Characterization of a *Giardia lamblia* WB C6 clone resistant to the isoflavone formononetin

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Giardia lamblia is a common intestinal-dwelling protozoan and causes diarrhoea in humans and animals worldwide. For several years, a small number of drugs such as the 5-nitroimidazole metronidazole (MET) or the thiazolide nitazoxanide (NTZ) have been used for chemotherapy against giardiasis. However, various pre-clinical and clinical investigations revealed that anti-giardial chemotherapy may be complicated by emergence of giardial resistance to these drugs. The present study addressed the question if isoflavones with anti-giardial activity, such as daidzein (DAI) or formononetin (FOR), may serve as alternative compounds for treatment of giardiasis. For this purpose, the potential of *G. lamblia* clone WB C6 to form resistance to FOR and related isoflavones was tested *in vitro*. In the line of these experiments, a clone (C3) resistant to isoflavones, but sensitive to MET and NTZ, was generated. Affinity chromatography on DAI-agarose using cell-free extracts of *G. lamblia* trophozoites resulted in the isolation of a polypeptide of approximately 40 kDa, which was identified by mass spectrometry as a nucleoside hydrolase (NH) homologue (EAA37551.1). In a nucleoside hydrolase assay, recombinant NH hydrolysed all nucleosides with a preference for purine nucleosides and was inhibited by isoflavones. Using quantitative RT-PCR, the expression of genes that are potentially involved in resistance formation was analysed, namely NH and genes encoding variant surface proteins (VSPs, TSA417). The transcript level of the potential target NH was found to be significantly reduced in C3. Moreover, drastic changes were observed in VSP gene expression. This may indicate that resistance formation in *Giardia* against isoflavones is linked to, and possibly mediated by, altered gene expression. Taken together, our results suggest FOR or related isoflavones as an alternative anti-giardial agent to overcome potential problems of resistance to drugs like MET or NTZ. However, the capacity of *Giardia* to develop resistance to isoflavones can potentially interfere with this alternative treatment of the disease.

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INTRODUCTION

Giardia lamblia can cause (human) giardiasis, which is a notorious health problem throughout the world (reviewed by Thompson, 2000). Infection by this parasite is one of the most important causes of diarrhoea in the world, and is especially prevalent in children in developing countries. Giardiasis is considered as a disease of the poor and has been included in the 'Drugs for Neglected Diseases Initiative' list (Savioli *et al.*, 2006). This initiative is focused, among others, on the urgent need for effective drugs with minor side effects. In the past, 5-nitroimidazole metronidazole (MET) (commercially known as Flagyl) and a few other drugs such as thiazolide nitazoxanide (NTZ)

(commercially known as Alinia) have been used as therapy against giardiasis (reviewed by Gardner & Hill, 2001; Upcroft & Upcroft, 2001). Although these compounds are able to efficiently kill *Giardia* trophozoites, treatment of giardiasis with these drugs may be associated with recurrence of symptoms (e.g. reviewed by Upcroft & Upcroft, 2001). Furthermore, drug resistance is one of the reasons for treatment failure. Resistance to MET and other nitroimidazoles has mostly been demonstrated or induced *in vitro*, but various genotypically distinct isolates with reduced drug susceptibility have also been isolated from human patients (reviewed by Upcroft & Upcroft, 2001; Upcroft *et al.*, 1999, 2006). In contrast, clinical isolates resistant to NTZ have not yet been found. However, our recent *in vitro* studies revealed that exposure of *G. lamblia* trophozoites to increasing concentrations of NTZ resulted in resistance formation, and at least one of the highly resistant clones isolated exhibited cross-resistance to MET (Müller *et al.*, 2007). This observation suggests a careful re-evaluation of NTZ regarding its

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Abbreviations: DAI, daidzein; FOR, formononetin; GEN, genistein; MET, 5-nitroimidazole metronidazole; NH, nucleoside hydrolase; NTZ, thiazolide nitazoxanide; PDI, protein disulfide isomerase; VSPs, variant surface proteins.

suitability as an alternative drug for treatment of MET-resistant giardiasis.

