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REVIEW PAPER

Antimicrobial resistance of the enteric protozoon *Giardia duodenalis* – A narrative review

ULRIKE LODERSTÄDT[1] and HAGEN FRICKMANN[2,3]*

[1] Institute for Infection Control and Infectious Diseases, University Medical Center Göttingen, 37075 Göttingen, Germany

[2] Department of Microbiology and Hospital Hygiene, Bundeswehr Hospital Hamburg, 20359 Hamburg, Germany

[3] Institute for Medical Microbiology, Virology and Hygiene, University Medicine Rostock, 18057 Rostock, Germany

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ABSTRACT

Introduction: As therapy-refractory giardiasis is an emerging health issue, this review aimed at summarizing mechanisms of reduced antimicrobial susceptibility in *Giardia duodenalis* and strategies to overcome this problem. *Methods:* A narrative review on antimicrobial resistance in *G. duodenalis* was based upon a selective literature research. *Results:* Failed therapeutic success has been observed for all standard therapies of giardiasis comprising nitroimidazoles like metronidazole or tinidazole as first line substances but also benznidazoles like albendazole and mebendazole, the nitrofuran furazolidone, the thiazolide nitazoxanide, and the aminoglycoside paromomycin. Multicausality of the resistance phenotypes has been described, with differentiated gene expression due to epigenetic and post-translational modifications playing a considerable bigger role than mutational base exchanges in the parasite DNA. Standardized resistance testing algorithms are not available and clinical evidence for salvage therapies is scarce in spite of research efforts targeting new giardicidal drugs. *Conclusion:* In case of therapeutic failure of first line nitroimidazoles, salvage strategies including various options for combination therapy exist in spite of limited evidence and lacking routine diagnostic-compatible assays for antimicrobial susceptibility testing in *G. duodenalis*. Sufficiently powered clinical and diagnostic studies are needed to overcome both the lacking evidence regarding salvage therapy and the diagnostic neglect of antimicrobial resistance.

KEYWORDS

giardiasis, therapy, resistance, epigenetics, posttranslational modification, resistance testing, *Giardia duodenalis*

INTRODUCTION

Giardia duodenalis (also termed *G. lamblia* or *G. intestinalis*) is an enteric protozoan parasite with a quite characteristic shape (Fig. 1) of etiological relevance. Human disease, mediated by damage of the enterocytes, loss of the brush border of the epithelial cells of the intestine, shortening of microvilli and altered epithelial barrier function, comprises acute to aqueous diarrhoea, flatulence, steatorrhea, nausea, abdominal pain, vomiting and, as complications in case of chronic disease, malabsorption and weight loss [1]. Especially in resource-poor high-endemicity settings, however, infections frequently stay asymptomatic [2, 3] as confirmed by studies indicating 50%–75% asymptomatic children in high-endemicity areas [4]. Transmission on the faecal-oral route makes the pathogen relevant for hospital and food hygiene [4]. In line with this, enforcement of strict food and drinking water hygiene precautions, e.g., by purification of water in endemicity settings, helps to prevent disease transmission [4]. In Germany, diagnostic proof of *G. duodenalis* is notifiable according to §7 of infection

*Corresponding author. Department of Microbiology and Hospital Hygiene, Bundeswehr Hospital Hamburg, Bernhard-Nocht-Str. 74, 20359 Hamburg, Germany.
E-mail: Frickmann@bnitm.de

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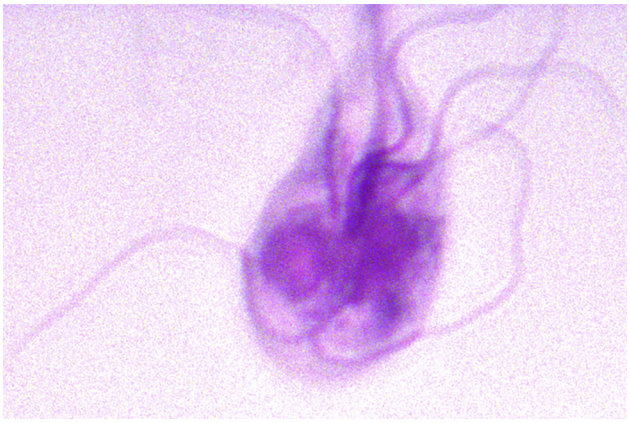

Fig. 1. Typical morphological features of a trophozoite of *Giardia duodenalis*

prevention law ("Infektionsschutzgesetz"). Identified risk factors, mostly identified in areas of endemicity, comprise day-care for children, working in child-care settings, status as institutionalized individual, travelling in endemic areas, ingestion of contaminated or recreational water, immunodeficiency, cystic fibrosis, and oral-anal sex techniques [4]. In Germany, round about half of detected *G. duodenalis* infections is imported from abroad, mostly due to travelling under poor hygiene conditions as described for soldiers and police officers without regular access to field camp infrastructure [5–8], for travellers visiting friends and relatives [9], and for migrants [10], respectively.

Microscopy and real-time PCR are the diagnostic procedures of choice [4] with modern real-time PCR assays being considerably more sensitive than microscopy [11] and quite stabile in inter-assay comparisons [11, 12]. Microscopy, in contrast, is investigator-dependent, which is associated with reduced diagnostic reliability even in reference centres as observed in the course of laboratory control trials [13].

Internationally applied treatment options comprise the application of azole compounds like metronidazole or tinidazole as well as nitazoxanide [4]. Although there is no internationally accepted general recommendation for the treatment of asymptomatic patients and there is a therapeutic neglect of this patient group in many countries [4], treatment of asymptomatically infected individuals is usually performed in Germany in order to interrupt transmission chains and infected individuals are prevented from commercial food handling by infection prevention law.

