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Udržování integrity chromosomů na modelu *Giardia intestinalis*

Maintenance of chromosome integrity in *Giardia intestinalis* as a model organism

Ph.D. Thesis

Supervisor: RNDr. Eva Nohýnková, Ph.D.

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Motto

„Each man's life represents the road toward himself, and attempt at such a road, the intimation of a path. No man has ever been entirely and completely himself. Yet each one strives to become that — one in an awkward, the other in a more intelligent way, each as best he can.“

Hermann Hesse (1877 - 1962)

Abstrakt

Giardia intestinalis je kosmopolitní jednobuněčný organismus způsobující průjmy. Kromě klinického významu, jsou tito dvoujaderní prvoci zajímaví také z hlediska postavení v rámci eukaryot. Jsou evolučně vzdálení obvyklým modelovým organismům a dokonale přizpůsobeni parazitickému způsobu života. Jejich genom je poměrně malý, obsahuje velice málo nekódujících oblastí a mnohé z genů známých u jiných organismů u giardií chybí, tyto vlastnosti z nich dělají atraktivní model pro studium schopnosti buňky fungovat s minimální výbavou. Předkládaná práce přináší nová dílčí zjištění o různých úrovních udržování chromozomové stability u tohoto parazita.

Jednou z nich je způsob ochrany konců chromozomů, tzv. telomer. Podařilo se nám lokalizovat telomery na koncích chromozomů v různých fázích buněčného cyklu a zpřesnit jejich přibližnou délku na 0,5 až 2,5 kb. Prokázali jsme existenci aktivního enzymu telomerázy odpovědného za přidávání telomerických repetic na konce chromozomů, ačkoliv se jedná o enzym strukturně odlišný od jiných eukaryot. Tyto poznatky ukazují, že giardie, stejně jako většina eukaryot, vyvinula konzervativní způsob, jak zamezit zkracování telomer.

Popisujeme také účinek léku pro léčbu giardiózy, metronidazolu, na DNA a buněčný cyklus citlivých a rezistentních buněčných linií giardií. Metronidazol způsobuje fosforylaci histonu H2A v jádrech a naštípání DNA. Subletální koncentrace prodlužuje replikaci, na rozdíl od letální dávky, která vede k rychlé ztrátě schopnosti buněk přilnout k povrchu přičemž buněčný cyklus není ovlivněn. Tato pozorování ukazují, že prvotní reakcí buněk na letální koncentraci metronidazolu není poškození DNA, ale spíše okamžité vzájemné působení mezi metronidazolem a nejbližšími biomolekulami v místě, kde dochází k tvorbě cytotoxické formy metronidazolu. U rezistentních linií je přibližně 40% buněk pozitivní na přítomnost fosforylované formy H2A histonu, což ukazuje, že v těchto buňkách je neustále přítomna poškozená DNA, která by mohla přispívat ke zrychlené mutagenezi a tedy také vzniku přirozené rezistence.

Je známo, že chromozomy giardií kondenzují. Popsali jsme celkovou morfologii chromosomů, stupně kondenzace a rozchod chromozomů v průběhu mitózy u tohoto parazita. Proces kondenzace chromozomů u giardií probíhá obdobně jako u jiných eukaryot, zvláštností oproti jiným organismům je způsob rozchodu chromozomů. U giardií se chromozomy neuspořádávají do ekvatoriální roviny, naopak dochází k průběžnému přesunu chromozomů k pólům vřeténka, což způsobuje opožďování přestupu chromatid do dceřiných buněk a mohlo by být vysvětlením pro aneuploidii, která je u tohoto parazita popisována. Na molekulární úrovni giardii chybí některé geny kódující proteiny kohesinového a kondenzinového komplexu, které mají roli v kondenzaci chromozomů a separaci chromozomů v mitóze. Nám se podařilo nalézt pravděpodobné členy rodiny kleisinů, kteří by mohli mít roli v tvorbě kondenzinového komplexu.

Konečně, studovali jsme vliv inhibitoru afidikolinu na DNA a buněčný cyklus giardií. Tato látka je využívána pro synchronizaci buněk giardií a vede k zastavení buněčného cyklu na rozhraní G1 a S fáze. Prokázali jsme, že afidikolin způsobuje fosforylaci histonu H2A, měl by proto být použit v minimálních koncentracích, po co nejkratší dobu a s vědomím, že u buněk je aktivována odpověď na poškození DNA.

Abstract

Giardia intestinalis is a protozoan causing diarrhea worldwide. Beside its medical importance, it is evolutionary distant protist with two nuclei within a cell adapted for parasitic life in the environment poor of oxygen. Its genome is small and compact in term of gene content and size. It is therefore an attractive model organism for studies of minimal requirements for cellular processes. Present work brings new partial information on different levels of chromosome integrity maintenance of this parasite.

Our study presents characteristics of chromosome termini and their protection. We localized telomeres during all stages of the trophozoite cell cycle and determined the length of *Giardia* telomeres ranging from 0.5 to 2.5 kb, we proved an existence of an active telomerase enzyme synthesizing telomeric repeats in in this parasite, despite the fact that giardial telomerase is structurally divergent. Present data support the view that the chromosomal termini in *Giardia* are maintained in a conservative manner that is common to other eukaryotes.

We described effects of commonly used drug for treatment of anaerobic infections, metronidazole, on DNA and cell cycle progression in susceptible and resistant cell lines. Incubation of cells with this drug causes phosphorylation of histone H2A in cell nuclei and fragmentation of DNA. Sublethal concentration affects the replication phase of the cell cycle and lethal drug concentration lead to rapid loose of adherence ability without any effect on cell cycle progression. Our results support the view that the early reaction of cells to lethal concentration of metronidazole is not primarily initiated by the reaction to DNA damage but rather by the immediate interaction of the drug with biomolecules where active form of metronidazole is generated. In resistant lines incubated in the presence of the drug, about 40% of cells remain permanently positive for H2A in nuclei without any effects on the cell cycle progression. This suggests that DNA damage caused by this drug treatment persists in these cells and may contribute to accelerated mutagenesis and consequently to the development of natural resistance.

Chromosomes in *Giardia* condense, we described the overall morphology, condensation stages, and mitotic segregation of these chromosomes, which is similar to other model eukaryotes. Differently, the anaphase poleward segregation of sister

chromatids is atypical and tends to generate lagging chromatids between daughter nuclei which could explain existence of aneuploidy in this parasite. On molecular level, *Giardia* lacks several genes involved in the cohesion and condensation pathways, in present study we identified two putative members of the kleisin family thought to be responsible for condensin ring establishment.

Lastly, we examined effects of synchronization agent aphidicolin on the nuclear cycle and cell cycle progression characteristics, as well as their reversibility. Treatment with aphidicolin leads to G1/S phase arrest and to phosphorylation of H2A histone. Thus, if aphidicolin is used for synchronization of *Giardia* trophozoites, this fact must be accounted for, and treatment with aphidicolin must be minimal.

List of publication

I. Uzlíková M, Fulnečková J, Weisz F, Sýkorová E, Nohýnková E, Tůmová P. Characterization of telomeres and telomerase from the single-celled eukaryote *Giardia intestinalis*. Mol Biochem Parasitol. 2017 Jan; 211:31-38. doi: 10.1016/j.molbiopara.2016.09.003. Epub 2016 Sep 13. PubMed PMID: 27638151.

Impact Factor: 2.158 (2018)

II. Uzlikova M, Nohynkova E. The effect of metronidazole on the cell cycle and DNA in metronidazole-susceptible and -resistant *Giardia* cell lines. Mol Biochem Parasitol. 2014 Dec; 198(2):75-81. doi: 10.1016/j.molbiopara.2015.01.005. Epub 2015 Feb 12. PubMed PMID: 25681616.

Impact Factor: 2.158 (2018)

III. Tůmová P, **Uzlíková M**, Wanner G, Nohýnková E. Structural organization of very small chromosomes: study on a single-celled evolutionary distant eukaryote *Giardia intestinalis*. Chromosoma. 2015 Mar; 124(1):81-94. doi: 10.1007/s00412-014-0486-5. Epub 2014 Aug 30. PubMed PMID: 25171919.

Impact Factor: 3.530 (2018)

IV. Hofstetrová K, **Uzlíková M**, Tůmová P, Troell K, Svärd SG, Nohýnková E. *Giardia intestinalis*: aphidicolin influence on the trophozoite cell cycle. Exp Parasitol. 2010 Feb; 124(2):159-66. doi: 10.1016/j.exppara.2009.09.004. Epub 2009 Sep 6. PubMed PMID: 19735659.

