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Research review paper

Drug resistance in *Giardia duodenalis*

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ABSTRACT

Giardia duodenalis is a microaerophilic parasite of the human gastrointestinal tract and a major contributor to diarrheal and post-infectious chronic gastrointestinal disease world-wide. Treatment of *G. duodenalis* infection currently relies on a small number of drug classes. Nitroheterocyclics, in particular metronidazole, have represented the front line treatment for the last 40 years. Nitroheterocyclic-resistant *G. duodenalis* have been isolated from patients and created in vitro, prompting considerable research into the biomolecular mechanisms of resistance. This class of compounds is redox-active and is believed to cause damage to protein and DNA after being activated by oxidoreductase enzymes in metabolically active cells. In this review, we explore the molecular phenotypes of nitroheterocyclic-resistant *G. duodenalis* described to date in the context of the protist's unusual glycolytic and antioxidant systems. We propose that resistance mechanisms are likely to extend well beyond currently described resistance-associated enzymes (i.e., pyruvate ferredoxin oxidoreductases and nitroreductases), to include NAD(P)H- and flavin-generating pathways, and possibly redox-sensitive epigenetic regulation. Mechanisms that allow *G. duodenalis* to tolerate oxidative stress may lead to resistance against both oxygen and nitroheterocyclics, with implications for clinical control. The present review highlights the potential for systems biology tools and advanced bioinformatics to further investigate the multifaceted mechanisms of nitroheterocyclic resistance in this important pathogen.

Contents

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1. Introduction

Giardia duodenalis (syn. *Giardia lamblia* or *Giardia intestinalis*) is a gastrointestinal parasitic protist that infects approximately one billion people world-wide and causes 200–300 million cases of disease (giardiasis) each year (Lane and Lloyd, 2002). Infection may be acute or chronic, and symptoms include nausea, vomiting, diarrhea and dehydration (Farthing, 1996; Robertson et al., 2010). *G. duodenalis* infection has been reported in approximately 15% of children aged 0–24 months in the developing world (McCormick, 2014) and contributes to the global burden of diarrheal diseases that collectively constitute the second-leading cause of death in children under five years of age (Kosek et al., 2003; Savioli et al., 2006). Infection can also cause malabsorption syndrome and failure to thrive. Indeed, as few as 3 episodes of chronic (>2 week duration) diarrheal disease per year in the first 24 months of life is associated with significant reductions in height (approximately 10 cm) and IQ (10 points) by 7–9 years of age (Guerrant et al., 2013). *G. duodenalis* infection induces physical stunting and modified gut physiology in laboratory animals (Bartelt et al., 2013). In humans, pathophysiological changes in the gut may persist long after *G. duodenalis* infection is cleared (Halliez, 2013), and so this parasite is also implicated in the etiology of major non-communicable diseases such as irritable bowel syndrome, chronic fatigue, obesity and type II diabetes (Mørch et al., 2013; Verdu and Riddle, 2012).

G. duodenalis is transmitted via environmentally resilient cysts shed in the feces of an infected individual, which are then ingested (e.g., in contaminated water or food). Upon ingestion and passage through the stomach, trophozoites emerge from cysts to colonize the small intestine. Treatment is largely limited to two drug classes: nitroheterocyclic compounds (e.g., metronidazole (MET), nitazoxanide and furazolidone) and benzimidazoles (e.g., albendazole (ALB) and mebendazole) (Escobedo and Cimerman, 2007; Wright et al., 2003). Paromomycin and quinacrine are also occasionally used to treat giardiasis, but pose problems due to low efficacy and high toxicity respectively (Escobedo and Cimerman, 2007). Treatment efficacy is 73–100% for MET, and 79–100% for ALB (Gardner and Hill, 2001; Solaymani-Mohammadi et al., 2010), suggesting that initial treatment failure is relatively common. Although non-compliance or immune deficiency may contribute to treatment failures, isolates from patients who were unsuccessfully treated with either MET or ALB have demonstrated resistance to the prescribed drug in the laboratory (Abboud et al., 2001; Adagu et al., 2002; Lemée et al., 2000; McIntyre et al., 1986). Whereas relatively few studies have focused on benzimidazole resistance in *G. duodenalis* (Argüello-García et al., 2009; Jiménez-Cardoso et al., 2004, 2009; Paz-Maldonado et al., 2012; Upcroft et al., 1996), nitroheterocyclic resistance has been extensively studied (Boreham et al., 1991; Gillin and Reiner, 1982; Smith et al., 1988; Townson et al., 1994a; Upcroft and Upcroft, 2001) and is thus the focus of the present review. Nitroheterocyclics enter the cell through passive diffusion, are generally activated by oxidoreductase enzymes and appear to induce oxidative stress, leading to protein and DNA damage (Edwards, 1993a; Leitsch et al., 2012a). Investigation of laboratory-derived nitroheterocyclic-resistant lines (Townson et al., 1992) has linked resistance to differential regulation of oxidoreductase enzymes (Leitsch et al., 2011; Müller et al., 2007a, 2008, 2013; Tejman-Yarden et al., 2011), and certain clinical isolates appear naturally resistant to nitroheterocyclics prior to treatment (Smith et al., 1988; Townson et al., 1994a). The present review considers the implications of differential oxidoreductase activity in the context of the multifaceted antioxidant system in *G. duodenalis*, and identifies points of convergence between the mechanisms of response and resistance to nitroheterocyclic compounds, and oxygen and its reactive metabolites. This work provides a timely consolidation of our understanding of nitroheterocyclic resistance in *G. duodenalis*, and highlights key areas and technologies for further exploration.