However, resistance or tolerance towards the antimicrobial agents of choice makes the therapy of infection or asymptomatic colonization with *G. duodenalis* challenging [4]. As recently summarized [14], up to 50% therapy-refractory courses of giardiasis after 5-nitroimidazole (like metronidazole, tinidazole) standard therapy have been reported in international literature [14, 15] with hints for an increase in the last decade, particularly in returnees from the Indian subcontinent [16]. Evidence levels of salvage therapy in case of such therapeutic failures like adding of a benzimidazole (like mebendazole, albendazole) to a new therapy course with a 5-nitroimidazole drug or prescribing the anti-malarial drug quinacrine, a substituted acridine with considerable side effects, instead are usually low, just based on small studies or expert opinions [16]. In contrast, sufficiently powered, well designed randomized, double-blinded, controlled trials are widely missing [14]. In a similar way, large predictor studies for therapeutic failure are unavailable so far [17].

The aim of this narrative mini-review is to summarize present knowledge on antimicrobial resistance in *G. duodenalis* as well as to highlight the way ahead with focus on therapeutic alternatives.

METHODS

A selective literature research based on the search words "*Giardia*" and "resistance" with the database NCBI (National Center for Biotechnology Information) pubmed (https://pubmed.ncbi.nlm.nih.gov/, last accessed at 11th May 2021) was conducted.

HISTORICAL BACKGROUND OF THE TIME BEFORE THE CHANGE OF THE MILLENNIUM

In the 1960s, nitroheterocyclic drugs including the 5-nitroimidazoles, which depend on reduction by ferredoxin or flavodoxin, became available for the treatment of anaerobic or microaerophilic protozoa lacking mitochondria like *G. duodenalis* [18, 19], but their use was early accompanied by the emergence of resistance. Indeed, resistance of *G. duodenalis* to 5-nitroimidazoles, but also to related nitrofurans requiring nitroreductase activity, has been known for decades [18, 20]. Thirty years ago, a patient with symptomatic chronic giardiasis was described, who had been cured by a combination of metronidazole and quinacrine after seven courses of monotherapy with metronidazole or quinacrine alone had failed to achieve clinical cure. Thereby, no increased susceptibility of the parasites to the drug combination compared to monotherapy could be shown *in vitro* but only reduced cellular cytotoxicity of the patient's macrophages for *G. duodenalis* [21]. Quinacrine, also called metacrine or by its trade name Atebrine™, was repeatedly successfully used in the 1980s to cure giardiasis patients who had failed to clinically respond to metronidazole [22]. As early as in the middle of the 1980s, when 5-nitroimidazoles like tinidazole and metronidazole still showed low minimum inhibitory concentrations (MIC) for many *G. duodenalis* isolates, about 50% of the strains already had increased MICs for alternative drugs like paromomycin, pyrimethamine, and chloroquine, while furazolidone was – in comparison – the most active nonimidazole agent [23]. A linkage between MIC increase for various chemically related substances suggested common mechanisms of resistance [23]. At the beginning of the 1990s, significant variability of *in*

vitro susceptibility of different *Giardia* spp. isolates towards metronidazole and ornidazole was shown. Thereby, decreased *in vitro* susceptibility was well correlated with therapeutic failure *in vivo* [24]. Interestingly, metronidazole, although considered as the therapeutic standard for giardiasis, did not have an Food and Drug Administration ("FDA") clearance for this indication in the early 1990s [25]. In 1991, a patient with multidrug-resistant giardiasis was treated at a Swiss hospital. After therapeutic failure with oral administration of metronidazole, tinidazole, ornidazole, and quinacrine, a combination of oral and intraduodenal administration of quinacrine finally cured the patient. Interestingly, no signs of immune deficiency or IgA deficit had been recorded for this patient [26]. In the middle of the 1990s, giardiasis experts called for action in order to prevent resistance dimensions as known from bacterial pathogens in *G. duodenalis* [27]. In a review published in 2001 [28], nitroimidazoles like metronidazole, tinidazole or ornidazole were still suggested as the treatment of choice due to the broadest respective experience with cure rates >90%. Quinacrine use was – in spite of comparably good efficacy – discouraged due to considerable side effects, furazolidone due its pharmacokinetics requiring application 4 times a day. Due to lack of enteric absorption, paromomycin was suggested for giardiasis requiring therapy in the early pregnancy in spite of its inconsistent therapeutic effectiveness [28].

RESISTANCE MECHANISMS

Details on the molecular mechanisms of resistance leading to 5-nitroimidazole refractory *G. duodenalis* infection as well as other resistance types are not yet completely deciphered [14], in spite of considerable effort both with laboratory and clinical strains [29]. Immunodeficiency and IgA deficit in particular as well as hypogammaglobulinaemia in general have been associated with increased risk of therapeutic failure in giardiasis patients [16, 26]. Also, the organochlorine DDT has been shown to increase *G. duodenalis*-associated disease severity due to aversive immunomodulating effects in animal experiments [30]. Further, an association of primary treatment failure and increased blood haemoglobin at the time of diagnosis but not with CD4-positive T-cell counts could be shown at least for the subpopulation of HIV-positive giardiasis patients [17].

Induced drug resistance in *G. duodenalis*

As early as in the 1980s, it has been described that susceptibility towards metronidazole can be reduced *in vitro* by constant exposure about several weeks towards sublethal concentrations by factor eight. However, it became also clear by those experiments that the so-induced resistance was unstable and that the protozoa tend to revert to their original metronidazole susceptibility after several weeks of growth without antibiotic pressure [31]. A similar mode of *in vitro* resistance induction in *G. duodenalis* has been described for other antiparasitic drugs like furazolidone [32], albendazole [33] and quinacrine [34] as well. Interestingly, it has been demonstrated *in vitro* that subpopulations of differing resistance levels may exist within defined strains, which show competition when cultured under various degrees of antimicrobial pressure [35]. Thereby, this variability seems to be bigger for nitroimidazoles than for benzimidazoles [35].