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1 INTRODUCTION

I would like to give you insight on this thesis. *Giardia* parasite is the scope of the research in the laboratory of Eva Nohynkova. At the early stage of my work, I collaborated with K. Jirakova (born. Hofstetrova). She used aphidicolin inhibitor for her experiments on synchronization of *Giardia* cells and as a side effect she detected DNA damage, more precisely - the phosporylation of H2A histone. This posttranslational modification was impressive. In regard to the detected modification I began experiments to study its induction by applying different agents, including gamma and UV radiation. We also tried to uncover some parts of DNA damage signaling pathway in *Giardia* by following DNA damage response genes, inhibition of H2A histone phosporylation and via localization of some chosen proteins. Much later we also used metronidazole, a common antigiardial drug, to induce H2A histone phosphorylation. Use of metronidazole to induce DNA damage was in fact an idea of professor Tachezy, who asked me that question during my presentation at the department meeting. Since that we focused on reaction of *Giardia* cells to this drug including cell cycle and DNA damage and my supervisor, Eva Nohynkova, had an idea to test also resistant cell lines and provided them from professor Müller laboratory, so we could include them to our experiments. The issue of chromosomes, their structures, numbers and their transmission from mother cell to daughter cell has been studies for a long time in the laboratory of Eva Nohynkova. The team concentrated on several topics. Klara studied transmission of genetic material during the process of cyst formation. Kristyna Markova focused on mitosis and its regulation. Pavla Tumova (born. Tesarova) was working on cell division and visualized condensed chromosomes on cytogenetic preparations during her graduate studies. After her maternity leave break she jumped in to the team and continued working on chromosomes. She established collaboration with professor Wanner in Munich and used high-resolution field emission scanning electron microscopy to describe the process of condensation of giardial chromosomes and chromosomal structures in more detail. In collaboration with Zuzana Vaitová (born. Zubáčová), she optimized fluorescence *in situ* hybridization for detection of genes on *Giardia* chromosomes spreads and her main interest has been aimed to study aneuploidy in this parasite. In this working atmosphere focused on chromosomes and

its structures I asked myself what is known about chromosome ends and their maintenance. Consequently, we started working on visualization of *Giardia* chromosome ends. At the same time, I contacted Jana Fulnečková and Eva Sýkorová from Institute of Biophysics, Academy of Sciences of the Czech Republic, where they work on plant telomerases. We planned experiments to verify if *Giardia* possesses an active telomerase enzyme.

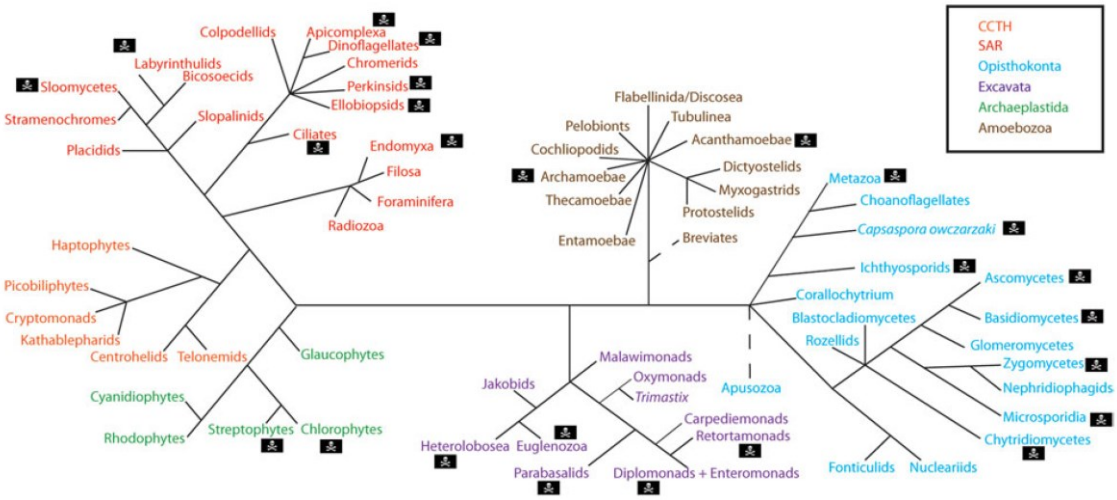
As described above, topics of my thesis progressed to certain extent based on actual situation in the lab and also during a long period of time. I believe that thanks to diverse topics, methods and collaboration with enthusiastic people, the present thesis brings several original findings which may help to better understand mechanisms of chromosome maintenance in *Giardia*.

1.1 *Giardia intestinalis* - phylogenetic position and speciation

For a long time, the genus *Giardia* together with *Trichomonas*, *Encephalitozoon* and *Trypanosoma* have been placed to the root of eukaryotes, often called „primitive“, „early branching“ or „amitochondriate“ eukaryotes. This view based on analysis of SSU rDNA genes and single protein genes in 1990s was rejected due to fact that the phylogenetic analysis were affected by „long branch attraction“. This artifact of the phylogenetic methods causes clustering of highly diverged sequences together, artificially suggesting their relation (Walker et al. 2011). And also due to the discovery of remnant, highly reduced mitochondria (organelles called hydrogenosomes or mitosomes) in majority of „amitochondriate“ eukaryotes (van der Giezen 2009).

The increase of gene sequences from genomes or expressed genes of multiple eukaryotes together with multi-gene concatenated phylogenies changed dramatically the view of evolution of eukaryotes. A framework of several unrooted supergroups of eukaryotes was given (see Fig. 1) and an independent evolution of parasitic lifestyle among eukaryotes is proposed (Walker et al. 2011).

Figure 1



Giardia belong together with e.g. *Hexamita, Enteromonas*, *Spironucleus, Trepomonas* and *Octomitus* to diplomonads, in higher hierarchy to Fornicata, Metamonads, Excavates group, according to Adl et al. (2019). To the common characteristics of Diplomonadida belong that they live in the environment poor of oxygen (they are both free-living protists and parasites), have a remnant mitochondria (mitosomes or hydrogenosomes) and in many of the representatives but not all a single cell contains a doubled set of cellular structures (two nuclei and doubled number of flagella with associated skeletal structures). Interestingly, on the basis of phylogenetic analyses on SSU rRNA, α-tubulin and HSP90 genes, this doubled state has probably evolved several times independently in the evolution or alternatively the secondary reduction to the single state occurred several times (Kolisko et al. 2008). Functional advantage of doubled cellular structures remains unknown.

Giardia intestinalis (synonyms: *Giardia lamblia, Giardia duodenalis*) is the only *Giardia* species infecting humans. Nowadays, this species consists of eight genetic groups (A – H) called assemblages which are morphologically more or less uniform, but most of them exhibit host specificity. The genomic heterogeneity among assemblages led to suggestion of re-evaluation of these assemblages as independent species of *Giardia* (Monis et al. 2009). Only assemblages A and B are known to be present in humans and have the broadest host specificity. Both assemblages are common in developed and developing countries worldwide. The question of differences in geographical or socioeconomic distribution of these two assemblages cannot be

resolved so far due to the small number of molecular epidemiological studies (Feng and Xiao 2011).

1.2 The disease and treatment

1.2.1 The life cycle and giardiasis

Infection caused by *Giardia* (term *Giardia* will be used within the following text instead of full name *Giardia intestinalis*) is the most common protozoan infection of the human intestine with worldwide distribution. The life cycle goes as follows: The host becomes infected by ingestion of cysts present in water, or less commonly by contaminated food or by fecal-oral route. After exposure to the acidic environment in the stomach, actively moving flagellated cells called trophozoites release from cysts in duodenum. Interestingly, a single cell with four nuclei excysts and then divide into two double- nucleated trophozoites (Jirakova et al. 2012). Trophozoites then multiply in the lumen, where they can move freely or attach to the mucosa by ventral adhesive disc and cause symptoms of the disease, giardiasis. After exposure to biliary fluid, new cysts are formed in the jejunum and are passed with feces to the environment, where they can complete the life cycle by infecting a new host (Adam 2001). Cysts are infectious immediately after release and can survive for weeks to months depending on the environmental conditions (Erickson and Ortega 2006).

The prevalence of giardiasis in humans varies from 0.4 to 7.5% in developed countries and from 8 to 30% in developing countries. It is also very common enteric parasite of domestic and wild animals (Feng and Xiao 2011). As mentioned above, humans are infected by *Giardia* assemblage A and B, where assemblage B has a higher prevalence (about 58%) then assemblage A (about 37%) (Feng and Xiao 2011). Mixed infections occur in about 5.2% of cases in developing countries and in about 3.2% of cases in developed countries, however the frequency seems to be underestimated due to the fact that conventional PCR diagnostic tools detect only the most abundant parasite population (Ryan and Caccio 2013). The transmission of infection is firmly believed to be zoonotic. Humans and chosen animals host the same *Giardia* assemblages. It was shown with increasing use of multilocus genotyping diagnostics

developed by Caccio et al. (2008) that humans and animals host the same genetic subgroups called subassemblages. This tool enables more detailed genetic analysis of *Giardia* isolates generated from infected humans, animals or environments and is therefore needed to be made in conjunction of any epidemiological study (Feng and Xiao 2011). Surprisingly, first study presenting a direct evidence for natural zoonotic transmission between a pet chinchilla and a human was published recently by Tumova et al. (2018).

