*	Glucuronidase activity (percentage of control)
Nitazoxanide	6,8,10	yes	0 ± 0
Tizoxanide	8,10	yes	0 ± 0
Tizoxanide glucuronide	8,10	no	32 ± 6
RM4802	8	yes	0 ± 0
RM4803	8	no	83 ± 8
RM4805	8	yes	2 ± 1
RM4806	8	no	65 ± 9
RM4807	8	yes	0 ± 0
Metronidazole	6,8,10	yes	0 ± 0
Albendazole	5,6	yes	0 ± 0
Praziquantel		?	81 ± 8
Pentamidine DB750	18	?	68 ± 3
Formononetin	7	yes	0 ± 0
Daidzein	7	yes	0 ± 0
Genistein	7	yes	0 ± 0
Mefloquine	19	?	0 ± 0
Dinitrotoluol	13	?	6 ± 1

Plates (96-well) were inoculated with 2×10^2 trophozoites per well and grown in the presence of the drugs listed (50 μM). After 72 h, growth of cells was monitored by a glucuronidase assay based on the formation of an insoluble, blue product from X-glucuronide. Glucuronidase activity is expressed as a percentage of the control mean value obtained in the presence of DMSO. Means ± SE are given for four replicates.

G. lamblia WB C6 wild-type and GusA transgenic strain exhibit similar vitality patterns in drug efficacy tests

A vitality assay based on the reduction of resazurin (Alamar Blue) quantified by fluorimetry had previously been established for testing drug efficacy against *G. lamblia* trophozoites.[11] It was thus tempting to compare trophozoites of *G. lamblia* WB C6 wild-type and GusA in this assay system. In order to investigate whether these trophozoites differ with respect to their metabolism of Alamar Blue, trophozoites of both strains were harvested, counted and subjected to an Alamar Blue assay at six different cell densities (ranging from 10^6 to 10^2 cells). The results were 3.4 ± 0.4 relative fluorescence units (RFU)/cell for the wild-type and 4.1 ± 0.7 RFU/cell for the GusA strain. In a second experiment, trophozoites of both strains were grown in 96-well microtitre plates in the presence of various concentrations of metronidazole. After 72 h, vitality was determined by an Alamar Blue assay. Both strains gave very similar IC$_{50}$ values, namely 3.1 ± 1.1 μM for the wild-type and 4.4 ± 1.2 μM for the GusA strain (Figure 2).

G. lamblia WB C6 GusA is suitable for drug efficacy tests

In order to investigate whether *G. lamblia* WB C6 GusA is suitable for drug efficacy tests in the microtitre format, trophozoites were grown in 24-well or 96-well microtitre plates in the presence of various concentrations of nitazoxanide and metronidazole, two drugs with proven effectiveness against *G. lamblia*.[8,10] After 72 h, growth was determined either by counting (24-well plates) or by a glucuronidase assay (96-well plates) adapted to the microtitre format. Both measurements yielded nearly identical results (Figure 3) with IC$_{50}$ values of ~3 μM of nitazoxanide and ~6 μM of metronidazole (Table 2). Both values were in good agreement with previously published data obtained with

untransfected WB C6.[8] The fact that these IC$_{50}$ values were slightly higher than the ones found with the Alamar Blue assay is in good agreement with previously published results.[11] Thus, *G. lamblia* WB C6 GusA is a suitable strain for use in drug testing.

In order to provide a proof of concept for the suitability of the transgenic strain for high-throughput drug screening purposes, a small screen was performed including compounds with known efficacy and some antiparasitic drugs with unknown efficacy against *G. lamblia*. For this screen, glucuronidase activity was detected using X-glucuronide. X-glucuronide as well as the resulting blue, insoluble product were stable for at least several weeks thus providing clear-cut results that were easily documented by photographs or simply by storing the dried plates. Concerning compounds with known efficacy against *G. lamblia*, such as the thiazolide tizoxanide and some of its derivatives, the screen provided similar results to those of previous reports.[8,10] Moreover, the screen showed that the antimalarial drug mefloquine inhibited trophozoite growth, as did dinitrotoluol, a substrate of a previously described *G. lamblia* nitroreductase.[13] Interestingly, the glucuronide of tizoxanide, which was ineffective against *G. lamblia* WB C6, showed some activity against WB C6 GusA, most likely due to a cleavage of the glucuronide due to the highly expressed glucuronidase (Table 3).

Taken together, *G. lamblia* WB C6 expressing GusA as a reporter gene constitutes a suitable tool for high-throughput anti-giardial drug screening.

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Transparency declarations

None to declare.

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