Resistance against 5-nitroimidazoles

Associated with reduced susceptibility, altered intracellular metronidazole concentrations were early recorded, resulting in speculation either on defective transport mechanisms across the cellular membrane or insufficient intracellular reduction of the substance to its biologically active metabolite [31, 36, 37]. The latter is due to modifications of proteins involved in drug activation [38]. In detail, influences of pyruvate:ferredoxin oxidoreductase, ferredoxin pathways and thiol-dependent peroxidase and reductase activities were shown to be of etiological relevance for resistance in *G. duodenalis* [39, 40]. Thereby, reduced cellular concentrations of pyruvate:ferredoxin oxidoreductase as well as downregulation of ferredoxin pathways in *G. duodenalis*' low-redox-potential anaerobic metabolism lead to decreased metronidazole uptake into the protozoa [40–44]. Also, reduced expression of the oxygen-insensitive nitroreductase-1 (*ntr-1*) gene of *G. duodenalis* may be associated with decreased drug activation, which in turn leads to tolerance towards metronidazole [44, 45]. NAD(P)H- and flavin-generating pathways and redox-sensitive epigenetic regulation can show similar effects [46]. As a consequence of reduced drug activation, reduced production of radicals that can form adducts with proteins such as thioredoxin reductase and α- and β-giardins as well as DNA damage follows, resulting in decreased probability of the trophozoites' death [47]. In addition, low expression of oxygen-detoxification enzymes can allow passive, non-enzymatic metronidazole detoxification mediated by futile redox cycling [44]. Such passive mechanisms are supplemented by active resistance strategies, comprising mechanisms of complete enzymatic detoxification of the pro-drug by nitroreductase-2 as well as enhanced repair of oxidized biomolecules mediated by thioredoxin-dependent antioxidant enzymes [44]. Of note, not all mechanisms are evenly expressed in all resistant strains. In a recent assessment with three different resistant *G. duodenalis* cell lines, common mechanisms comprised up-regulating of genes encoding for variant-specific surface proteins, a high cysteine membrane protein, calcium and zinc channels, a Mad-2 cell cycle regulator and a putative fatty acid α-oxidase as well as down-regulated genes encoding nitroreductase-1, putative chromate and quinone reductases, as well as numerous genes that act proximal to the gene encoding the pyruvate:ferredoxin oxidoreductase. In contrast to those similarly regulated genes, a cell line with increased passive resistance mediated by a nonsense mutation in nitroreductase-1 transcripts showed increased transcription of nitroreductase-2 and a MATE transmembrane pump system, supporting active drug detoxification and

efflux, respectively. Lines without this mutation had to cope with a higher oxidative stress load caused by metronidazole- and oxygen-derived radicals [44]. In addition, it has been suggested that posttranslational modifications like protein acetylation, methylation, ubiquitination, and phosphorylation play a role in metronidazole resistance [48]. More than this, it has been shown that metronidazole fails to arrest the cell cycle progression in resistant strains while it shows this effect in susceptible ones [49]. Other than reported for metronidazole resistance in *Trichomonas vaginalis*, however, measurably defective O_2-scavenging capabilities compared to metronidazole-sensitive isolates were not reported for *G. duodenalis*, although elevated NADPH-oxidase activities have been shown for metronidazole-resistant *G. duodenalis* strains [50]. Of note, metronidazole resistance in *G. duodenalis* is accompanied by a glucose metabolism-related attachment defect to mucosal cells. So, metronidazole-resistance appears to be evolutionarily balanced against the infectious potential of a *G. duodenalis* strain [51]. Altogether, polygenically mediated changes in the antioxidant network, glycolysis, and electron transport affect metronidazole resistance, which is also influenced by protein acetylation as indicated by cross-resistance to the deacetylase inhibitor trichostatin A [48].

Co-resistance against metronidazole and nitozoxanide

Co-resistance against metronidazole and nitazoxanide was found to be associated with changed expression of stress response-related and heat shock proteins (HSP70 B2, HSP40), major surface antigens such as the variant surface protein (TSA417, AS7), nitazoxanide-binding proteins like nitroreductase 1 (GlNR1) and the protein disulphide isomerase PDI4 [43, 52, 53]. Interestingly, as observed with a *G. duodenalis* strain expressing this resistance type, a cycle of en- and excystation leads to vanishing or resistance, suggesting epigenetic changes rather than changes of the DNA sequence to be responsible for the resistance pattern [52]. Further, in a cell line with stabile co-resistance to the nitrocompounds metronidazole and nitazoxanide, multiple metabolic adaptations were observed. They comprised a reduction of the activities of FAD-dependent oxidoreductases, lower nitroreductase activities, lower oxygen consumption and resazurin reduction rates, lower ornithine-carbamyl-transferase activity, reduced FAD and NADP(H) pool sizes and higher ADP/ATP ratios, respectively, compared to the wildtype [54]. As resistance against nitro drugs is mediated by several distinct mechanisms and not the consequence of a directed process, it is consequently not correlated with a specific pattern of differentially expressed proteins as demonstrated with mass spectrometry shotgun analysis of the proteomes of distinct nitro drug-resistant strains [55].

Resistance against benzimidazoles

Albendazole resistance, which is associated with parbendazole cross-resistance, is reported to be mediated by alterations of the cytoskeleton of *G. duodenalis* with particular emphasis on the median body [56]. In particular, alterations of the so-called ROD-domain of the beta-giardin protein have been associated with albendazole resistance [57]. Further, efflux of both albendazole and nitazoxanide from *G. duodenalis* cells has been shown to be mediated by proteins of the ATP-binding cassette (ABC) transporter superfamily, in particular with the ABC-C1 transporter, also known as multidrug resistance protein 1 [58]. In addition and next to β-tubulin changes in albendazole-resistant *G. duodenalis*, pro-oxidant cytotoxicity of albendazole is counteracted by an increased antioxidant response involving reactive oxygen species-(ROS-)metabolizing enzymes (NADH oxidase, peroxiredoxin 1a, superoxide dismutase and flavodiiron protein) and higher levels of intracellular free thiols [59].