Clinical manifestation of the infection includes watery diarrhea, epigastric pain, nausea and vomiting. This acute phase lasts typically 1-3 weeks, in some patients may persists for months. The majority of infection resolves spontaneously, but recurrences are common in endemic areas (Certad et al. 2017). Chronic infections may lead to malabsorbtion and weight loss and consequently to growth slowdown and cognitive impairment in children in developing countries. Asymptomatic infections are common; however the factors determining the course of infection are currently unknown (Certad et al. 2017). Giardiasis is also a common cause of diarrhea in travelers (Zimmermann et al. 2016), where particularly those returning from Asian countries suffer with refractory giardiasis (Nabarro et al. 2015).

The development of disease depends on interaction between the parasite and the host. The known key *Giardia* virulence factors include (1) strong attachment of trophozoites to enterocytes by cytoskeletal structure, the ventral disc, to avoiding peristaltic elimination, (2) induction of disruption of tight-junction affecting the epithelial barrier and leading to increased intestinal permeability, (3) low infective dosage, when 10 cyst were reported to cause infection, (4) ability to induce apoptosis in enterocytes (Certad et al. 2017) and (5) ability to disrupt innate mucosal protective barriers which consequently leads to altering commensal microbiota biofilm integrity (Allain et al. 2017).

1.2.2 Treatment of giardiasis

The most commonly used drugs for giardiasis treatment targets either (1) metabolic pathways specific for microaerophilic life style of *Giardia* in case of nitroheterocyclics metronidazole (MTZ), tinidazole (TIN) and nitazoxanide (NTZ) or (2)

Giardia- specific cytoskeletal structures in case of benzimidazoles albendazole or mebendazole. Except for these, less commonly used are another 5- nitroimidazole ornidazole, acridine drug quinacrine, the aminoglycoside paromomycin, nitrofuran furazolidone or polypeptide antibiotic bacitracin in combination with zinc (Leitsch 2015). In most analyses, MTZ and TIN have similar efficacies, with parasitological cure rates and symptom relief in more than 90% of patients (Minetti et al. 2016). Combination therapies comprise the use of MTZ or TIN and another agent. This cure strategy combining drugs with different mechanisms of action is used in cases when initial therapy fails (Escobedo et al. 2016; Lalle and Hanevik 2018; Nabarro et al. 2015). In the following text, mainly MTZ will be addressed.

In the context of metabolism, *Giardia* as a microaerophilic protist lives in very low but fluctuating oxygen condition in the small intestine (Leitsch et al. 2018). This parasite lost mitochondria and harbors a remnant organelle – the mitosome (Dolezal et al. 2005). Thus the most metabolic pathways are present in cytosol and they are not sub-compartmentalized. Energy metabolism is minimalistic, with enzymes likely acquired by lateral gene transfer (Ansell et al. 2015), and consists mainly of glycolytic and arginine dihydrolase pathways. The preferred sugar glucose is catabolized via glycolysis into pyruvate which is then converted into acetate, ethanol and alanine as nongaseous end products and CO_2. However, the small amounts of oxygen may affect the end product generation. The parasite also lacks pathways for *de novo* synthesis of pyrimidines and purines (Einarsson et al. 2016).

Metronidazole and some other 5-nitroimidazoles are gold standards for treatment of not only giardiasis but many other anaerobic and microaerophilic infections of protozoan and bacterial origin (Brook 2007; Brook 2011; Brook 2016). The strong reductive environment in cells of these microorganisms enables reduction of a nontoxic prodrug into toxic intermediates which interacts with surrounding biomolecules. On the other hand, the ability of aerobic cells to re-oxidize the nitro radical anion to non-toxic nitro group enables this drug to be non-toxic to host cells (Ansell et al. 2015).

Several enzymes can reduce MTZ within *Giardia* cells. In addition to pyruvate:ferredoxin oxidoreductase (PFOR), three other reductases, specifically thioredoxin reductase (GlTrxR) and nitroreductases 1 and 2 (GlNR1 and GlNR2), were

identified as drug targets (Leitsch et al. 2011; Muller et al. 2013; Nillius et al. 2011). Interestingly, *Giardia* GlNR2 appears to detoxify MTZ by converting it into a non-toxic end product via complete reduction of the drug (Muller et al. 2013). Results from a proteomic analysis suggest that MTZ forms adducts with several unrelated *Giardia* proteins, among which only thioredoxin reductase (TrxR) is directly involved in redox processes. Some of the other proteins including glycolytic enzymes pyruvate phosphate dikinase (PPDK) and alcohol dehydrogenase, and *Giardia* trophozoite antigen (GTA-2) are closely associated with PFOR and the remaining proteins (e.g. elongation factor-1γ, alpha-11 giardin, β-giardin) are involved in other cellular processes (Leitsch et al. 2011).

Regarding the cytotoxic effect of MTZ, it is widely accepted that reduction of its 5-nitro group yields a nitro anion radical and nitroso derivatives, that are presumed to cause DNA damage (Edwards 1993). Previous electrochemical studies have demonstrated that *in vitro,* the radical formed via reduction of the nitro group interacts with DNA bases (Declerck 1987). Drug target studies conducted *in vitro* have shown that oxidative damage to DNA resulting in strand breaks occurs in the presence of reduced MTZ (Edwards 1993). However, a more complex response appears to occur *in vivo,* as suggested by recent findings in anaerobic protists (Leitsch et al. 2011; Leitsch et al. 2018; Muller and Hemphill 2013; Muller et al. 2013). *In vivo* detection of nitro radical anions have only been described in *Trichomonas vaginalis* (Lloyd and Pedersen 1985) whereas the electron paramagnetic resonance signal from MTZ nitro anion radical could not be demonstrated in *Giardia* (Lloyd and Williams 2014).

These findings indicate that the drug may affect different metabolic pathways in *Giardia* and that DNA damage may be secondary to the drug´s direct attack on specific protein(s). Several hints showed that MTZ treatment may induce DNA damage *in vivo*. Incubation of cells with different concentration of this drug led to positive signals on TUNEL assay which detects DNA fragmentation (Bagchi et al. 2012). Another study describes fragmentation of DNA visualized on agarose gel together with prolonged replication phase of the cell cycle (Ghosh et al. 2009). Apart from our study (Uzlikova and Nohynkova 2014), the most recent results show that the response of cells to DNA damage caused by this drug involves homologous recombination repair

machinery. Its components, giardial MRE11 and DMC1B are upregulated in reaction to MTZ (Ordonez-Quiroz et al. 2018).

Taken together, the above observations support the view that in *Giardia*, 5-nitroimidazole drugs are metabolized in different way when compared to other pathogenic microaerophilic protists *Entamoeba histolytica* and *T. vaginalis,* and the cytotoxic impact on cells is not restricted only to inhibition of an essential TrxR enzyme activity but has a broader effect including DNA damage and non-protein thiols binding (e.g. cellular redox buffer cystein in *Giardia*) which all together lead to cell death (Leitsch et al. 2018).

1.2.3 Treatment-refractory giardiasis

Metronidazole was introduced more than 50 years ago and nowadays it seems that five decades of susceptibility to MTZ treatment was replaced by increasing resistance to this and also to the other drugs (Lalle and Hanevik 2018). The drug-resistant giardiasis is termed when stool samples remain positive for *Giardia* more than 1 week after treatment completion and when other reasons for treatment failure have been excluded and the risk of re-infection is low (Lalle and Hanevik 2018). The retrospective study summarizing the period of time from 2008 to 2013 at the Hospital of Tropical Diseases in London manifests increase of nitroimidazole treatment-refractory cases from 15% in 2008 to 45% in 2013 (Nabarro et al. 2015). The authors suspect that the reason for treatment failure is increasing nitroimidazole resistance, predominantly in India, which is in agreement with findings that 70% of patients with refractory giardiasis had this travel history. The authors also summarize factors leading to spread of resistance in India which include sale of antibiotics without prescription, limited excess to healthcare and microbiological diagnostics together with overprescribing of antibiotics by physicians. In a similar study, 22% of Spanish travelers had refractory giardiasis, particularly those travelling in Asia (Munoz Gutierrez et al. 2013). The only study from the Czech republic summarizing data from 2004 to 2014 at the Hospital Na Bulovce showed that treatment of travelers suffering from giardiasis failed in 29% (37 of 127) (Stejskal F. 2015).