In order to improve the situation in chemotherapy of resistant giardiasis, new potential pharmaceuticals from natural sources – such as isoflavones isolated from the bark of *Dalbergia frutescens* – have been evaluated regarding their anti-giardial activity *in vitro* (Khan *et al.*, 2000). Isoflavones are mainly found in Leguminosae where they have anti-oxidant, anti-microbial and signalling functions (reviewed by Dakora & Phillips, 1996; Dixon & Steele, 1999). Within this group of compounds, Khan *et al.* (2000) identified formononetin (FOR), a major isoflavone of *Ononis* sp., as the most potent anti-giardial agent (Fig. 1). *In vitro*, FOR has an IC₅₀ value more than 5 times lower than MET, and more than 150 times lower than genistein (GEN), a major isoflavone of soybean (Khan *et al.*, 2000). Furthermore, even at high concentrations this compound did not exhibit a cytotoxic effect in a Vero cell culture and had no detectable adverse effects in mice (Khan *et al.*, 2000). Due to the observations that FOR and MET have differential kinetics of anti-giardial activity *in vitro* and possess a differential spectrum of antiprotozoal activity, the mode of action of these two drugs was suggested to be completely different (Khan *et al.*, 2000).

It is evident that an eventual chemotherapeutical application of FOR against giardiasis can be complicated by the capacity of *Giardia* to develop resistance to this alternative drug. In the present study, we have established an *in vitro* cultivation assay to find out if FOR treatment against giardiasis promotes formation of anti-giardial drug resistance. In particular, we have generated FOR-resistant trophozoites by increasing stepwise the amount of FOR in the culture and have characterized a resistant clone with respect to growth in the presence of isoflavones, NTZ and MET. In order to identify isoflavone-binding proteins, we have developed an affinity chromatography approach (see Müller *et al.*, 2007) coupling the simplest isoflavone, daidzein (DAI) to agarose and succeeded in isolating a polypeptide of approximately 40 kDa, which was identified by mass spectrometry as a nucleoside hydrolase (NH) homologue (EAA37551.1). The resistant clone was

analysed regarding NH gene expression. Furthermore, since upon selective drug pressure *G. lamblia* trophozoite populations may undergo antigenic variation (Müller *et al.*, 2007), variant surface protein (VSP) gene expression was also analysed in this study.

METHODS

Biochemicals and drugs. If not otherwise stated, all biochemical reagents including the isoflavones FOR, GEN and DAI were from Sigma. For structural formulas see Fig. 1. They were kept as 100 mM (FOR, GEN) or 40 mM (DAI) stock solutions in DMSO at 4 °C.

Drug treatment assays. Trophozoites from *G. lamblia* WB C6 were grown under anaerobic conditions in 10 ml culture tubes (Nunc) containing modified TYI-S-33 medium as described (Keister, 1983). The effects of drugs in axenic *G. lamblia* trophozoite cultures were assayed as previously described (Müller *et al.*, 2007).

Generation of FOR-resistant *Giardia lamblia* trophozoites.

FOR-resistant *G. lamblia* trophozoites were generated using parasites with the same genetic background (i. e. WB C6). In order to obtain drug resistant trophozoites, *G. lamblia* WB C6 wild-type parasites grown to confluency were initially cultured in the presence of sublethal concentrations (100 nM) of FOR. After 3 days, the medium was removed, viable trophozoites remained attached to the tube walls, and fresh medium was added, containing 250 nM FOR. Living (attached) trophozoites were counted under the microscope as previously described (Müller *et al.*, 2006). When the respective numbers remained constant or increased, fresh medium was added, containing similar or slightly higher FOR concentrations. In case the number of living trophozoites decreased, medium was replaced by fresh medium without the corresponding drug (see Megraud *et al.*, 1998). This operation was repeated during eight passages until levels of 500 nM FOR were reached. Clones from the resistant cell line were generated by two rounds of limited dilution of trophozoites (Müller *et al.*, 2007). In total, 12 clones having maintained their resistance were subcultivated. Among these clones, clone 3 (C3) exhibited the shortest generation time and no visible morphological alterations in the presence of the drug, and was selected for detailed characterization. C3 was kept in long-term cultures by periodic passaging in modified TYI-S-33 medium containing 500 nM FOR.