Resistance against other therapeutic drugs for giardiasis

The thiol cycling enzymes mediate furazolidone resistance [41], while metronidazole only slightly reduces the thiol pool even within susceptible *G. duodenalis* strains [60]. Quinacrine resistance has been reported to be associated with active exclusion from resistant trophozoites. Of note, *G. duodenalis* strains with pre-existing furazolidinone resistance tend to adapt more quickly to increasing quinacrine concentrations as well [34].

HINTS FOR GENETIC VERSUS EPIGENETIC DETERMINANTS OF RESISTANCE IN *G. DUODENALIS*

There are no well-defined resistance genes to be used for diagnostic purposes like, e.g., for methicillin-resistance in bacteria of the species *Staphylococcus aureus* [61]. This is well in line with lacking stability of resistance induction in the absence of selective pressure by the antimicrobial drug as early observed [31]. Instead, redox-sensitive epigenetic regulation is discussed [46] and molecular resistance mechanisms are likely to be largely founded on reversible transcriptional changes. This, in addition to post-translational modifications, best explains the observed phenomena that resistant lines revert to drug sensitivity during drug-free culture *in vitro* or during passage through the life cycle [44, 48].

Nitroimidazole resistance

Matching the abovementioned, metronidazole resistance induced by long-term growth of *G. duodenalis* with sublethal metronidazole doses can occur completely without mutations in metronidazole resistance-associated genes like *pfor*, *fd*, *nr-1* or *trxr* [62]. Nevertheless, some associations have been described. Genomic sequencing of various strains of the *G. duodenalis* assemblages A and B showed that, even irrespective of individual metronidazole resistance, genetic variability is common in important genes in metronidazole metabolizing pathways and in the management of oxidative

and nitrosative stress, including high numbers of non-synonymous (amino acid-changing) single nucleotide polymorphisms [63]. Also, rearrangements on the chromosome and repetitive DNA level have been early seen in metronidazole resistant *G. duodenalis* strains [41]. In particular, the loss of the 3000-base pair-sequence G6/1 on chromosome 4 of *G. duodenalis*, which seem to interfere with cell division of the parasite, has been associated with the onset of metronidazole resistance [64]. Increased levels of expression of the gene for protein disulphide isomerase 2 (PDI2) has been described for strains resistant against either metronidazole or nitazoxanide, while combined resistance was also associated with PDI4 expression. Also, drastic changes in the expression of genes for variant surface proteins (VSP) in strains resistant against those substances have been reported [65]. Altered expression of *pfor* RNA, coding for the pyruvate:ferredoxin oxidoreductase, was identified in metronidazole resistant strains [66]. However, altered *pfor* RNA expression levels were only found in case of severe metronidazole resistance and the association was generally weak, so *pfor* expression is a poor diagnostic marker of metronidazole resistance [67]. More than this, this mechanism does not seem to be a necessary condition for metronidazole resistance at all, as it was found to be completely absent in highly 5-nitroimidazole resistant *G. duodenalis* strains [68, 69]. Indeed, a multi-factorial nature of metronidazole resistance was confirmed by the identification of strains, in which impaired flavin metabolism played the major role for nitroimidazole resistance, mediated by the flavin-dependent *G. duodenalis* thioredoxin reductase (GlTrxR) and the NADPH oxidase [69]. Interestingly, resistance associated changes in the expression of stress response-related and heat shock proteins (HSP70 B2, HSP40), major surface antigens such as the variant surface protein (TSA417, AS7) as well as the nitazoxanide-binding proteins nitroreductase and protein disulphide isomerase PDI4 can vanish after an en- and excystation cycle, confirming epigenetic changes rather than persistent changes of the DNA sequence [52]. Metronidazole-induced cellular stress leads to downregulation of the antioxidant system and α-giardins, while associated resistance-development is believed to be influenced by multiple epigenetic mechanisms of transcriptional control as suggested by antisense de-repression and differential regulation of RNA [70]. However, it has been suggested that accelerated mutagenesis resulting from metronidazole-induced DNA damage might also contribute to resistance development [71], mediated by triggering the parasite's DNA homologous recombination repair pathway [47]. In line with this, in a metronidazole-resistant *G. duodenalis* cell line, a nonsense mutation in nitroreductase-1 transcripts has been observed, which supports metronidazole resistance but is not a necessary condition for such resistance by itself [44].

Benzimidazole resistance

Associated with albendazole resistance [56], chromosome rearrangements affecting the cytoskeleton structure were observed, but without coding for a tyrosine for phenylalanine amino acid exchange at position 200 in beta-tubulin, which has been described for benzimidazole-resistance in helminths and fungi, or other consistent sequence changes in the target structure beta-tubulin [56, 59, 72]. However, permanent sequence changes in the beta-giardin gene, resulting in amino acid changes in the protein's ROD domain from TIARERA to IDRPRE, have also been associated with reduced susceptibility to albendazole [57]. Further, RNA of the variant surface protein ARR-VSP was found to be upregulated in albendazole resistant *G. duodenalis* clones [66]. Altogether, the differential expression of several genes for proteins playing a role in maintaining cell structural stability, coping with oxidative stress and adapting energy supply to altered metabolic states is crucial for albendazole resistance. The affected proteins comprise proteins with functional roles not only in the cytoskeletal system (alpha 2-giardin and RanBP1) but also in the antioxidant metabolism (NADH oxidase) and in the energy metabolism (triosephosphate isomerase, phosphoglycerate kinase and ornithine carbamoyltransferase), respectively [73].

Resistance against other drugs

Changes in the expression of the variant surface protein as well as reduced expression of the parasite's nucleoside hydrolase (NH) are considered to be responsible for resistance against isoflavones like daidzein and formononetin [74]. Increased transcription of the neomycin phosphotransferase (*neo(r)*) gene is known to mediate resistance against the aminoglycoside G418 (geneticin) in *G. duodenalis* [75].

TREATMENT ATTEMPTS ALTERNATIVE TO THE "5-NITROIMIDAZOLES STANDARD"

Natural and synthetic substances with giardiacidal effects were early tried to identify [76, 77] and those efforts proceeded with ongoing resistance issues [78].

