Infection, Genetics and Evolution 15 (2013) 10–17

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Proteomic and transcriptional analyses of genes differentially expressed in *Giardia duodenalis* clones resistant to albendazole

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ARTICLE INFO

Article history:
Available online 26 September 2012

We dedicate this work to the memory of Dr. Guillermo Mendoza-Hernández

Keywords:
Giardia
Albendazole
Proteomics
Transcriptional analysis
Drug resistance

ABSTRACT

In this study we performed proteomic and transcriptional analyses to identify and characterize genes differentially expressed in *Giardia duodenalis* clones resistant to albendazole. The expression of proteins and their corresponding mRNAs was analyzed in clones resistant *in vitro* to different concentrations of albendazole (1.35, 8.0 and 250 μM) and these were compared with albendazole-sensitive clones using two approaches: (1) two-dimensional protein electrophoresis to analyze the proteome by the LC-MS/MS technique, and (2) semi-quantitative RT-PCR to assess the mRNA levels of proteins with the highest levels of differential expression .This strategy allowed the identification of eight proteins differentially expressed in albendazole resistant clones with roles in: (a) the cytoskeletal system (alpha 2-giardin and RanBP1), (b) the antioxidant metabolism (NADH oxidase) and (c) energy metabolism (triosephosphate isomerase, phosphoglycerate kinase and ornithine carbamoyltransferase). Gene expression analyses of these genes correlated well with the proteomics results. These observations suggest that resistance to albendazole in *Giardia* encompasses a complex response involving an altered expression of genes regulated at the transcriptional level that might have an important role in maintaining cell structural stability, coping with oxidative stress and adapting energy supply to a new metabolic status. These molecules are indeed promising targets for drug development.

1. Introduction

Giardia duodenalis (syn: *lamblia*, syn: *intestinalis*) is an early-divergent protist but highly evolved member of Diplomonadida adapted to parasitism causing giardiasis, a leading cause of parasitic diarrhea producing retardation of growth and development in infants (Farthing, 1989). *G. duodenalis* infects about 200 million people worldwide each year and a broad spectrum of domestic, livestock and wild species. Giardiasis has been included in the 'Drugs for Neglected Diseases Initiative' list since it impairs the host ability to achieve full physiological and cognitive potential (Savioli et al., 2006). Effective antimicrobial chemotherapy for giardiasis initiated in 1940s with quinacrine (Thomas, 1953). However, quinacrine was no longer used due in part to the introduction and wide availability of metronidazole (Darbon et al., 1962) and other nitroimidazole derivatives (tinidazole, ornidazole, secnidazole), nitrofuranes (furazolidone) and aminoglucosides (paromomycin) that were developed later on (Fox and Saravolatz, 2005). Benzimidazoles such as albendazole (Abz) and mebendazole (Mbz) were introduced by late 1980s and in the mid-1990s the nitrothiazole nitazoxanide was also introduced to the market as an antigiardial agent (Gardner and Hill, 2001).

Regarding the clinical efficacy of antigiardial drugs, *in vitro* and *in vivo* studies have reported that Abz and other related benzimidazoles are more effective against *G. duodenalis* than are metronidazole and tinidazole (Reynoldson et al., 1991). However, treatment failure is an issue of growing concern as up to 20% of patients treated with metronidazole may not become parasitologically cured (Upcroft and Upcroft, 2001). Likewise unsuccessful therapies with Abz have been reported (Lindquist, 1996). It is thought that Abz primarily acts by binding to parasite β-tubulin which affects microtubule dynamics and secondarily by impairing glucose uptake (Venkasetan, 1998) indeed affecting trophozoite morphology, motility, adherence and viability Meloni et al., 1990). Regarding benzimidazole resistance, these compounds were suggested to be glycoprotein P (GpP) substrates as GpP activity increases drug efflux to avoid drug accumulation and belongs to the multidrug resistance phenotype (Mottier and Lanusse, 2001). However Merino et al. (2002) observed that Abz is neither a substrate nor an inhibitor of GpP albeit GpP inhibitors blocked Breast Cancer

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http://dx.doi.org/10.1016/j.meegid.2012.08.021

Resistance Protein (Bcrp)-mediated albendazole sulfoxide (AbzSO) transport (Muenster et al., 2008).

Resistance to Abz in helminth models has been related to changes in cytoskeletal molecules, especially β-tubulin where there may be one or two single amino acid substitutions from phenylalanine to tyrosine at positions 167 or 200 (Katiyar et al., 1994; Schwab et al., 2005).

In previous studies from our group and others showed that modifications in the β-tubulin gene sequence in Abz-resistant clones (up to 8 μM) are absent (Upcroft et al., 1996; Argüello-García et al., 2009) suggesting that alternative mechanisms of Abz resistance might be working in *Giardia*. On the other hand, Abz is also potentially able to inhibit glucose absorption selectively and irreversibly, which in turn might alter glycogen storage and glycolytic pathways in helminth parasites (Venkatesan 1998; Vinaud et al., 2008) and moreover drug metabolization itself may be altered in benzimidazole-resistant parasites (Alvarez et al., 2005). However, these issues have not yet been assessed in Abz-sensitive protists to date. In this complex context, advances in proteomic technology have opened new opportunities to study parasite biology (Longley and Johnston, 2005) and demonstrate that proteomic analysis is valuable for the essential study of potential drug targets and drug resistance mechanisms (Drummelsmith et al., 2003; Prieto et al., 2008; Lin et al., 2008). In this study we used proteomic and transcriptional analyses to identify proteins and their corresponding genes which were differentially expressed in *G. duodenalis* clones resistant or sensitive to Abz, in order to begin elucidating molecules and mechanisms directly or concurrently involved in the development of Abz resistance in this organism.