Preparation of crude extracts from *G. lamblia* trophozoites.

Crude extracts were prepared by suspending 10⁸ trophozoites in 2 ml extraction buffer, i.e. PBS containing 0.1 % Triton X-100 and 1 mM phenyl-methyl-sulfonyl fluoride followed by vigorous vortexing and centrifugation (10 000 g, 4 °C). The pellet was re-extracted once with 2 ml and once with 1 ml of extraction buffer. The supernatants were pooled, yielding approximately 5 ml of crude extract.

Affinity chromatography using DAI-agarose.

In order to produce DAI-agarose, 0.75 g lyophilized epoxy-agarose with a C-12 spacer was suspended in 15 ml H₂O and centrifuged at 300 g for 5 min. Washes in water were repeated twice, and once using coupling buffer (NaHCO₃ 0.1 M, pH 9.5). After the last wash, 20 mg DAI was added and coupling buffer was added to a maximum volume of 5 ml. The mixture was incubated for 3 days at 37 °C under slow but continuous shaking in order to allow coupling of the epoxy group to DAI via one of its OH groups. The resulting column medium (approx. 2 ml) was then transferred to a chromatography column (Novagen, Merck) and the column was washed with coupling buffer (20 ml). This was followed by ethanolamine (1 M, pH 9.5) for 4 h at 20 °C in the absence of light in order to block residual reactive groups. Finally, the column was extensively washed with PBS and PBS/DMSO (1:1) in

Fig. 1. Structural formulas of isoflavones used in this study. DAI, daidzein; FOR, formononetin; GEN, genistein.

order to remove unbound DAI. The DAI column was stored in PBS containing 0.02 % NaN₃ at 4 °C.

Prior to affinity chromatography, the DAI column was washed with 50 ml PBS equilibrated at 20 °C. Crude extracts (5 ml) of *Giardia* trophozoites prepared as described above were loaded with a flow rate of approximately 0.25 ml min⁻¹. The column was washed with PBS until the baseline was flat (8 column volumes, corresponding to about 24 ml). Proteins binding to the DAI column were eluted with 0.1 mM FOR in PBS followed by elution with a pH shift (glycine Cl⁻ 100 mM, pH 2.9) in order to remove non-specifically bound proteins. Moreover, fractions were taken before elutions with FOR (pre-FOR) or pH shift (pre-pH shift). Sizes of these fractions ranged between 3 and 5 ml. From all fractions, 0.05–0.2 ml aliquots were taken for analysis by SDS-PAGE. SDS-PAGE was performed according to Laemmli (1970) using a Hoefer Minigel 250 apparatus (Amersham). Silver staining was performed according to Blum *et al.* (1987).

Protein sequencing by mass spectrometry. For protein sequencing, the FOR eluates with the highest amounts of binding protein were pooled and dialysed against ammonium bicarbonate (1 g l⁻¹) for 4 h, then against 0.4 g l⁻¹ overnight at 4 °C in the dark. The dialysed fraction was then lyophilized. Aliquots of the lyophilized binding protein (approx. 200 ng) were suspended in SDS-PAGE sample buffer, loaded on a 12 % acrylamide gel and subjected to electrophoresis. After staining with colloidal Coomassie (0.1 % Coomassie brilliant blue G 250 in 34 % methanol with 0.5 % acetic acid and 17 % ammonium sulfate), a band of approximately 40 kDa was excised and processed for mass spectrometry analysis performed by Core Facility Proteomics (Centre Médical Universitaire, Geneva, Switzerland).