Alternative nitroheterocyclic substances

Although there is considerable cross-resistance between nitroimidazoles in *G. duodenalis*, there are also substance-specific differences, making it worth testing various substances of the group for potential therapeutic success [51, 79–81]. The ongoing effort for optimizing nitroimidazoles in order to overcome metronidazole resistance have shown that easier redox activation is positively correlated with greater giardicidal activity. For example, olefins with a conjugated bridge connecting the core and a substituted phenyl or heterocyclic ring were identified as promising options [82]. *In vitro* assessments also suggested high effectiveness of 2-lactam-substituted 5-nitroimidazoles for the treatment of *G. duodenalis* [79]. A modification based on a side chain carrying a remote phenyl group in the 2-position of the imidazole ring was found to be 14-fold more active against *G. duodenalis* than metronidazole [61]. Metronidazole-triazole conjugates and nitroimidazole carboxamides were also able

to overcome some types of metronidazole resistance *in vitro* [83, 84]. Parasite cell vesicle trafficking, autophagy, and triggered differentiation into cysts are processes which are targeted by modern metronidazole derivates for giardiasis treatment [85]. In silico drug design offers new options of designing nitroheterocyclic drugs with therapeutic effects against giardiasis. In addition to nitroimidazoles, such nitroheterocyclic drugs also comprise nitropyrroles and nitrofurans [86]. The nitrofuran furazolidone has been shown *in vitro* to provide more severe damage than metronidazole to cyst stages of *G. duodenalis*, associated with morphological alterations like the presence of cavities, lamellar bodies as well as thread-like structures and inhibition of *in vitro* cyst differentiation [87]. Similar like metronidazole but other than nitazoxanide, furazolidone uses the parasite's antioxidative enzyme thioredoxin reductase to be converted into its active form which is toxic to *G. duodenalis* [88].

Benzimidazoles

In vitro experiments with *G. duodenalis* suggested 30- to 50-fold higher activity of albendazole and mebendazole compared to metronidazole and 4- to 40-fold higher activity compared to quinacrine, respectively, while thiabendazole scored poorer. Static *in vitro* effects have been reported for mebendazole, which shows poor intestinal absorption and interacts with the microtubules of the parasite even at lower concentrations than required for the standard agent metronidazole [89]. In detail, microtubule polymerization is inhibited through selective binding to the β-tubulin subunit [72]. Of note, the giardicidal effects of different benzimidazoles and tubulin inhibitors vary. While mebendazole, albendazole and fenbendazole have been described to induce irreversible effects on the cells, only transient effects have been reported for nocodazole, oxfendazole, and albendazole sulfoxide, respectively [90]. At least in calves, effective eradiation of *Giardia* cysts from stool due to fenbendazole therapy has been shown *in vitro*, but reoccurrence within 4 weeks after treatment suggests occasional need for repeated therapy [91]. Further, *in vitro* experiments suggested increased activity of S-substituted 4,6-dibromo- and 4,6-dichloro-2-mercaptobenzimidazoles against *G. duodenalis* compared to metronidazole [92]. Therapeutic activity of benzimidazoles against *G. duodenalis* can further be increased by the combination with phenyl-carbamates, which has been discussed as an option for strains with reduced susceptibility for benzimidazoles [93]. Albendazole, in particular, also partially inhibits encystation and, to a lower degree, excystation [94]. Such interruption of the parasitic life cycle is considered to be important for the prevention of the spread of giardiasis [95]. While showing therapeutic effects on giardiasis comparable to metronidazole, albendazole has the advantage of lower side effect rates [96]. Chemically modified benzimidazole derivates have been shown to allow higher activity against *G. duodenalis* and may be suitable therapeutic alternatives for the future [51]. In benzimidazole-susceptible *G. duodenalis* cells, albendazole induced oxidative stress results in DNA damage as indicated by 8OHdG adducts, DNA degradation and histone H2AX phosphorylation, partial arrest within the cell cycle, and subsequent induction of apoptosis associated with phosphatidylserine exposure on the parasite surface [97].

Nitazoxanide

In vitro activity of the thiazolide nitazoxanide [2-acetoloxy-N-(5-nitro 2-thiazolyl) benzamide] against *G. duodenalis* has been thoroughly assessed since 2001. Nitazoxanide induced changes in trophozoite volume, loss of characteristic shape and swelling, but the effects were less severe in direct comparison to tinidazole [98]. Both nitazoxanide and its metabolite tizoxanide were shown to be considerably more active than metronidazole in metronidazole-susceptible *G. duodenalis* strains and at least slightly more active in metronidazole resistant strains [99], which is not surprising as resistance formation in *G. duodenalis* against nitazoxanide and metronidazole is linked, most likely due to altered gene expression [65]. In spite of this, successful treatment of a giardiasis patient with HIV co-infection and a *G. duodenalis* isolate resistant to metronidazole and albendazole by application of nitazoxanide could be demonstrated [100]. Nitazoxanide is known to inhibit the protein disulphide isomerases PDI2 and PDI4 of *G. duodenalis* [65]. Nitroreduction and free radical production are the likely modes of giardicidal action, because analogues lacking the reducible nitro-group showed reduced activity [99]. Further, the giardicidal effect of nitazoxanide was shown to be partially mediated by activation of the nitroreductase GlNR1 [101].

Quinacrine

In spite of considerable side effects, the antimalarial drug quinacrine had a renaissance in the treatment of nitroimidazole-resistant giardiasis. In a retrospective assessment at the London School of Hygiene and Tropical Medicine, London, United Kingdom, 100% of cases of metronidazole-refractory giardiasis could be successfully cured with quinacrine. Of note, however, those data were from a retrospective assessment and the sample size (n = 20 patients) was small [16]. However, 100% therapeutic success with quinacrine in 13 giardiasis patients, infected with strains of the assemblages A and B as identified by PCR, after failed nitroimidazole therapy was reported from Spain as well [102]. Similarly, 15 out of 15 patients with nitroimidazole-refractory giardiasis were successfully treated with quinacrine in Cuba [103].