To study resistance of *Giardia* to MTZ is complicated by the fact that mainly laboratory-derived resistant isolates are used for research and clinically resistant isolates do not display the same sensitivity to the drug *in vitro* (Leitsch 2015). It is relatively easy to induce nitroimidazole resistance *in vitro* by increasing concentration of the drug or by exposure to UV light and the mechanism of resistance has been widely studied using such isolates. However, as summarized by Leitsch (2015) the observations were often conflicting and suggested that the MTZ resistance in *Giardia* is complex and polygenic. Recent studies employing new approaches, in this case quantitative transcriptomics and proteomics on three laboratory-derived MTZ-sensitive and – resistant cell lines confirmed substantial changes in the antioxidant network, glycolysis and electron transport and interestingly and newly, indicated changes on post-translation level, especially acetylation in studied lines (Ansell et al. 2017; Emery et al. 2018). Another study analyzing proteomes of susceptible and resistant *in vitro* derived lines by mass spectrometry showed that 200 to 500 proteins were expressed differentially. The authors conclude that resistance to nitro drugs evolves independently for each strain and each drug and does not have a common pattern. Based on these findings they substitutes the term “drug resistance” for more accurate “drug tolerance” when compared to concept generated from antibiotic resistance (Muller et al. 2019).

1.3 Cell cycle

1.3.1 Cell cycle and its control in eukaryotes and in *Giardia*

In general, the process of duplication of single cell content and its division into two daughter cells is called the cell cycle. The main events are replication of DNA (S phase or DNA synthesis) and precise segregation of replicated DNA between progeny (M phase or mitosis). Most cells require time to grow and double their mass of proteins and organelles that they require to replicate the DNA and divide. These phases are called gap phases- a G_1 phase between M and S phase and G_2 phase between S and M phase. The traditional cell cycle is then divided into four phases – G_1, S, G_2 and M.

Each *Giardia* cell accommodates two approximately diploid nuclei, so the cell alternates between two diploid (2 x 2N, tetraploid per cell) and two tetraploid (2 x 4N, octoploid per cell) states corresponding to G_1 and G_2 phases of the cell cycle (Bernander et al. 2001). The duplicated DNA is then segregated into daughter cells by formation of two separated spindles (Nohynkova et al. 2000; Sagolla et al. 2006). Thus similarly to other eukaryotes, *Giardia* cell cycle consists of four cell cycle phases with G1 lasting about 0.7 hour, S phase 1.3 hours and G2/M/cytokinesis about 4 hours for the most commonly used WB isolate (Reiner et al. 2008). Unlike the most eukaryotes, *Giardia* longest cell cycle phase is G2 which is also the phase from which *Giardia* cell enters to the process of cyst formation (Reiner et al. 2008). The key events of mitosis and cytokinesis last a few minutes. This process is tightly connected with simultaneously ongoing reorganization of adhesive disc, when the parent disc disappears and daughter discs become newly formed. The parasite needs to detach from the epithelium for a short time and attach using newly formed discs to finish division (Tumova et al. 2007b).

The *Giardia* cell cycle is poorly defined at molecular level with some orthologues identified and many orthologues missing in the parasite genome (Davids et al. 2008; Eme et al. 2011; Morrison et al. 2007; Reiner et al. 2008). Functional studies are scarce. Generally, central components of cell-cycle control system in eukaryotes are members of protein kinases known as cyclin-dependent kinases (Cdks). The activities of these kinases cyclically change throughout the cell cycle. Their most important regulators are cyclins which, if tightly bound to Cdks, are necessary for protein kinase activity. While cyclins undergo cycles of synthesis and degradation during the cell cycle, the levels of Cdks remain constant (Alberts 2008). In *Giardia*, among others, three mitotic cyclin (A/B) genes and also three genes for cyclin-dependent kinases (CDK1) are present in the genome (Manning et al. 2011; Morrison et al. 2007). The most studied giardial cyclin B was followed on mRNA level and also on protein level, where the protein and mRNA levels reach the maximum in G2/M and minimum in G1/S (Gourguechon et al. 2013; Horlock-Roberts et al. 2017; Reiner et al. 2008). Its binding to giardial Cdk1 and activity of the complex was proved (Gourguechon and Cande 2011; Gourguechon et al. 2013). These findings indicate that cyclin B similarly to other eukaryotes binds to Cdk that stimulates entry into mitosis

and is degraded lately in mitosis. Unlike in other eukaryotes, *Giardia* mitotic cyclin B lacks the degradation motif, is not ubiquitinylated and the process of degradation is independent on anaphase promoting complex, which components are completely missing in *Giardia*. It is therefore of interest to investigate how *Giardia* cells provide the active degradation of cyclin B in late mitosis using a novel unknown mechanism unique to eukaryotes (Gourguechon et al. 2013).

The cell cycle must be very precisely organized and controlled. Errors, if not recognized, may have fatal consequences for a cell, daughter cells or for a body of human where an uncontrolled cell line progresses. In eukaryotes, the key cell cycle checkpoints control conditions for DNA replication start (G1/S or Start checkpoint), if DNA is completely and correctly replicated and the cell may enter mitosis (G2/M checkpoint) and finally checkpoint guarding if all chromosomes are attached to spindle and the cell may progress to anaphase (metaphase-to-anaphase checkpoint, spindle assembly checkpoint or mitotic checkpoint). It should be noted that one of the most serious influences on the cell cycle progression is DNA damage which appears spontaneously during cellular processes or by extracellular effects like radiation or chemicals. The cell cycle control system can readily detects DNA damage and arrest the cell cycle at either of two checkpoints – the G1/S which prevents enter to replication and G2/M which prevents enter to mitosis (Zhou and Elledge 2000).

In *Giardia*, studies on cell cycle checkpoints are rare. Inhibition study employing DNA synthesis inhibitor aphidicolin showed that cells incubated with this drug accumulate at G1/S border (Hofstetrova et al. 2010; Reiner et al. 2008). The same reaction was described, when trophozoites were irradiated with UV-C light (Einarsson et al. 2015). These results indicate the presence of functional checkpoint at G1/S border activated by DNA damage. The affected cells were positive for H2A histone phosphorylation and dramatically delayed their cell cycle progression until the DNA damage became repaired. In model organisms, DNA damage checkpoint activation is mediated by the signaling cascade where ATM, ATR or DNAPK members of the phosphatidyl-inositol-3-kinase related kinases (PIKKs) play a central role (Bartek and Lukas 2001). These kinases also provide phosphorylation of H2A histone in place of DNA lesions and are responsible for downstream signaling. It is therefore of interest to understand what are molecular players in *Giardia* cell signaling pathway in reaction to

DNA damage, because *Giardia* is the only known eukaryote, though it has one gene with weak sequence similarity to PIKKs domain, that lacks all three PIKKs (Manning et al. 2011; Morrison et al. 2007). Despite these facts, recent studies suggest existence of functional DNA double-strand break repair machinery, namely homologous recombination. Some members of signaling pathways have been described. Giardial human ortholog of Rad52 is upregulated in trophozoites in reaction to DNA damage. This protein also binds to dsDNA and ssDNA *in vitro* and interacts with RPA1 and DMC1B proteins (Martinez-Miguel et al. 2017). Moreover, an upregulation of DMC1B and Mre11 was observed in cells exposed to MTZ (Ordonez-Quiroz et al. 2018).

Indications pointing out possible anomalies in *Giardia* mitotic checkpoint were brought with studies on *Giardia* chromosome numbers showing that in chosen isolates, each nucleus of this binucleated parasite contains various number of chromosomes (Tumova et al. 2007a). Therefore, the opinion based on flow cytometry analysis showing that each nucleus is diploid (Bernander et al. 2001) had to be modified. Recent findings demonstrate that diploid set of chromosomes is rather exceptional state in this parasite and naturally occurring state is aneuploidy exhibiting extraordinary dynamics (Tumova et al. 2016; Tumova et al. 2015). While the functional advantage of this feature remains unknown, the question of the structural and molecular cause for unequal distribution of chromosomes into daughter cells led to the focus on spindle assembly checkpoint functioning. As noted above, in human cells, functional spindle assembly checkpoint prevents metaphase-anaphase transition until each sister chromatid is attached via kinetochore to spindle microtubules and aligned at metaphase plate (Lara-Gonzalez et al. 2012). For the sister chromatids separation in metaphase-anaphase transition, enzyme separase has to cleave cohesin complex which holds sister chromatids together. Separase becomes active when inhibitory protein securin is targeted for destruction by ubiquitinylation carried out by the active anaphase-promoting complex (APC/C). Finally, the APC/C in its inactive state is stably bound to complex of proteins termed the mitotic checkpoint complex (MCC, composed of Mad2, BubR1-Bub3 and Cdc20) and when all kinetochores are attached to microtubules, this complex disassembles enabling release of Cdc20 protein for APC/C activation (Lischetti and Nilsson 2015). In other words, SAC checkpoint is “turned on” starting from the nuclear breakdown when the kinetochores are

unattached (the APC/C forms a stable complex with MCC proteins) and becomes "turned off" when all kinetochores are attached to microtubules and MCC complex disassembles thus freeing Cdc20 for APC/C activation and consequent downstream signaling.