Cloning and heterologous expression of *Giardia lamblia* nucleoside hydrolase (NH). In order to clone NH into the His-tag-expression vector pET151 directional TOPO (Invitrogen), a forward (NHfullF; see Table 1) and reverse (NHfullR, see Table 1) primer (MWG Biotech) were created for the amplification of gene fragments encoding the NH polypeptide. CACC at the 5' end of NHfullF was added in order to allow directional cloning. The cyclic NH gene amplification reaction, cloning of the NH gene amplification product into pET151 directional TOPO, as well as bacterial expression and subsequent His-tag purification of recombinant NH was done according to the procedure previously applied for production of recombinant protein disulfide isomerase (PDI) 4 of *G. lamblia* WB clone C6 (Müller *et al.*, 2007). Purified recombinant *G. lamblia* NH protein (recGINH) was stored in 50 % glycerol at -20 °C.

NH enzyme assay. To determine NH K_m value, activity was measured by a direct assay based on the hydrolysis of inosine (Parkin *et al.*, 1991). The assay was performed in a quartz cuvette containing HEPES 50 mM, pH 7.3 with inosine in concentrations ranging from 0 to 2 mM in a dual-beam spectrophotometer (UV 1700; Shimadzu). After initiation of the reaction by adding recGINH (1 to 5 µM), absorption was continuously read at 280 nm. The values were corrected by a corresponding substrate blank inserted into the second beam. The values obtained were fitted to the Lineweaver–Burk transformation of the Michaelis–Menten law in order to calculate the K_m.

For the determination of substrate specificity and for inhibition studies, recGINH was assayed using an indirect colorimetric assay of the ribose released based on the cuproin method as described previously (Parkin *et al.*, 1991). Eppendorf tubes containing 0.1 ml HEPES 50 mM, pH 7.3 with substrates and/or isoflavones as indicated were incubated for 30 min to 1 h at 37 °C in a water bath. Reaction was stopped by adding 0.4 ml water, 0.15 ml cuproin reagent I (Na₂CO₃ 4 %, glycine 1.6 %, CuSO₄ · 5 H₂O 0.05 %) and 0.15 ml cuproin reagent II (cuproin, 2,9-dimethyl-1,10-phenanthroline 0.12 % suspended in water) followed by 10 min incubation at 95 °C. After cooling to room temperature, absorption was measured at 450 nm. Blanks for enzyme, substrate and isoflavones were included and subtracted from the reaction. A standard curve (glucose from 0 to 100 nmol) made in HEPES buffer under reaction conditions was included in each assay.

Processing of RNA samples and quantitative reverse transcription (RT)-PCR. For quantification of gene expression by real-time RT-PCR, trophozoites of wild-type WB C6 and isoflavone resistant clone C3 were grown in the absence of drugs until near confluency was reached. Cells were harvested as described, and RNA was extracted using a Qiagen RNeasy kit (Qiagen) and including a DNase I digestion (to remove residual genomic DNA) according to the instructions provided by the manufacturer. RNA was eluted with 50 µl RNase free water and stored at -80 °C.

First-strand cDNA synthesis using a polyT-ANC primer (von Allmen *et al.*, 2004; primer sequences, see Table 1) and subsequent quantitative ACT-, VSPtot- and TSA417-PCR (primer sequences, see Table 1) were done as previously described (Müller *et al.*, 2007). In parallel runs, forward (NHquantF) and reverse (NHquantR) primers (see Table 1) were applied for the quantitative NH-PCR. From the quantitative RT-PCR, mean values (± SEM) from triplicate determinations were assessed and expression levels of NH, VSPtot and TSA417 were given as values in arbitrary units relative to the constitutively expressed 'house keeping' gene ACT.

Table 1. Overview of primers used in this study

CDS, coding sequence; utr, untranslated.

