International Journal for Parasitology: Drugs and Drug Resistance 7 (2017) 147–158

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

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

Proteomic and functional analyses reveal pleiotropic action of the anti-tumoral compound NBDHEX in *Giardia duodenalis*

Serena Camerini [a], Alessio Bocedi [b], Serena Cecchetti [a], Marialuisa Casella [a], Miriam Carbo [c], Veronica Morea [c], Edoardo Pozio [d], Giorgio Ricci [b, **], Marco Lalle [d, *]

[a] Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, viale Regina Elena 299, 00161 Rome, Italy
[b] Department of Chemical Sciences and Technologies, University of Rome "Tor Vergata", via della Ricerca Scientifica 1, 00133 Rome, Italy
[c] Institute of Molecular Biology and Pathology, CNR - National Research Council of Italy, Department of Biochemical Sciences "A. Rossi-Fanelli", "Sapienza" University, 00185 Rome, Italy
[d] Department of Infectious Diseases, Istituto Superiore di Sanità, viale Regina Elena 299, 00161 Rome, Italy

ARTICLE INFO

Article history:
Received 3 February 2017
Received in revised form
25 March 2017
Accepted 27 March 2017
Available online 29 March 2017

Keywords:
Giardia
NBDHEX
Thioredoxin reductase
Elongation factor 1Bγ

ABSTRACT

Giardiasis, a parasitic diarrheal disease caused by *Giardia duodenalis*, affects one billion people worldwide. Treatment relies only on a restricted armamentarium of drugs. The disease burden and the increase in treatment failure highlight the need for novel, safe and well characterized drug options. The antitumoral compound NBDHEX is effective *in vitro* against *Giardia* trophozoites and inhibits glycerol-3-phosphate dehydrogenase. Aim of this work was to search for additional NBDHEX protein targets. The intrinsic NBDHEX fluorescence was exploited in a proteomic analysis to select and detect modified proteins in drug treated *Giardia*. *In silico* structural analysis, intracellular localization and functional assays were further performed to evaluate drug effects on the identified targets. A small subset of *Giardia* proteins was covalently bound to the drug at specific cysteine residues. These proteins include metabolic enzymes, e.g. thioredoxin reductase (gTrxR), as well as elongation factor 1B-γ (gEF1Bγ), and structural proteins, e.g. α-tubulin. We showed that NBDHEX *in vitro* binds to recombinant gEF1Bγ and gTrxR, but only the last one could nitroreduce NBDHEX leading to drug modification of gTrxR catalytic cysteines, with concomitant disulphide reductase activity inhibition and NADPH oxidase activity upsurge. Our results indicate that NBDHEX reacts with multiple targets whose roles and/or functions are specifically hampered. In addition, NBDHEX is in turn converted to reactive intermediates extending its toxicity. The described NBDHEX pleiotropic action accounts for its antigiardial activity and encourages the use of this drug as a promising alternative for the future treatment of giardiasis.

1. Introduction

Giardiasis is an ubiquitous gastrointestinal disease caused by the flagellated protozoan parasite *Giardia duodenalis* (syn. *lamblia* or *intestinalis*) infecting the gut of mammals, including humans. Almost one billion people are estimated to be infected worldwide, including Europe, with a higher disease burden in developing countries (over 200 million symptomatic cases/year) (Lane and Lloyd, 2002). Infection occurs following ingestion of parasite cysts present either in contaminated water or food or via the fecal-oral route by host-to-host contact. Trophozoites, released from the cysts, multiply and colonize the upper part of the host small intestine, adhere to enterocyte surface and cause the symptoms, such as acute and chronic diarrheal disease. Infections may be also asymptomatic (Buret, 2008). Giardiasis is prevalent in children (other risk groups include returning travellers and immigrants/refugees) (Shah et al., 2009; Staat et al., 2011), with prolonged/recurrent infections correlated to failure to thrive syndrome in malnourished children. Post-infectious long term consequences of giardiasis, including post-infectious irritable bowel syndrome, have been also reported (Buret, 2008). Consequently, treatment of clinical giardiasis is recommended. Nitroimidazoles, whose prototype is metronidazole (MTZ), and other nitrocompounds, e.g. nitazoxanide, are the most used therapeutic agents (Lalle, 2010; Ansell et al., 2015). Benzimidazoles, including the anthelmintic