The increasing evidence shows that conventional spindle assembly checkpoint (SAC) is not present in *Giardia*. The *Giardia* genome lacs all APC/C components, suggesting that besides of giardial cyclin B, also aurora kinase, an upstream SAC component is regulated independently on ubiquitinylation pathway (Gourguechon et al. 2013). Only reduced repertoire of SAC proteins have been found in *Giardia* and their function remains unclear. SAC and SAC-interacting proteins including Cdc20, securin, Knl1, Bub1 or Mad1 were not found in *Giardia* genome (Eme et al. 2011; Manning et al. 2011). Mps1 and Bub3 are associated with chromatin and centromeres during mitosis, while Mad2 has a cytoplasmic location (Vicente and Cande 2014). The localization of Mad2 on both caudal axonemes and weak effect of its downregulation or overexpression on the course of mitosis and cells population growth led to speculation about a role of this protein in cytoplasmic spindle microtubule system rearrangement in semi-open mitosis of this parasite (Markova et al. 2016; Vicente and Cande 2014). The localization of Mad2 different from the canonical nuclear placement has been observed also in trypanosomes, where Mad2 localizes to basal bodies of flagella. These findings open an interesting question if the ancient function of SAC component Mad2 might have been to monitor segregation of flagella/basal bodies (Akiyoshi and Gull 2013).

It is especially interesting to point out that the dynamic karyotype variability in *Giardia* might arise due to the altered checkpoint control present in this parasite. Although the true mechanisms remain to be uncovered, some explanations have been suggested by Tumova et al. (2016). One of them might be merotelic kinetochore-microtubule attachment, in which the same kinetochore binds to microtubules emanating from both poles. This attachment is not detected by SAC, because merotelic attachment generates tension and is therefore not sensed as erroneous. It is the most common cause of aneuploidy in cancer cells. Nonetheless, merotelic attachment is frequently observed also in healthy human cells in anaphase, where correct segregation is provided by aurora kinase or as recently suggested it is also influenced

by forces generated by different number of microtubules occupying the kinetochore (Dudka et al. 2018). Furthermore, the absence, altered canonical checkpoints including SAC and DNA damage checkpoint or another unexpected mechanism should not be excluded.

1.3.2 Synchronization of the cell population

Synchronization of cell population is widely used for studying the cell cycle in model organisms. It enables analysis of cellular processes dependent on specific cell cycle phase or processes with very short duration causing its very rare observation in unsynchronized cell population. Generally, synchronization methods are based on starvation of cells or usage of inhibitors specifically blocking DNA replication or mitosis. All methods have to be used with care, because each manipulation may have adverse effects on the cell population and may influence results of the original research. In fact, three criteria should be fulfilled to speak about synchronized cells, including these: (1) DNA pattern should be uniform in initial cells, (2) size distribution in initial cells should be narrower than size distribution in unsynchronized cells and (3) synchronized cell population should exhibit synchronized divisions (Cooper 2003).

Studies on giardial cell cycle have been hampered by inability to synchronize trophozoite population. A few studies on *Giardia* synchronization applying aphidicolin (Reiner et al. 2008) or combination of nocodazole and aphidicolin (Poxleitner et al. 2008) have been used to synchronize *Giardia* cultures at G1/S border. Aphidicolin is a mycotoxin from a group of tetracyclic diterpenoids produced by fungi *Cephalospora aphidicola* or *Nigrospora oryzae* that reversibly inhibits replication of DNA due to its competitive binding with dNTP to eukaryotic DNA α polymerase and has been used in various eukaryotic cell lines (Spadari et al. 1984). In both studies, cells were incubated with an effective dosage of the drug for a period of time corresponding to the duration of one cell cycle and with or without pretreatment with nocodazole, spindle assembly checkpoint activator. Cells were then released from the block by overlaying them with fresh drug-free media. Beside aphidicolin and nocodazole, other drugs including hydroxyurea, colchicine or demecolcine and also nutrient deprivation were applied on

Giardia cells with no cell cycle arrest observation or cell synchrony lost after short time for nutrient deprivation (Reaume et al. 2013).

The side effect of aphidicolin was studied in great detail in our laboratory. It was shown, that higher dosages or long incubation with the drug causes massive DNA damage and cells, though blocked in G1/S or S phase regarding their DNA content, increase significantly cell size in comparison to control cells and form median body, a structure typical for G2 cells. These observations suggest dissociation of nuclear and cytoplasmic cycle upon aphidicolin treatment and make this method for synchronization rather problematic (Hofstetrova et al. 2010).

Recently, a new approach for studying cell cycle regulated processes was used in *Giardia*. The counterflow centrifugal elutriation (CCE) is a size-based separation technique enabling to obtain fractions of cells enriched in G1, S and G2 from unsynchronized cell culture. The advantages of this method are that it is possible to use unsynchronized drug-free cells, to load a large numbers of cells (10^7 to 10^9 for a standard elutriation chamber) and the procedure takes a short separation time (Horlock-Roberts et al. 2017). Up to date, this is the only drug-free method for cell cycle dependent processes analysis in trophozoites, because as authors state, the usage of flow cytometry for cell sorting is not possible likely due to the lysis of the cells during the process.

1.4 Chromosomes organization

1.4.1 Packing of the DNA into chromosomes – histones and condensins

Condensed chromosomes of *Giardia* in the course of mitosis visualized by classic cytogenetic techniques were first observed by Tumova et al. (2007a). Similarly to other eukaryotes giardial chromosomes condense, sister chromatids are formed and mitotic segregation of chromosomes in to daughter nuclei proceeds.

In eukaryotes, the DNA in nucleus is divided into a set of different chromosomes which store and transmit the hereditary information in to next generations. Each chromosome consists of a linear molecule of DNA associated with proteins which function is to pack the fine DNA into more compact structure. The core

histone proteins are the building blocks of nucleosomes. These proteins are the most conserved proteins and in eukaryotes are classified in to five groups H2A, H2B, H3, H4 and H1. These histones are further divided into canonical and variant histones. It is widely accepted that canonical histones are present within cells in equimolar amounts, their transcription is tightly coupled with S phase, are arranged in tandem repeats in genome and their mRNAs consist of a conserved hairpin structure on 3´UTR instead of poly(A)tail. Histone variants are paralogs of canonical histones, their transcription is independent on replication and their mRNAs are polyadenylated. The H2B and H4 histones are markedly poor on variants, while in H2A and H3 histones large number of variants has been described. The presence of histones and its variants in protozoan parasites was summarized by Dalmasso et al. (2011). In *Giardia*, the core histones H2A, H2B, H3 and H4 were described with H1 histone missing in parasite genome. Interestingly and in contrary to human core histones, all the histones in *Giardia* possess polyadenylation signal on 3´ noncoding sequences of core histone genes (Yee et al. 2007). The two copies of H2A histone in *Giardia* genome contain the characteristic H2AX motive. This motif is known to undergo phosphorylation upon DNA damage in eukaryotes. This reaction was first observed in *Giardia* in response to aphidicolin inhibitor (Hofstetrova et al. 2010) and then to metronidazole (Uzlikova and Nohynkova 2014) or UV irradiation (Einarsson et al. 2015). Most eukaryotes have three H3 histones, the canonical H3, H3.3 and centromeric-specific, CenH3. *Giardia* possesses two copies of canonical H3 histone and a single copy of CenH3. The observation of giaridal recombinant CenH3 in discrete foci in interphase and in spindle poles in mitosis suggests its centromeric localization (Dawson et al. 2007). Surprisingly, the linker H1 histone is missing in *Giardia* with persisting ability of this parasite for chromosome condensation (Morrison et al. 2007; Wu et al. 2000; Yee et al. 2007). The absence of linker histone is not exceptional in *Giardia* as it has not been found in Apicomplexa or Microsporidia (Dalmasso et al. 2011).