Antibiotics and anti-viral substances

As predicted from the sequence of ribosomal RNA and subsequently confirmed *in vivo*, the aminoglycosides hygromycin and paromomycin can successfully inhibit the growth of wild-type strains of *G. duodenalis*. This is, however, not a group-specific, but a substance-specific effect, as growth inhibition cannot be achieved with other aminoglycosides like kanamycin or apramycin in the same way [104]. Altogether, sequences within domain V of

G. duodenalis' large-subunit rRNA resemble archaebacterial rRNA, suggesting a high level of evolutionary conservation [105]. Paromomycin susceptibility of *G. duodenalis* is considered to be associated with the presence of a C:G base-pair near the decoding region of the small subunit ribosomal RNA [106].

In vitro, ciprofloxacin as well inhibits *G. duodenalis* growth, adherence and O_2 uptake in a concentration-dependent manner and finally leads to death of the *G. duodenalis* trophozoites due to a necrosis process. Based on those *in vitro* results, ciprofloxacin has been discussed as a potential option for therapy-refractory giardiasis [107].

While high concentrations of the macrolide azithromycin are required of achieve growth inhibition of *G. duodenalis*, hundred-fold lower concentrations are sufficient for adherence inhibition [108]. Thereby, however, azithromycin susceptibility varies considerably in different strains [41].

Bacitracin shows *in vitro* inhibiting effects on *G. duodenalis* which can be increased by zinc substitution [109].

Interestingly, also some anti-retroviral protease inhibitors, in particular ritonavir-boostered lopinavir (trade name Kaletra™), show therapeutic effect against *G. duodenalis*. While standard dosage of ritonavir alone can also inhibit *G. duodenalis*, lopinavir alone is insufficient for complete inhibition but at least results in blockage of cytokinesis in *G. duodenalis* trophozoites [110].

Anti-rheumatic and anti-tumoral substances

The antirheumatic drug auranofin eradicated *G. duodenalis* in different rodent models by blocking the activity of giardial thioredoxin oxidoreductase and thus by interfering with normal protein function and with combating oxidative damage [111]. The membrane-active alkylphospholipid hexadecylphosphocholine (miltefosine), which has been developed as an anti-tumoral drug and which is nowadays applied to cure visceral leishmaniasis, eliminates giardiasis in the mouse model by affecting the parasites' cellular membrane and adhesive disc [112]. Another anti-tumoral compound with *in vitro* giardicidal effects is NBDHEX, which is active on several levels in *G. duodenalis* trophozoites, comprising inhibition of glycerol-3-phosphate dehydrogenase, binding to metabolic enzymes like thioredoxin reductase (gTrxR), elongation factor 1B-γ (gEF1Bγ), and structural proteins like α-tubulin. Thioredoxin reductase, in particular, is able to nitroreduce NBDHEX leading to drug modification of catalytic cysteines in thioredoxin reductase, with concomitant disulphide reductase activity inhibition and NADPH oxidase activity upsurge, resulting in increased toxicity of the compound [113].

Synergistically acting drugs, drug combinations and hybrid compounds

Synergistic effects on adherence inhibition of *G. duodenalis* were observed for dyadic combinations of azithromycin-furazolidone, doxycycline-mefloquine, doxycycline-tinidazole, and mefloquine-tinidazole, respectively, suggesting increased therapeutic effects of such combinations [108]. Further *in vitro* synergistic action could be shown for albendazole and the synthetic derivatives 2-aryl-3-hydroxymethylimidazo[1,2-a]pyridine and -pyrimidine against *G. duodenalis* trophozoites [114].

Synergisms have not only been shown *in vitro*. Also, there are hints for clinical superiority of combination therapy in patients with therapy-refractory giardiasis, although evidence is scarce [115, 116]. In a small group of giardiasis patients who could not be cured with metronidazole therapy, combination of albendazole plus metronidazole was superior to albendazole alone [117]. In a review from 2001, prolonged application of a combination of nitroimidazole and quinacrine was recommended for patients with resistant giardiasis [28], as therapeutic success was repeatedly observed with this approach [28, 118]. Combination therapy of mebendazole plus secnidazole was successfully applied as a salvage therapy for patients with nitroimidazole-resistant giardiasis as well [103].

As a comparatively new approach, hybrid compounds created by combining different giardicidal drugs within one molecule have been introduced [119]. For example, CMC-20, a nitazoxanide and N-methyl-1H-benzimidazole hybrid molecule, showed giardicidal effects in *G. duodenalis* strains resistant against albendazole or nitazoxanide alone by affecting the parasite's microtubule reservoir, triggering the parasite's encystation and alpha-7.2 giardin co-localization with CWP-1 protein *in vitro* and in the murine model [120].

Herbal preparations

Herbal treatment approaches have been assessed as promising options for therapy-refractory giardiasis as well [121]. In India, Pippali rasayana, prepared from *Piper longum* and *Butea monosperma*, has been tested for giardicidal effects in mice. The substance did not affect the parasite itself but increased both the macrophage migration index (MMI) and the macrophages' phagocytic activity, resulting in 98% recovery of the mice from *G. duodenalis* infection [122]. The component (-)-epigallocatechin from the plant *Helianthemum glomeratum*, which had already been used in Mayan traditional medicine for the treatment of diarrhoea, shows growth inhibiting potential against *G. duodenalis in vitro* [123]. *Lippia beriandieri* (oregano) has been reported to lead to giardicidal effects *in vitro* even more pronounced than observed with tinidazole, resulting in damage of nucleo-skeleton proteins associated with an altered structure of the nucleus as well as in deterioration of size and shape of *G. duodenalis* trophozoites [124]. Reduction of the MTT-tetrazolium salt levels is believed to be one mode of giardicidal action of oregano. Naringenin, thymol, and pinocembrin were identified among oregano's giardicidal components [125]. Polyphenolic-rich blueberry extract leads to a dose-dependent reduction of *G. duodenalis* trophozoite viability; the polyphenols are considered as the components in charge of the reduced survival of the parasites [126]. In mouse experiments, dichloromethane extracts of *Zingiber officinale* (ginger) and *Curcuma longa* (curcumin) showed