Gene Accession number	Region of CDS	Primer (5' → 3')
Actin (ACT) EAA39190	CDS 715–933	ACTquantF ACATATGAGCTGCCAGATGG; ACTquantR TCGGGGAGGCCTGCAAAC
Nucleoside hydrolase (NH) EAA37551.1	CDS 1–1097 plus 7 of 3' utr	NHfullF CACCATGGCTGTACAAAGCAC; NHfullR GAATTCATTATTCGTTAGG;
	CDS 751–1037	NHquantF GGGCGCTTCCACCGATC; NHquantR GAATTCATTATTCGTTAGGGT
Variant surface protein TSA417 U89152.1	CDS 127–335	TSA417quantF TGTGGAACGTGTGCCAATAG; TSA417quantR AGACACGTAGTACAGTCGG
Variant surface protein (total)	see von Allmen <i>et al.</i> (2004)	VSP _{tot} quantF (MM16) GGCTTCCTCTGCTGGTGGTTC; VSP _{tot} quantR (ANC) GACCACGCGTATCGATGTCCA

Statistical methods. The significance of the differences of the NH, VSptot and TSA417 gene, and gene expression between wild-type clone WB C6 and isoflavone resistant clone C3 were determined by Student's *t*-test using Microsoft's Excel program. *P* values of <0.01 were considered statistically highly significant.

RESULTS

Generation of FOR-resistant (FORr) *Giardia lamblia* trophozoites

By axenic culture of *G. lamblia* WB C6 trophozoites in the presence of slowly and continuously increasing concentrations of FOR (see Methods), a drug-resistant trophozoite line was generated. This trophozoite line was subcultured in the presence of up to 500 nM FOR. In total, 12 clones from the resistant cell line were generated and one FORr clone (C3) was further investigated. Regarding the growth kinetics in normal medium, clone C3 did not significantly differ from wild-type clone WB C6 (Fig. 2). Upon prolonged growth of C3 in drug-free medium for more than 10 generations, resistance was maintained. The clone C3 encysted as well as the wild-type when put on encystation medium (not shown).

Cross-resistance

In order to determine IC_{50} values of the FORr clone C3 in the presence of FOR and the related isoflavone DAI, trophozoites of the respective strains were grown for 3 days

Fig. 2. Growth of *Giardia lamblia* WB C6 wild-type (WT) and FOR-resistant (C3) trophozoites. At day 0, 10^4 trophozoites were inoculated to normal culture medium and trophozoite numbers were determined daily during a period of 4 days. Trophozoite numbers per culture tube are given as mean values ($\pm$ SEM) from triplicate determinations.

Fig. 3. Growth of *Giardia lamblia* WB C6 wild-type (WT) and isoflavone resistant (C3) trophozoites in the presence of for-mononetin (FOR, a) or daidzein (DAI, b). At day 0, 10^4 trophozoites were inoculated to normal culture medium. Trophozoites were harvested and counted at day 4. Trophozoite numbers are given as mean values ($\pm$ SEM) from triplicate determinations.

at various drug concentrations (Fig. 3). At 0.5 μ M FOR, C3 had about 75 % of control growth, whereas the wild-type was decreased to approximately 1.5 % (Fig. 3a). On 10 μ M DAI, C3 reached approximately 58 % of the control level whereas the wild-type was decreased to approximately 1 % (Fig. 3b). Growth on GEN essentially corresponded to growth on DAI (growth curves not shown). C3 could thus be regarded as isoflavone-resistant.

In order to investigate whether resistance formation of C3 was a multidrug-resistance phenomenon, C3 and wild-type cells were grown in the presence of the commonly used anti-giardial nitro drugs NTZ and MET. Both wild-type and C3 trophozoites were equally sensitive to these compounds (growth curves not shown).

A nucleoside hydrolase homologue is a major DAI-binding protein in *Giardia lamblia*

Following DAI-affinity chromatography, a major *Giardia* protein of approximately 40 kDa was eluted with 0.1 mM FOR (Fig. 4a, lane FOR). FOR was used as eluent since this