The higher-scale condensation of chromosome is dependent on the presence of large protein complexes called cohesins and condensins. While cohesins are required for sister chromatids cohesion and is partly degraded in mitosis, condensins play role in chromosome assembly and segregation. Condensin complexes are present in all eukaryotes, most of them have two different types of condensins, known as condensin

I and II. Roles of these two types of condensins vary in different organisms with condensin I mostly playing the role in mitosis while condensin II is used for specialized functions in interphase or meiosis. It is nonetheless remarkable, that condensin II is enriched at the centromere/kinetochore region in mitotically dividing cells including human, fruit fly or red alga *C. merolae*. This indicates that the ancient role of condensin II could have been to resolve sister centromeres. In eukaryotes lacking genes for condensin II subunits, including fungi or ciliates, the condensin I took over the function of condensin II at centromeres. It is believed that these organisms lost one of the condensins during the evolution secondarily (Hirano 2012). Condensins I and II share the heterodimer SMC2 and SMC4, that have an ATP-binding domain modulating engagement and disengagement of the two head domains. Re-closing of the ring leads to entrapment of DNA. The non-SMC subunits of condensins belong to kleisin family of proteins. They are also conserved in eukaryotes and are predicted that bridge the two head domains of SMCs. In eukaryotes, the kleisin subunits are bound by distinct pairs of large subunits containing HEAT repeats. The function of these subunits is not fully known, however their role is apparently in chromosome axis assembly forming a backbone of mitotic rod-shaped chromosomes (Hirano 2016). In *Giardia*, the SMC2 and SMC4 condensin core subunits were found in the genome of this parasite (Malik et al. 2007). In our study, we found two putative members of kleisin family which could associate with SMC2 and SMC4 core subunits of giardial condensin I or condensin II. Moreover, a putative HEAT-domain containing protein which may act in condensin I or II complex was described (Tumova et al. 2015). Nothing has been known about localization or function of these orthologs so far.

1.4.2 Centromeres and segregation of chromosomes in *Giardia*

Both the assembly of the kinetochore on centromeric part of chromosomes and binding of kinetochores to spindle microtubules is required for chromosome segregation in mitosis. The presence of about 10 of cenH3 foci in interphase nuclei (Dawson et al. 2007) together with the observation of approximately the same amount of spindle microtubules entering semi-closed nuclear membrane at the spindle pole in *Giardia* suggest that each chromatid binds to only one microtubule (Tumova et al.

2007a). Despite the presence of the canonical equipment for chromosome segregation in *Giardia*, divergences from the normal course have been found in this parasite resulting very likely to aneuploid karyotype in clinical isolates and laboratory lines (Tumova et al. 2007a; Tumova et al. 2016; Tumova et al. 2015)). The cause for this phenomenon is slowly getting uncovered with increasing amount of findings. The metaphase plate with aligned chromosomes has never been observed (Sagolla et al. 2006; Tumova et al. 2007b). As a consequence of the missing canonical anaphase/metaphase, the assembly of chromatids into rows between spindle poles and lagging chromatids between already formed telophase nuclei are frequently observed (Tumova et al. 2015). This is in agreement with above noticed, that the spindle assembly checkpoint is not present in *Giardia* (Markova et al. 2016) and reduced repertoire of mitotic checkpoint proteins in this parasite play atypical roles (Vicente and Cande 2014).

1.4.3 Telomeres and telomerases in eukaryotes and in *Giardia*

The evolution of linear chromosomes led to the necessity of managing the end-replication problem of telomere shortening and distinguishing the natural chromosome ends from DNA damage. Both of these issues are resolved by the activity of telomerase. This special reverse transcriptase regulates the telomere length and, together with telomere-specific proteins, binds to telomere repeats preventing the ends of chromosomes from being recognized by DNA damage response proteins (de Lange 2004). The functional telomerase is composed of the telomerase reverse transcriptase (TERT) and telomerase RNA (RT), which provides the template for telomere repeat synthesis (reviewed in Blackburn and Collins (2011). In addition to common eukaryotic model organisms, such as yeast and vertebrates, telomeres or telomerase has been identified in phylogenetically diverse eukaryotes, including ciliates (Greider and Blackburn 1989), plants and green algae (Fulneckova et al. 2013), dinoflagellates (Fojtova et al. 2010), trypanosomes (Cano et al. 1999) and the apicomplexan *Plasmodium* (Aldous et al. 1998). However, the composition of both core telomerase subunits varies significantly among different groups of eukaryotes (Beilstein et al. 2012; Chen and Greider 2004; Malik et al. 2000). In addition, extensive

sequence heterogeneity of terminal telomeric repeats was found across eukaryotes (Fulneckova et al. 2013). Nevertheless, the telomerase mechanism of chromosome termini maintenance seems to be evolutionary conserved because developing alternative methods to telomerase is an exception rather than a rule (Blackburn and Collins 2011).

Similarly to other eukaryotes, *Giardia* chromosomes include telomeric repeats on both their ends. The chromosome termini in *Giardia* are built from repetitions of the pentanucleotide TAGGG, which was identified in cloned fragments (Adam et al. 1991) and localized by hybridization experiments on both PFGE-separated chromosomes (Le Blancq et al. 1991) and condensed chromosomes in microscopic preparations (Tumova et al. 2015). Folding of TAGGG telomeric repeats into G-quadruplexes *in vitro* was experimentally demonstrated (Tran et al. 2011). The *Giardia* telomere length was predicted not to exceed 1 kb (Le Blancq et al. 1991). When compared to other eukaryotic telomeric repeats, the *Giardia* telomeric sequence is shorter and seems to be unique (Fulneckova et al. 2013). In its genome, *Giardia* encodes the telomerase catalytic subunit TERT, which was shown to be rather sequence-divergent and lacking the N-terminal T motif that is required for telomerase RNA subunit binding (Malik et al. 2000). The absence of a T motif together with the finding of transposable elements joined to the telomeric repeats in *Giardia* have led to speculations that retrotransposons may have overtaken the function of telomere maintenance (Arkhipova and Morrison 2001; Mason et al. 2016).

2 AIMS OF THESIS

I. To characterize chromosome ends and their maintenance mechanism in *Giardia*

II. To determine the effect of metronidazole on the cell cycle and DNA

III. To describe structural organization of *Giardia* chromosomes

IV. To determine side effects of synchronization agent aphidicolin on *Giardia* cells

3 LIST OF PUBLICATIONS

I. Uzlíková M, Fulnečková J, Weisz F, Sýkorová E, Nohýnková E, Tůmová P. Characterization of telomeres and telomerase from the single-celled eukaryote *Giardia intestinalis*. Mol Biochem Parasitol. 2017 Jan; 211:31-38. doi: 10.1016/j.molbiopara.2016.09.003. Epub 2016 Sep 13. PubMed PMID: 27638151.

II. Uzlikova M, Nohynkova E. The effect of metronidazole on the cell cycle and DNA in metronidazole-susceptible and -resistant *Giardia* cell lines. Mol Biochem Parasitol. 2014 Dec; 198(2):75-81. doi: 10.1016/j.molbiopara.2015.01.005. Epub 2015 Feb 12. PubMed PMID: 25681616.

III. Tůmová P, **Uzlíková M**, Wanner G, Nohýnková E. Structural organization of very small chromosomes: study on a single-celled evolutionary distant eukaryote *Giardia intestinalis*. Chromosoma. 2015 Mar; 124(1):81-94. doi: 10.1007/s00412-014-0486-5. Epub 2014 Aug 30. PubMed PMID: 25171919.

IV. Hofstetrová K, **Uzlíková M**, Tůmová P, Troell K, Svärd SG, Nohýnková E. *Giardia intestinalis*: aphidicolin influence on the trophozoite cell cycle. Exp Parasitol. 2010 Feb; 124(2):159-66. doi: 10.1016/j.exppara.2009.09.004. Epub 2009 Sep 6. PubMed PMID: 19735659.

4 RESULTS, DISCUSSION AND PERSPECTIVES

4.1 Paper I.

Chromosome ends are widely studied in model eukaryotes. Beside the medical importance of telomere biology in humans it is exciting to follow the diversity of mechanisms which different eukaryotes uses to figure out the end replication problem and see the variability of chromosome-end organization. Chromosome-end biology in *G. intestinalis* remains poorly understood. Characteristics including extremely small linear chromosomes, minimalistic genome with often missing or highly diverged canonical nuclear proteins or 5 bp telomeric repeat TAGGG led to the suggestions that telomere biology will be rather alternative than conservative in *Giardia*. Using traditional approaches including FISH, TRF and BAL-31 digestion we aimed to determine telomeres localization during the cell cycle, their length and its variability in different cell lines. Importantly, we aimed to reveal if *Giardia* telomerase is an active enzyme sharing conserved function in telomeric repeat addition to chromosome ends and also what is the localization of the TERT subunit of this enzyme in *Giardia* cells.

FISH signal number corresponding to telomeres varied in cells depending on the cell cycle phase. In small compact nuclei representing the G1 cell cycle phase, the number of FISH signals was distinguishably lower, around than in G2 cells with around 12 signals/nucleus and on mitotic chromosomes. In G1 cells, telomere clustering to two foci localized at opposite nuclear poles was observed. Moreover, the signal intensity within a single nucleus varied in all interphase cells and on individual condensed mitotic chromosomes. Such a variation may be attributable to variable telomere length as well as telomere clustering. Telomere clustering in G1 phase to usually two spots in *Giardia* trophozoites and cysts was also observed by Carpenter et al. (2012) and was hypothesized to be a prerequisite for chromatin recombination.