therapeutic effects against giardiasis. Thereby, the giardicidal effect of ginger was more pronounced than the effect of curcumin [127]. *In vitro* assessment also indicated a dose-dependent giardicidal effect of a chloroformic extract of *Artemisia annua* [128, 129]. Olive leaf extracts and extracts of *Satureja khuzestanica* (a plant used for medical purposes by nomads in southwestern Iran) show *in vitro* giardicidal effects in the range of metronidazole or better [130]. In contrast, *Allium sativum* (garlic) scores poorer *in vitro*, discouraging its therapeutic use [130]. Also, methylgerambullin, a sulphur-containing amide in *Glycosmis* spp. (family *Rutaceae*) [131], 2,3-Dihydroxyphenyl B-D-glucopyranosiduronic acid as well as the tannins gallic acid and chebulic acid extracted from *Terminalia ferdinandiana* in combination with ascorbic acid, respectively [132], and crude *Ageratum conyzoides* extracts [133] showed giardicidal effects *in vitro* and partly also in animal experiments. The giardicidal effects of the latter were associated with changes in the flagella and the ventral discs of *G. duodenalis* trophozoites [133].

Milk and milk components

In the 1980s, a giardiacidal effect of non-heated human but not of non-heated cow's or goat's milk on *G. duodenalis* trophozoites has been observed, most likely mediated by the heat-sensitive fatty acid esterase bile salt-stimulated lipase (BSL) [134]. However, a clinical application has never been successfully implemented. Further, it is believed that both human and bovine lactoferrin, particularly the N-terminal peptides, may be of relevance as a nonimmune component of host mucosal defence against *G. duodenalis* due to its giardicidal activity. However, the presence of Fe^{3+} ions can protect *G. duodenalis* trophozoites from this effect *in vitro* [135].

Others

G. duodenalis' glycolytic enzyme triosephosphate isomerase (GlTIM), next to other glycolysis-specific enzymes of the parasite, have been proposed as potential drug targets. For GlTIM, thiol-reactive compounds are under investigation as potential therapeutic drugs [136]. The giardial glycolytic enzyme triosephosphate isomerase is dose-dependently inhibited by the proton pump inhibitor omeprazole, leading to cell death of *G. duodenalis in vitro* [137]. The chemically modified proton pump inhibitors BHO2 and BHO3 showed even stronger inhibition of triosephosphate isomerase and associated giardicidal effects, which are mediated by chemical modification of Cys222 and associated structural changes of the enzyme as well as by adducts linked to cysteine residues [138]. Also, disulfiram shows giardicidal effects *in vitro* mediated by Cys222-modifications of the triosephosphate isomerase of *G. duodenalis*, thus deteriorating its stability [139]. *Giardia* carbamate kinases (glCK), which have no equivalent in human cells, are affected by disulfiram and are targets of interest for drug development [139, 140]. KH-TFMDI, a 3-arylideneindolin-2-one-type sirtuin inhibitor, shows *in vitro* giarcidal effects by inhibiting sirtuins, which are class III NAD+-dependent histone deacetylases, in the parasite. Associated micromorphological changes comprise multinucleated cell clusters suggesting compromised cytokinesis in treated trophozoites, cell rounding, concomitantly with the folding of the ventrolateral flange and flagella internalization, and finally cell death associated with DNA/nuclear damage, formation of multi-lamellar bodies and annexin V binding on the parasite surface [141]. Also, the five long-chain fatty acyl-CoA synthetases (GiACS1 to GiACS5) of *G. duodenalis* have been identified as potential drug targets, since the acyl-CoA synthetase inhibitor triacsin C was shown to inhibit giardial growth *in vitro* [142]. Giardicidal effects in animal models were recently also demonstrated for selected Hsp90 (heat shock protein 90) inhibitors [143]. Deconjugated bile salts as produced by bacterial bile-salt-hydrolases of the probiotic bacterial strain *Lactobacillus johnsonii* La1 show giardicidal effects as well, suggesting potential protective effects of probiotic applications [144]. Isoflavones like daidzein and formononetin inhibit *G. duodenalis* growth [74]. Aminoguanidine compounds like robenidine are associated with *in vitro* giardicidal effects, inhibiting *in vitro* adherence of the parasite and damages to the parasite's plasma membrane [145]. New therapeutic substances under investigation comprise so-called "bumped" kinase inhibitors (BKIs). BKIs target protein kinases of *G. duodenalis*, which have an expanded active site pocket. The latter results from an atypically small gatekeeper residue. Inhibition of those small-gatekeeper kinases is a completely new mode of action, which does not overlap with modes of action exerted by current giardicidal drugs [146]. Even therapeutic effects of the beta receptor blocker propranolol on metronidazole-resistant giardiasis have been assessed [147]. Salinomycin, an ionophore therapeutic drug, shows adherence reduction of *Giardia* trophozoites *in vitro* but the development of natural *in vivo* resistance limits its suitability for the control of giardiasis [148]. The giardicidal effects of triazolyl-quinolone-based chalcone derivatives depends on the presence or absence of oxygen [149]. Also, giardicidal effects of substances like allicin, propolis, tenatoprazole, fabomotizole, tenatoprazole and ipriflavone have been suggested [150, 151]. Some giardicidal substances have been newly identified from collections of bio-active compounds like the malaria venture's pathogen box. Components of such collections have been specifically chosen to stimulate infectious disease drug discovery. Various drugs of interest with potential giardicidal effects are only coded so far, like, e.g., MMV676358 and MMV028694 [152].