* Corresponding author.
** Corresponding author.
E-mail addresses: riccig@uniroma2.it (G. Ricci), marco.lalle@iss.it (M. Lalle).

http://dx.doi.org/10.1016/j.ijpddr.2017.03.006

albendazole, are also used with lower efficacy. Despite their therapeutic value, all these compounds provoke from mild to serious side effects. Treatment failures with MTZ have been reported in 10–20% of patients, especially returning travellers from poor endemic countries (Muñoz Gutiérrez et al., 2013; Yadav et al., 2014; Ansell et al., 2015). Therefore, an implementation of the available anti-giardial arsenal with more potent, safe and well tolerated drugs are desirable. Novel classes of compounds, some already approved for unrelated human diseases treatment, are effective against *Giardia* (Tejman-Yarden et al., 2013; Hahn et al., 2013; Kulakova et al., 2014) and potential drug targets have been also identified (Reyes-Vivas et al., 2014; Debnath et al., 2014; Galkin et al., 2014).

We have proven that the anticancer agent 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX) is five times more potent than MTZ in killing *Giardia* trophozoites (NBDHEX IC_{50}: 0.3 ± 0.1 μM; MTZ IC_{50}: 1.5 ± 0.1 μM) (Lalle et al., 2015). NBDHEX is a "mechanism-based inhibitor" of human glutathione-S-transferases (GSTs) promoting apoptosis in a variety of cancer cell lines with good tolerability and safety profile in mouse model (Ricci et al., 2005; Turella et al., 2005, 2006; Sau et al., 2012). Since neither GST and glutathione cycling nor canonical apoptotic pathways are present in *Giardia* (Bagchi et al., 2012; Ansell et al., 2015), our data indicate that reduction of NBDHEX nitro moiety in the parasite environment, associated with ROS generation, is likely involved in cytotoxicity (Lalle et al., 2015).

MTZ and other nitrocompounds (i.e. nitroimidazoles, nitrofurans and nitrothiazolides) are enzymatically nitroreduced to nitroradical anions, forming adducts with DNA, proteins and free thiols leading to DNA damage, protein inactivation and generating oxidative stress (Müller, 1983; Ansell et al., 2015). In *Giardia*, pyruvate ferredoxin oxidoreductase (PFOR) and its ferredoxin substrate, thioredoxin reductase (TrxR), nitroreductase1 (NR1) and the NADH oxidase, have been implicated in the activation of nitrodrugs (Ansell et al., 2015).

In *Giardia* trophozoites, NBDHEX administration induces a significant reduction of the FAD-dependent glycerol-3-phosphate dehydrogenase (gG3PD) activity (Lalle et al., 2015). *In vitro*, gG3PD is able to nitroreduce NBDHEX, but not MTZ, resulting in the formation of covalent adducts between nitroreduced NBDHEX and several enzyme cysteine residues. Additionally, spectroscopic analyses suggest that oxidized NBDHEX, which is intrinsically fluorescent, can bind gG3PD, likely via σ-complexes (Lalle et al., 2015). Since small molecule drugs can target multiple proteins in the same organisms, the identification of all possible binding partners is an essential step to fully understand the mechanism(s) of activity and advance the drug development process.

Taking advantage of the intrinsic NBDHEX fluorescence properties, we present a proteomic and functional analysis of NBDHEX-treated *Giardia* trophozoites aimed at detecting additional protein targets.