Fig. 4. (a) Affinity chromatography of cell-free extracts from 3×10^8 *Giardia lamblia* trophozoites. Aliquots of fractions were separated by SDS-PAGE and a silver staining of the gel was performed. M, marker proteins (92, 66, 45, 31, 22.5 and 14 kDa); CE, crude extract; FOR, elution with FOR (0.5 mM in PBS); HT, recombinant EAA37551.1 eluted after His-tag affinity purification. (b) Alignment of EAA37551.1 (*Giardia*) with its closest homologues, the *Trichomonas vaginalis* nucleoside hydrolase EAY18059 (*Trichomonas* 1), the *Trichomonas vaginalis* inosine-uridine preferring nucleoside hydrolase family protein (*Trichomonas* 2), the *Xenopus tropicalis* protein containing an inosine-uridine preferring nucleoside hydrolase domain NP_001039191 (*Xenopus*), the *Danio rerio* LOC402865 protein AAH56692 (*Danio*) and the *Entamoeba histolytica* inosine-uridine preferring nucleoside hydrolase XP_654837 (*Entamoeba*). The alignment was produced by CLUSTAL W (Huang & Miller, 1991).

compound showed the best growth effects on *Giardia* trophozoites and since the blocking of one OH group corresponded best to the ligand DAI bound via a C12-spacer to one of its OH groups to the matrix. By mass spectrometry, the 40 kDa protein was identified as a nucleoside hydrolase (NH) homologue (accession EAA37551.1; orf 13272 in the *Giardia* genome database; approximately 30 % coverage of the sequence by peptides from MS sequencing). Protein–protein BLAST (Altschul *et al.*, 1997) revealed that the closest homologues of EAA37551.1 were NHs from *Trichomonas vaginalis* (two enzymes), *Entamoeba histolytica*, *Danio rerio* and *Xenopus tropicalis*. Besides the conserved domains, EAA37551.1 possessed, however, short peptide motives (between aa residues 61 and 226) not found in the other NHs (Fig. 4b), a phenomenon that has been observed in many other *Giardia* orthologues (see *Giardia* genome database). Like

its homologues, EAA37551.1 had no signal peptide and was considered as cytosolic.

EAA37551.1 exhibits nucleoside hydrolase activity and is named GINH

The coding sequence of EAA37551.1 was amplified by PCR and cloned and expressed in *E. coli*. The resulting product migrated at around 40 kDa by SDS-PAGE. The recombinant protein was the major protein in IPTG-induced *Escherichia coli* and could be further purified by His-tag affinity chromatography (Fig. 4a). The purified protein was subjected to an indirect nucleoside hydrolase assay based on the determination of riboside released from various nucleosides. The recombinant protein hydrolysed the purine nucleosides adenosine, guanosine and inosine much better than the pyrimidine nucleosides uridine or cytidine (Fig. 5a).

Fig. 5. Nucleoside hydrolase activity of EAA37551.1. (a) Activity on various nucleosides (2 mM each) was measured in an indirect assay system at 37 °C. (b) Direct assay for K_m determination with various concentrations of inosine. (c) Indirect assay with inosine (Ino, 0.1 or 0.5 mM) as a substrate and the isoflavones daidzein (DAI) or formononetin (FOR) as inhibitors. Values correspond to triplicates (mean values $\pm$ SEM).

In order to determine the kinetic constants of GINH, recGINH was subjected to a direct enzyme assay using inosine as a substrate. In this system, the K_m value was 70 ± 11.5 μ M and the V_m was 185 ± 32 μ kat g⁻¹ as determined after a Lineweaver–Burk transformation of the curve shown in Fig. 5(b). These values were in good

agreement with previously published values from NHs from *Crithidia fasciculata* (Parkin *et al.*, 1991) and *Trichomonas vaginalis* Gero *et al.* (2000). Thus, we propose that EAA37551.1 should be named *G. lamblia* purine nucleoside-preferring nucleoside hydrolase (GINH).

Recombinant nucleoside hydrolase (NH) is inhibited by isoflavones

When an indirect NH assay was performed in the presence of DAI and FOR, NH activity decreased in a concentration-dependent manner. The inhibition was more pronounced at 0.1 mM than at 0.5 mM of the substrate inosine (Fig. 5c). GEN behaved similarly to DAI (data not shown). Assuming a competitive inhibition, the K_i values were 3.8 ± 0.7 μ M for DAI and 11 ± 2.8 μ M for FOR as calculated according to Parkin (1996).