The minimal length of telomeres 0.5 kb determined in our study is in agreement with the previously published estimation in *Giardia* to be 0.6 kb (Adam et al. 1991). The longest TRF products were 2.5 kb in most lines or 4.5 kb in one line derived from the same isolate. The relatively short telomere length in *Giardia*, which has also been described in other unicellular organisms (Dreesen and Cross 2008; Figueiredo et al. 2002; Fulneckova et al. 2012), validates the question of how much the

telomere length and reshuffling within dynamic subtelomeric regions constitute an evolutionary advantage for organismal adaptation.

BAL-31 nuclease treatment of a high-molecular weight DNA is widely used to reveal the terminal position of telomeric repeats because it shows progressive shortening of genomic DNA fragments at both chromosome termini and the loss of the probe-specific signal. The results of the TRF analysis and BAL-31 digestion experiment demonstrated that the *Giardia* genome does not contain long interstitial telomeric sequences as suggested by Arkhipova and Morrison (2001) and Upcroft et al. (1997). In organisms in which these regions were found (He et al. 2013; Uchida et al. 2002), telomeric repetitions point on chromosome rearrangement loci, including telomere-telomere fusions of chromosomes. The absence of intrachromosomal telomeric repetitions indicates that chromosome fusions are rare in *Giardia*. This result agrees with findings proposing that chromatid missegregations are causative of aneuploidy and karyotype variation (Tumova et al. 2016).

The existence of functional telomerase that is responsible for DNA repetition synthesis in *Giardia* was demonstrated by the TRAP assay and nuclear localization of recombinant GiTERT. The presence of active telomerase is especially interesting because the *Giardia* TERT protein sequence is highly divergent and lacks the typical telomerase-specific T moti that is important for binding the RNA subunit (Arkhipova and Morrison 2001; Malik et al. 2000). The results of the TRAP assay clearly demonstrated the activity and heat sensitivity of the *Giardia* telomerase, similar to other model telomerases. Although the canonical function of *Giardia* telomerase that is typical for other constantly dividing cells was demonstrated, it would be of interest to determine RNA subunit of this enzyme or to test whether *Giardia* telomerase also has other roles, such as in chromosome healing in karyotype stabilization for irradiated holocentric chromosomes of the plant *Luzula* (Jankowska et al. 2015).

The prevailing telomeric repeat across eukaryotes is the human variant TTAGGG. This repeat also holds for the majority of Excavata representatives in which telomeric repetitions have been studied (Fulneckova et al. 2013). In contrast, *Giardia* linear chromosomes end with TAGGG repeats, and an alternative repeat TAAGG has been described (Adam et al. 1991; Le Blancq et al. 1991). In the present study, beyond the mentioned sequence variants, a novel TAAGGG variant was found. Such a finding

suggests that *Giardia* telomeres may consist of variant telomeric repeats synthesized by telomerase. It is tempting to speculate that the short length of telomere moti and periodicity is a sturdier parameter in forming functional telomere chromatin (Fajkus and Trifonov 2001). Organisms possessing telomere lengths of at least several kb have the majority of their telomeric DNA packed in nucleosomes and they can form the T-loop structure (Griffith et al. 1999). Organisms with short telomeres, e.g., yeast or ciliates, form special structures, such as the telosome or G-quadruplex, that may be present at the tips of longer telomeres. Biophysical studies showed that the *Giardia*-type motif is able to form a special structure of a G-quadruplex with intermolecular multi-strand structure (Hu et al. 2009; Tran et al. 2011) in contrast to the *Bombyx*-type motif that probably forms more different structures *in vitro* and the human-type motif that forms an intramolecular G-quadruplex (Tran et al. 2011). Thus, *Giardia* has a boundary-type telomere to balance short/long telomere length and biophysical parameters for the formation of special G-quadruplex structures.

The present study reports novel information concerning the telomeres and telomerase biology of *Giardia*. The presence of an active enzyme that is responsible for telomere repeat addition to chromosome ends together with the presence of telomeric repeats on chromosome ends forming a stable mean length support the existence of conserved mechanisms of chromosome end maintenance in this parasite.

4.2 Paper II.

Metronidazole (MTZ) is used as the drug of choice to treat *Giardia* infections. It is believed that the prodrug is transformed intracellularly into toxic intermediates that interact with cellular components, leading to cell death. The present study aimed to describe the effects of MTZ treatment on DNA and cell cycle progression in MTZ-sensitive and *in vitro*-derived MTZ-resistant cell lines.

Detection of the phosphorylated form of histone H2A in cell nuclei as an indicator of DNA damage together with electrophoresis of genomic DNA, flow cytometry analysis and incubation of cells with other drugs (albendazole or neomycin) demonstrated that DNA damage in MTZ-treated cells is clearly conditioned by the presence of this drug. The concentrations 1.25, 2.5, 5, 10, 20, 50 and 100 μM MTZ

were chosen for experiments, since 7.8 μM MTZ is the concentration required for 50% inhibition of growth (IC_{50}) of the WBc6 clone (Muller et al. 2006). After 24 hours of exposure, the percentage of cells positive for γH2A increased from approximately 15% in cells treated with 1.25 μM MTZ to approximately 80% in cells treated with 5 μM MTZ. In cells treated with 10 μM MTZ or higher, a decrease in γH2A positivity was observed; however, only a fraction of the cells remained attached to the slide and could be examined. In untreated cells, γH2A positivity varied from 1% to approximately 20% depending on the age of the cell culture. When analyzing fragmentation of gDNA isolated from cells incubated with increasing concentrations of the drug (5 to 100 μM) for 24 hours, no gDNA fragmentation was observed in cells exposed up to 20 μM MTZ and highly fragmented DNA was evident in cells treated with 50 and 100 μM MTZ. The reversibility of the effect of MTZ on the phosphorylation of H2A histone in cells was determined by first incubating the cells with medium containing 5 μM MTZ for 24 hours and then replacing the medium and overlaying the cells with fresh drug-free medium for another 24 hours. A decrease in γH2A positivity after 24 hours in fresh medium was observed. Similar experiments were carried out with the MTZ-resistant lines 106-2ID10 and 713-M3 and the corresponding sensitive isolates (the parent isolates) from which the resistant lines had been derived *in vitro*. During the cultivation of MTZ-resistant cells in the presence of the drug (both resistant lines continuously cultured in 5 μM MTZ), approximately 40% of the cells were permanently positive for γH2A. The sensitive isolates responded similarly to MTZ treatment as the susceptible WBc6 isolate.

The flow cytometry analysis and a BrdU labeling assay showed that the sublethal drug concentration affects the replication phase of the cell cycle. Concentrations of 1.25 and 2.5 μM MTZ slightly affected cell population growth but had no obvious effect on the cell cycle. Effects of the drug on cell cycle progression were observed beginning at a concentration of 5 μM MTZ. Treatment with 5 μM MTZ led to an increase of the number of cells in S phase after 12 and 24 hours of incubation. Similar situation was observed for cells incubated with 10 μM MTZ after 12 hours of incubation. After 24 hours, these cells exhibited G2/M DNA content. Cells incubated with lethal drug concentration exhibit unchanged DNA profile, only about 50% of cells were positive for γH2A and lose an ability to attach to a surface after few

hours of incubation. The MTZ-resistant lines cultured in the presence of the drug did not demonstrate any significant differences in cell cycle progression compared with untreated cells.