2. The microaerophilic lifestyle of *G. duodenalis*

G. duodenalis belongs to a phylogenetically diverse 'group' of microaerophilic protists and bacteria, which thrive in environments with low dissolved oxygen (dO_2) of approximately 5–80 μM, but fail to survive under atmospheric dO_2 concentrations (298 μM) (Krieg and Hoffman, 1986; Lane and Lloyd, 2002). Other medically important microaerophiles include the parasitic protists *Trichomonas vaginalis* and *Entamoeba histolytica*, and bacteria such as *Helicobacter pylori* and *Campylobacter jejuni* (Lloyd, 2004; Lloyd et al., 1989). The microaerophilic metabolism of *G. duodenalis* appears to have been shaped by fluctuating oxygen levels and commensal bacteria in the host intestine. In natural infections, *G. duodenalis* trophozoites attach to the intestinal mucosa, and detach periodically, especially during division. Concentrations of dO_2 in this environment fluctuate from 0 to 80 μM depending on the metabolic activity of host enterocytes; the oxygen affinity of other commensal microbes (Espey, 2013); the antioxidant richness of host bile, and proximity to the anoxic luminal mid-point (Espey, 2013; Mastronicola et al., 2011).

Products of lateral gene transfer (LGT) from anaerobic bacteria, allow *G. duodenalis* to maximize energy production under low dO_2 conditions (Morrison et al., 2007; Nixon et al., 2002; Pal et al., 2009). For example, the remnant mitochondria of *G. duodenalis* lack a tricarboxylic acid cycle and oxidative phosphorylation, but the organism has acquired enzymes of likely LGT origin that support fermentative glycolysis (Lindmark, 1980), the arginine dihydrolase pathway (Schofield et al., 1990, 1992) and substrate-level ATP generation (Adam, 2001; Han and Collins, 2012; Mendis et al., 1992) (Supplementary Fig. 1). Many LGT-derived *G. duodenalis* enzymes contain catalytic iron, which renders them liable to inactivation under higher dO_2 concentrations (Dan et al., 2000; Gillin and Reiner, 1982; Lloyd, 2004; Lloyd et al., 2002). Notable among these oxygen-sensitive enzymes are two pyruvate ferredoxin oxidoreductases (PFOR-1 and -2) that decarboxylate pyruvate to acetyl-CoA and shuttle the resultant electrons to ferredoxin via iron–sulfur clusters (Chabriere et al., 2011; Nixon et al., 2002; Townson et al., 1996) (Fig. 1). In *E. histolytica*, oxidation of PFOR iron–sulfur clusters is associated with enzyme inactivation and the generation of reactive oxygen species (ROS) (Pineda et al., 2010), which in *G. duodenalis*, can lead to disruption of the plasma membrane potential and parasite death (Lloyd et al., 2000). In contrast, other iron-dependent enzymes (aka metalloenzymes) in the antioxidant network of *G. duodenalis* (including LGT products), appear to be more resistant to dO_2 inactivation, and may therefore be involved in dO_2 detoxification processes (Goncalves et al., 2014; Mastronicola et al., 2010; Müller et al., 2013; Nixon et al., 2002; Pal et al., 2009; Rafferty et al., 2010; Testa et al., 2011; Vicente et al., 2009).

2.1. The antioxidant system

G. duodenalis lacks superoxide dismutase, catalase (Brown et al., 1995; Morrison et al., 2007), and glutathione cycling (Brown et al., 1993), but expresses a number of oxidoreductases, likely derived through LGT, that consume dO_2 and produce water. A 46 kDa enzyme termed 'NADH oxidase' was the first of these oxidoreductases to be identified, although subsequent studies showed that it preferentially utilizes NADPH as a source of electrons to reduce oxygen (Brown et al., 1996a) (see Table 1 for gene identifiers). More recently, a NADH-dependent flavodiiron protein with water-forming activity has been characterized (Di Matteo et al., 2007; Mastronicola et al., 2011).

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