Gene expression

In order to determine gene expression of NH identified as potential target of isoflavones (see above) and thus potentially involved in resistance formation, we developed a real-time RT-PCR assay quantifying reverse transcripts of the NH gene. In this assay, reverse transcripts of ACT gene were also quantified as reference for the amount of total cDNA. ACT gene expression had been previously revealed to be constant in WB C6 wild-type and drug-resistant strains (Müller *et al.*, 2007). Respective analysis demonstrated that gene expression of NH was significantly decreased in C3 (approx. 41%) compared with the corresponding wild-type WB C6 expression level (Fig. 6). However, the differences between the wild-type and the resistant *G. lamblia* clone were much more pronounced when expression of VSP genes was investigated. For respective analyses, cDNA was synthesized issuing from polyT-ANC primers (see Methods). This experimental strategy allowed RT-PCRs to be performed with a general forward primer (MM16 primer) targeted to a highly conserved VSP gene region and a reverse primer targeted to the 3' terminal anchor (ANC) sequence adjacent to the polyT stretch of the cDNA molecules (Table 1). By comparative quantitative RT-PCR, the cDNA synthesized with polyT-ANC was also amplified by including a primer pair specific for the gene TSA417 that encodes the major VSP in WB C6 (Fig. 6). While wild-type clone WB C6 exhibited TSA417 gene expression, corresponding expression levels were dramatically reduced and virtually non-detectable in FORr clone C3. Conversely, the overall expression of VSP genes (VSP_{tot}) remained constant, suggesting that resistance was associated with an antigenic switch from TSA417 to one, or several unknown, VSPs

DISCUSSION

In the past, various antiparasitic drugs have been developed but only a few compounds such as MET and NTZ turned

Fig. 6. Quantification of NH and variant surface protein (VSP) gene expression by real-time RT-PCR. Trophozoites were grown in normal culture medium until near confluency was reached. RNA was extracted and reverse transcribed to cDNA. Transcripts of NH, VSP gene encoding TSA417, and of total VSPs (VSPtot) were quantified in relation to transcripts from constitutively expressed actin (ACT) gene. The relation is expressed in arbitrary units (AU) as a percentage of the wild-type (WT) mean values ($\pm$ SEM).

out to be effective for treatment of giardiasis (reviewed by Gardner & Hill, 2001; Upcroft & Upcroft, 2001). In the field of anti-giardial drug development, the possibility of resistance formation has been recognized as an important problem and was the subject of various preclinical and clinical investigations (reviewed by Upcroft & Upcroft, 2001). Formation of resistance to MET is a major constraint for efficient treatment of human giardiasis, and therapeutic failure due to giardial resistance to this compound is occurring at an increasing frequency (Townson *et al.*, 1994; Wright *et al.*, 2003). Furthermore, our recent investigations revealed that *G. lamblia* trophozoites grown *in vitro* were able to develop resistance to NTZ, and at least one of the resistant clones exhibited cross-resistance to MET (Müller *et al.*, 2007).

Under the circumstances outlined above, the isoflavones previously described as a new group of antimicrobial compounds (reviewed by Dakora & Phillips, 1996) have been investigated as alternative drugs for the treatment of giardiasis (Khan *et al.*, 2000). Among the different isoflavones tested *in vitro*, FOR turned out to be the most efficacious anti-giardial compound (Khan *et al.*, 2000) and was also demonstrated to efficiently inhibit growth of MET- and/or NTZ-resistant *G. lamblia* strains (Müller *et al.*, 2007). This observed lack of cross-resistance *in vitro* certainly suggested a further evaluation of FOR regarding its applicability as an alternative anti-giardial agent to overcome potential problems of resistance to conventional drugs. Conversely, it is evident that this concept of treatment may be complicated by the capacity of *Giardia* to develop high-level resistance to this drug. In fact, in our study, resistance to FOR was already detectable upon relatively short exposure of *in vitro*-cultivated trophozoites to the drug, suggesting that genetic predispositions allow

the parasite to immediately adapt to an isoflavone-enriched host intestinal environment, e.g. emerging upon uptake of an isoflavone-rich vegetarian diet by the host.