CONCLUSIONS

Several conclusions can be drawn from the available data on antimicrobial resistance in *G. duodenalis*. The recorded multiple realizability of resistance, which can be mediated by various factors influencing the expression of multiple effector proteins without specific target gene mutations [62], is most likely due to epigenetic or posttranslational modifications [44, 46, 48, 52, 70] and does not require

unambiguously identifiable patterns in proteomic assays [55]. Based on these findings including the results of comparative proteomics [55], it is highly unlikely to assume that rapid and easy-to-perform molecular assays for the detection of antimicrobial resistance will be available in the near future. Hence, testing of antimicrobial resistance in human *G. duodenalis* strains in order to allow resistance-based treatments will remain challenging. Although animal models for the assessment of *G. duodenalis* resistance against metronidazole and albendazole have been introduced to replace even more laborious culture procedures [153], resistance testing applying mouse models will nevertheless be poorly suitable for the diagnostic routine situation under the most circumstances. Standardization of resistance testing for *G. duodenalis* in microtiter plates about 3 days has been attempted as well [154]. For example, the trophozoite adherence inhibition method can be applied after *in vitro* excystation and axenical cultivation in TYI-S-33 modified medium as described [155]. But again, broad diagnostic implementation never succeeded. As recently shown, results of culture-based resistance testing also depend on whether the test is performed under anaerobic or microaerophilic conditions [149] and are affected by cysteine concentrations in the growth medium [156]. Further, phenotypic resistance testing is challenged by subpopulations of differing resistance levels even within the same *G. duodenalis* strain [35]. Thus standardization of both methodology and interpretation will be crucial if implementation in the routine laboratory diagnostic setting shall succeed.

Because culture of clinical *G. duodenalis* isolates is quite challenging and poorly compatible with diagnostic routine procedures so far, whole genome sequencing of parasite genomes has been proposed as a screening option for resistance determinants. In order to do so, purification of *G. duodenalis* from stool samples based on immunomagnetic separation after sucrose gradient flotation has been introduced [157]. However, as long as resistance determinants are poorly characterized, phenotypic resistance shows lacking stability [31] and epigenetic or post-translational modifications are often more relevant than DNA sequence changes [44, 46, 48, 52, 70], a practical value of solely sequence-based resistance testing for the diagnostic routine as guidance for antimicrobial therapy of giardiasis remains doubtful.

Without reliable *in vitro* diagnostic assays indicating antimicrobial resistance or susceptibility, however, therapy of chronic therapy-refractory giardiasis remains a considerable challenge. Accordingly, a whole bundle of management efforts has been suggested, comprising the recognition of the known modifiable causes of this health condition, the assessment of symptoms and potential complications as well as – if necessary - their treatment utilizing a multidisciplinary therapeutic team and an ongoing monitoring of the effects of therapy [158]. However, also multidisciplinary therapeutic teams will have to rely on evidence, but evidence for the treatment of therapy-refractory giardiasis is scarce.

In order to provide such evidence, sufficiently powered, randomized controlled trials should be conducted as suggested previously [16, 102, 103]. In order to include sufficient numbers of patients with therapy-refractory giardiasis, multicentric studies will be preferable. Although a lot of potentially promising therapeutic approaches have been introduced above, focus on already available drugs will most likely be the best strategy to provide evidence in the near future. Next to the choice of optimal substances or substance combinations, optimal dosages for the treatment of *G. duodenalis* should be addressed in future studies, as suboptimal dosing regimens are likely to contribute to the resistance problem [1, 159]. Further, a study focus should be on predisposing factors of therapy failure [17]. For example, studies on the effects of therapy adherence on therapy failure and resistance development might provide beneficial insights.

Next to sufficient numbers of included patients, adequate case definitions [160], precise knowledge of diagnostic characteristics of applied diagnostic tests [161] and appropriate surrogate parameters of the outcome [162] are critical in order to achieve meaningful study results by applying accuracy-adjusted estimators [163, 164]. If, as shown before [13], reliability of microscopy-based parasite diagnostics is limited, molecular assays should be considered [11, 12], although molecular diagnostic tests for parasitic disease are still scarcely applied [165]. Thereby, PCR-based therapy control has to consider that DNA clearance from stool, either by excretion or nucleases, is a stochastic process. In literature, contradicting reports of DNA persistence after cleared infections between few days and several weeks within the complex stool matrix exist [166, 167]. Based on the experience at the German National Reference Centre for Tropical Pathogens Bernhard Nocht Insititute for Tropical Medicine Hamburg, *G. duodenalis* DNA should be cleared from stool at least 14 days after the end of a successful therapy. However, spontaneous cure of giardiasis has to be considered as well when estimating the patient numbers to be included [4]. Suggestions to overcome obstacles for the study design are included in Table 1. A major challenge for

Table 1. Suggestions to circumvent obstacles for therapeutic studies on optimal treatment of therapy-refractory giardiasis

Obstacle	Circumvention strategy
Low numbers of patients with therapy-refractory giardiasis	Multi-centric trials and focusing on limited numbers of treatment arms with drugs, dosages and drug combinations
Lacking standardized diagnostics for antimicrobial resistance in *G. duodenalis*	Combination of culture-based, sequence-based and comparative proteomics approaches as well as clinical and diagnostic case definitions
Lacking reliability of microscopy for the outcome assessment	Use of more sensitive real-time PCR approaches in combination with clinical case definitions
Imperfect accuracy of case definitions	Assessment of sensitivities and specificities in pilot studies, then application of accuracy adjusted estimators

studies on the optimal treatment of giardiasis is the underlying economics, as it is considered as a neglected disease with low funding priority and limited commercial interest [159, 168]. Accordingly, the topic will most likely have to remain in the scope of not-for-profit research activities.

Last not least, next to the development and implementation of new therapeutic strategies, infection prevention should not be neglected. Therefore, studies on successful enforcement of adequate hygiene standards to reduce both the transmission of *G. duodenalis* and the infectious burden will remain an issue of relevance [169]. Further, due to the difficult-to-control resistance conditions including the risk of multidrug resistance in *G. duodenalis* [170], also vaccine development remains an ongoing issue for the control of giardiasis [171].

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