2. Materials and methods

2.1. Parasite cultivation and drug treatment

Giardia WB-C6 was used and cultivated as described (Lalle et al., 2015). Ethanol-dissolved NBDHEX (50 μM), synthetized as described (Ricci et al., 2005), or solvent alone, was added to confluent culture of *Giardia* trophozoites for 2 h at 37 °C (Lalle et al., 2015). Parasite soluble proteins were prepared as described (Lalle et al., 2015) from 2×10^9 trophozoites. Protein concentration was determined by Bradford methods (Thermo Fisher Scientific).

2.2. Vector construction, expression and purification of the recombinant proteins

The full-length coding sequences of gTrxR and gEF1Bγ (GiardiaDB accession number GL50803_9827 and GL50803_12102, respectively) were PCR amplified from the *Giardia* WB-C6 genomic DNA using primers reported in Supplemental Table S1. PCRs were performed on a T-Personal Thermocycler (Biometra, Göttingen, Germany) using 100 ng of gDNA, 10 units of high fidelity Pfu turbo DNA polymerase (Agilent Technologies, Santa Clara, CA, USA), 50 μM dNTP, 20 pmol of each primer in 50 μl of reaction mixture. Amplification conditions were: 1 cycle at 95 °C for 2 min; 30 cycles at 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s; and 1 cycle at 72 °C for 7 min. The coding sequence of g14-3-3 was excised from p14-X vector3. For the expression of N-terminal 6xHIS-tagged fusion protein in *Escherichia coli*, the *Bam*HI/*Not*I digested fragments were cloned in *Bam*HI/*Not*I linearized pQ30 vector (Qiagen, Germany) and transformed in M15 strain. Expression of recombinant proteins and purification under native conditions by metal affinity chromatography (Qiagen, Germany) were performed as described (Lalle et al., 2015).

2.3. Production of polyclonal antibodies

BALB/c mice (Charles River Laboratories International, Inc., MA, USA) were intraperitoneally immunized with either purified HIS-gTrxR or HIS-gEF1Bγ. Fifty μg of protein were inoculated on day 0 in 300 μl of emulsified 1:1 PBS/Freund's complete adjuvant (Sigma Aldrich), on day 21 in 1:1 PBS/Freund's incomplete adjuvant (Sigma Aldrich) and without adjuvant on day 42. Blood samples were collected before initial immunization and after each boost from the tail vein. Sera fractions were assayed for specific antibody content.

2.4. Western blot analysis

Proteins were separated on a NuPAGE 4–12% (Novex, Invitrogen, Carlsbad, CA, USA) as described (Lalle et al., 2015). Antibodies used were: mouse polyclonal sera anti-gTrxR and anti-gEF1Bγ, 1:3000 (see Supplemental Information); mouse polyclonal (pAb) anti-gADI and anti-gOCT (Ringqvist et al., 2008), 1:2000; mouse monoclonal (mAb) anti-HA (Sigma-Aldrich), 1:3000; mouse anti-αTubulin (clone B-5-1-2, Sigma-Aldrich), 1:10000; mouse anti-HIS mAb (Qiagen), 1:2000. Interaction was revealed by incubation with HRP-cojugated secondary Ab (1:3000) followed by chemiluminescence (Millipore, France).

2.5. Confocal laser scanning microscopy (CLSM)

CLSM analyses of trophozoites were performed on a Leica TCS SP2 AOBS apparatus (Leica Microsystems, Germany) as described15. Antibodies used were: mouse polyclonal anti-gTrxR and anti-gEF1Bγ sera, 1:50; mouse anti-gADI and anti-gOCT4, 1:100; mouse anti-αTubulin mAb (clone B-5-1-2), 1:200; FITC-conjugated mouse anti-HA mAb (Miltenyi Biotec, Germany), 1:50; Alexa-Fluor 647- and 488-conjugated anti-rabbit and anti-mouse secondary Ab (Invitrogen), 1:500. Image deconvolution was performed with Huygens software (Scientific Volume Imaging BV, The Netherlands).

2.6. Enzymatic assay for gTrxR, spectrophotometric and fluorimetric analysis

HIS-gTrxR activity was assayed in continuous at 412 nm (25 °C) with Uvikon 941 Plus spectrophotometer (Kontron Instruments,