In the present study, trophozoites from FORr clone C3 were found to be cross-resistant to other isoflavones DAI and GEN, but not to the nitro compounds MET or NTZ. This indicated that resistance was not caused by a general mechanism like multi-drug transporters, but rather by an as yet unknown mechanism that specifically concerns isoflavones. This conclusion was also supported by our previous observation demonstrating that NTZ-resistant *G. lamblia* trophozoites were not cross-resistant to isoflavones (Müller *et al.*, 2007).

One, but not the only, effect of isoflavones could be the inhibition of the isoflavone-binding protein NH. This inhibition occurs in a range of concentrations where DAI and GEN inhibit trophozoite growth *in vivo*. FOR is inhibitory at much lower concentrations, suggesting other mechanisms to be involved. In *Giardia*, hydrolysis of exogenous nucleosides by NH has been previously described as the first step of one of two distinct salvage pathways to form purine (see e.g. Wang & Aldritt, 1983; Aldritt & Wang, 1986) and pyrimidine ribonucleotides (Aldritt *et al.*, 1985; for review see Jarroll *et al.*, 1989). Moreover, a low-affinity transporter for purine nucleobases has been described as a potential adenine and guanine donor for the second salvage pathway (Ey *et al.*, 1992). In *Crithidia*, *Leishmania* or *Trypanosoma*, the function of NH is central in salvaging purine nucleosides from the host and essential since the parasites lack purine *de novo* synthesis (Parkin *et al.*, 1991; Parkin, 1996; Shi *et al.*, 1999). Inhibition of NH by a drug would thus lead ultimately to a block of DNA synthesis and thus cell division. Parasites could overcome this inhibition by synthesizing more NH. An evaluation of the gene expression pattern revealed, however, a significant reduction of NH transcripts in the isoflavone-resistant clone C3 rather than an increase. This pattern is expected if the target enzyme activates a (non-toxic) prodrug, e.g. pyruvate-oxidoreductase in the case of MET (see Müller *et al.*, 2007 and references therein). One explanation could be that NH is downregulated and another, unrelated scavenging enzyme such as purine phosphorylase (Parkin *et al.*, 1991; Parkin, 1996) is upregulated, thus compensating NH inhibition.

In comparison to NH gene expression, the gene regulatory effects associated with antigenic variation of the parasite were much more pronounced. Here, expression of TSA417 representing the major surface antigen (VSP C6) of WB C6 was massively downregulated in isoflavone-resistant clone C3. This finding is in agreement with results from our recent study that demonstrated a striking reduction of the TSA417 gene expression in trophozoites that had developed resistance to NTZ and/or MET (Müller *et al.*, 2007). It is known from current literature that *G. lamblia* is able to escape host immunological defence mechanisms by changing the expression of its surface antigens (reviewed by

e.g. Müller & Gottstein, 1998; Müller and von Allmen, 2005). Furthermore, it was found that, besides the immunological pressure, physiological factors such as intestinal proteases also act as driving forces for antigenic diversification of the parasite (Nash *et al.*, 1991). Finally, we now realized that drug pressure is able to select for (a) new surface antigen variant(s) within a *G. lamblia* trophozoite population (Müller *et al.*, 2007 and present study). Taking all these observations into account, antigenic variation seems to be part of a versatile adaptive mechanism that allows the parasite to respond to a broad spectrum of environmental stress factors. As reported recently, an epigenetic mechanism, probably including gene acetylation, seems to be responsible for antigenic switching of the parasite (Kulakova *et al.*, 2006). Possibly, this epigenetic mechanism involves pleiotropic gene-regulatory effects that mediate, or at least participate in, anti-giardial drug resistance formation. Future studies based on differential and functional genomics and proteomics approaches will reveal if surface antigen alterations per se or rather pleiotropic gene-regulatory processes associated with a surface antigen switch are responsible for *in vitro*-induced drug resistance in *G. lamblia*.

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