2. Materials and methods

2.1. Parasite culture and induction of albendazole resistance

G. duodenalis trophozoites of the WB strain (ATCC#30957) and derived Abz-resistant cultures were maintained in TYI-S-33 medium supplemented with 10% adult bovine serum, penicillin (50 U/ml) and streptomycin (50 g/ml) at 37 °C (Keister, 1983) in 4.5 mL screw-capped vials. Abz-resistant trophozoites were selected by continuous subculture under increasing sub-lethal concentrations of Abz [Sigma^R] and once parasites adapted to each increase of drug concentration, cultures were cloned by limiting dilution (Baum et al., 1988) using the corresponding Abz concentration. Trophozoites were subcultured three times a week always in the presence of drug (Abz-resistant clones) or vehicle [*N*,*N*-dimethylformamide, Sigma^R] (DMF) for the Abz-sensitive clones. Stock solutions (0.01–25 mM) of Abz in DMF or DMF alone were used in drug susceptibility test and cultures. Cell viability was assessed by subculture in liquid medium after 24 h of exposure to Abz and the corresponding minimal lethal concentrations (MLC) were calculated by the least-square method using the Microsoft Excell^R software.

2.2. Protein extraction of sensitive and Abz-resistant G. duodenalis clones

Trophozoites of *G. duodenalis* were harvested at the exponential phase of growth (72 h after incubation) by chilling in a water–ice bath for one hour. After this parasites were centrifuged at 440×*g* for 10 min, washed three times with PBS pH 7.2 and counted in a haemocytometer. Afterwards 1.7×10^7 trophozoites were resuspended in PBS (pH 7.2) with 1X Complete Mini Protease Inhibitors cocktail (Roche^R) and sonicated six times with 15 s-pulses at 60% amplitude, then the extract was mixed with a 4-fold volume of trichloroacetic acid-acetone (TCA-A) modified lysis buffer (10% TCA and 2% β-mercaptoethanol in ice-cold acetone) (Saravanan and Rose, 2004). The mixture was homogenized, proteins were precipitated at −20 °C overnight and the pellet was recovered by centrifugation at 10,000×*g* for 30 min.

2.3. SDS–PAGE analysis

Solubilized proteins (10 μg protein per lane) were separated by one dimension SDS–PAGE using a Miniprotean II System (BioRad^R) and separating gels containing 15% acrylamide at 110 V for approximately 2 h. The gels were then stained with Coomassie Blue R-250 (BioRad^R). All one dimension SDS–PAGE gel separations were repeated at least 3 times for each of the four extracts of every clone.

2.4. Sample preparation for 2DE

The protein pellets from the four *Giardia* clones were washed with ice-cold methanol followed by multiple ice-cold acetone washes, dried at 25 °C and resuspended in IEF buffer (7 M urea, 2 M thiourea, 2% w/v CHAPS, 65 mM DTT, 2% w/v carrier ampholytes pH 3–10 (Amersham Biosciences^R) and 0.002% w/v bromophenol blue. Protein concentrations were quantified using the Folin–Ciocalteus reagent (Lowry et al., 1951) with BSA as a standard.

2.5. IEF and Two-dimensional gel electrophoresis

Typically, 250 μg of protein taken from each sample were dissolved in a 250 μl mixture of IEF solution, placed at room temperature for 1 h, transferred to Immobiline DrySrip Reswelling Tray (Amersham Bioscience, Piscataway, NJ, USA) in which IPG dry gel strip (13 cm, pH 3–10) was put and covered with mineral oil. The isoelectric focusing program was as follows: held at 500 V over 500 Vh; linear increase from 800 V over 1000 Vh; linear increase from 8000 V over 11300 Vh and then held at 8000 V over 4400 Vh, for a total of 17 kVh. Following IEF, the proteins were reduced by incubating the IPG strips with 1% w/v DTT for 15 min and alkylated with 2.5% w/v iodoacetamide in 10 ml of equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS and a trace amount of bromophenol blue) for 15 min. SDS–PAGE was performed using 12.5% acrylamide slab gels running at a constant current of 70 V/gel for 12–16 h until the bromophenol blue dye reached the end of the gel. A protein marker was applied at the other terminus of the gel. After electrophoresis, gels were stained with silver nitrate using a standard protocol that is compatible with mass spectrometry (MS) analysis (Shevchenko et al., 1996).

2.6. Image analysis and spot identification

The stained 2-DE gels were scanned with LabScan software on Imagescanner and analyzed using the Image Master v 5.0 system (Amersham Biosciences^R, Piscataway, NJ, USA), according to the protocols provided by the manufacturer. To minimize the contribution of experimental variations, three separate gels were prepared for each clone. The following criteria for differential protein expression were used: a% volume n-fold >1.0 of spot was considered as increase of expression and a% volume n-fold <1.0 of spot was considered as decrease of expression in Abz-resistant clones in comparison to sensitive ones.

2.7. MS analysis and protein sequence determination

The protein spots identified as differentially expressed were excised from the silver-stained gels, destained, reduced, carbamidomethylated and digested with modified porcine trypsin (Promega^R, Madison, WI). Peptide mass spectrometric analysis