The present data clearly demonstrate that exposure of *Giardia* cells to MTZ causes phosphorylation of histone H2A. Drug-sensitive cells when incubated with 5 µM MTZ also prolong the replication phase of the cell cycle, as determined by flow cytometry and BrdU incorporation assays. These results suggest that MTZ in concentration near or lower than IC50 affect the replication phase (DNA synthesis or S phase) of the cell cycle. Because the DNA has to be repaired, the replication phase is prolonged and, as a consequence, multiplication slows. These findings, together with the observed reversibility of the effects of MTZ treatment indicate that non-lethal concentrations of this compound induces reversible damage of DNA, moreover it demonstrates that *Giardia* possesses a functional DNA damage response network, that enables recognition and repair of tolerable DNA damage. These results are in agreement with our study demonstrating that in this parasite, H2A phosphorylation occured in response to DNA damage when replication in cells was blocked by the presence of aphidicolin (Hofstetrova et al. 2010). Although the above observation implicates MTZ as a DNA damaging agent, other findings contribute to the view that demonstrates complexity of the cytotoxic effect of MTZ treatment. As shown also by other authors, lethal concentration of MTZ lead to the rapid loss of cell adherence and to cell death in *Giardia* (Muller et al. 2006; Sandhu et al. 2004). In the present study, it was further found that the percent positivity for γH2A in cells incubated with 10 µM MTZ was lower compared to cells treated with the 5 µM MTZ, but the fragmentation of DNA increased with MTZ concentration and time of incubation. Further, only about 50% of cells treated with a lethal MTZ concentration (100 µM) were positive for γH2A after 4.5 hours incubation, when almost all cells were detached, whereas the cell cycle of these cells was not affected. These particular results suggest that activated MTZ affects number of biomolecules with DNA being only one of its targets. The DNA damage may hardly be the cause for the loss of adherence ability that is characteristic for *Giardia* early response to lethal concentration of MTZ. More likely, the interactions between MTZ and biomolecules where activated MTZ is generated may be the critical event. Described formation of covalent adducts between MTZ and proteins of redox

system that accompanied MTZ cytotoxicity to a human parasite *Trichomonas vaginalis* and a fish parasite *Spironucleus vortens* led authors to similar assumptions, that rapid cellular response to high concentrations of nitroimidazoles cannot be conclusively explained by DNA damage response, given the necessity of MTZ intermediates to cross the nuclear membrane and the relatively slow DNA damage response (Leitsch et al. 2009). The same authors introduce redox imbalance as the mode of action of MTZ for a diplomonad *Spironucleus* (Williams et al. 2012), however, in contrast to *Trichomonas* or *Spironucleus*, a different mode of action has been suggested in *Giardia* with elongation factor 1γ as a possible MTZ target (Leitsch et al. 2012). The disrupted redox balance as the overall consequence responsible for cell death upon MTZ treatment has been drown up recently (Lloyd and Williams 2014).

Interestingly, in MTZ-resistant lines incubated in the presence of the drug, about 40% of cells remain permanently positive for γH2A without any effects on the cell cycle progression suggesting that DNA damage caused by MTZ treatment persists in these cells. Accelerated mutagenesis caused by MTZ-induced DNA damage may therefore be an important factor for resistance development and from a practical point of view, a possible contribution of MTZ underdosing to the development of treatment-refractory giardiasis should not be overlooked.

4.3 Paper III.

Life of unicellular eukaryotes depends on the ability to store the hereditary information and on passing this data from a cell to its daughter cells at cell division. This process must be extremely accurate to keep the information almost unchanged for millions of replication and cell divisions. From this point of view the carriage of this genetic information, the DNA, on chromosomes during mitosis and cell division is one of the crucial steps. The packaging of DNA in to mitotic chromosomes and global structure of such chromosomes are notoriously known in model eukaryotes. In *Giardia*, the study of its chromosomes is limited due to the small size of the chromosomes (300 nm to 1.5 μm). In our study we decided to complement molecular and sequencing data on *Giardia* chromosomes with cytological and ultrastructural

information to show if *Giardia* shares the morphological features of DNA compaction process and metaphase chromosomes with other eukaryotes.

Standard cytogenetic chromosome spreading technique (Tumova et al. 2007a) followed by high-resolution field emission scanning electron microscopy (FESEM) following correlative light and electron microscopy (CLEM) was used to visualize chromatin condensation levels and condensed chromosomes in *Giardia*. CLEM enables positioning of chosen mitotic figures on chromosome preparation prior to SEM, which increases the effectiveness of time-consuming metaphase chromosome observation, which is important especially in *Giardia* parasite with limited synchronization methods. High-resolution FESEM uses low voltage and no additional metal coating to investigate chromosome substructures with highest possible resolution (Wanner and Schroeder-Reiter 2008). On *Giardia* chromosomes we observed 10-nm fibrils and 30-nm fibrils similarly as in other eukaryotes. The presence of only up to five chromomeres per chromosome, which are the most efficient structures in condensation process, with the size of 100 nm suggests lower chromatin condensation level in comparison to other eukaryotes where the number of chromomeres is about 350 per chromosome and the size ranges from 200 to 350 nm (Wanner and Formanek 2000). These differences are however apparently due to the small size of *Giardia* chromosomes and fast mitosis phase where there is a pressure on a quick condensation process.

Based on morphological observation we asked if *Giardia* possesses the orthologs of proteins involved in DNA compaction process. The linker histone H1 important for nucleosome joining is missing in *Giardia* (Dawson et al. 2007; Yee et al. 2007) suggesting that an alternative mechanism for DNA compaction may exist in this parasite. The higher-order compaction of DNA is in general provided by condensin comlexes, which contain SMC, kleisin and HEAT domain containing accessory subunits (CAP-D2 and CAP-D3) which together ensure the DNA trapping and compaction dynamics (Hirano 2016). The SMC2 and SMC4 are present in *Giardia* (Malik et al. 2007). We therefore searched for condensin subunits in *Giardia* genome. We found one putative homologs (GL50803_90044) of CAP-D2/CAP-D3 of condensin I/II subunits and two putative kleisin orthologs (GL50803_15239, GL50803_102874) which may act as CAP-H/CAP-H2 in condensin I/II. Theoretically giardial genome contain all crucial

subunits of condensin I or II and it would be of interest to experimentally verify this hypothesis.

Giardia metaphase chromosomes have two sister chromatids closely aligned together and a slight groove is visible between them. Surprisingly, only reduced protein inventory for sister chromatids cohesion including SMC1 and SMC3 proteins are present in *Giardia* with kleisin Rad21/Scc1 missing in this parasite (Eme et al. 2011; Malik et al. 2007). Also the APC complex, important in other eukaryotes for anaphase-metaphase transition is absent in *Giardia* (Gourguechon et al. 2013). All these information together with our findings that the spindle-assembly checkpoint is absent in this parasite (Markova et al. 2016) helps to explain our observation of lagging chromatids between already formed telophase nuclei and corroborates our hypothesis of the mechanism for aneuploidy generation in *Giardia* (Tumova et al. 2016).

Although we observed neither primary, nor secondary constriction on metaphase chromosomes in our study, we assume that giardial chomosomes are monocentric. The localization of centromeric histone H3 in *Giardia* is to approximately 10 foci in interphase nuclei and to one spot at each spindle pole in anaphase nuclei (Dawson et al. 2007) and the study by Tumova et al. (2007a) showed a presence of approximately 10 kinetochoral microtubules penetrating closed nuclear membrane at the spindle pole in *Giardia,* both corresponding to the number of chromosomes. The localization of telomeres on *Giardia* metaphase chromosomes was shown to be conservative. The telomeric repeats (TAGGG)n (Adam et al. 1991) were observed on all chromosome ends of a single metaphase chromosome using fluorescence in situ hybridization (FISH). Detection of relatively short telomeres of predicted size up to 1 kb was carried out using slightly modified protocol by Zubacova et al. (2011) employing FISH signal amplification by deposing an extra tetramethylrhodamine substrate in the area of the antidigoxigenin antibody. Finding of telomeres on metaphase chromosome ends is in agreement with previous study where telomeres were detected on both chromosome ends by southern blot hybridization (Le Blancq and Adam 1998).

Study of chromosomes of this parasite belonging to distant group of eukaryotes and characteristic with its small chromosomes, reduced protein inventory employed in condensation or cohesion of chromosomes as well as short telomeres led us to adjust

methods for chromosome observation. Interestingly, the higher structure chromosome organization seems to be conservative in this parasite despite the absence of some canonical proteins. In contrary, an increasing number of evidence suggests that metaphase chromosome segregation to daughter nuclei is processed by an unknown mechanism unique to *Giardia*. This process progresses independently on anaphase-promoting complex and spindle mitotic checkpoint, lagging chromatids are frequently observed in mitotic cells as well as tolerance of aneuploidy. All these features make *Giardia* an interesting model for future work. I may among others reveal a diversity of mechanisms which could use our unicellular ancestors to store and pass genetic information from one generation to another or alternatively which could be an adaptation of *Giardia* parasite to its specific lifestyle persisting to present times.

4.4 Paper IV.

Synchronization methods are needed for studying the cell cycle regulated processes. Our study is examination of side effects of synchronization agent, aphidicolin, on DNA synthesis, DNA damage and characteristics of the cell cycle progression of *Giardia* unsynchronized population. Using flow cytometry analysis and immunofluorescence labeling it was possible to determine cell cycle progression characteristics, as well as their reversibility upon aphidicolin treatment.

Aphidicolin is DNA polymerase alpha inhibitor which arrests susceptible eukaryotic cells at G1/S border. Both approaches used in our study, the BrdU incorporation and flow cytometry, confirmed that this drug arrests *Giardia* trophozoites at the beginning of replication similarly to other susceptible eukaryotic cells. Majority of *Giardia* cells share G1/S DNA content (Poxleitner et al. 2008; Reiner et al. 2008); however, a small fraction of cells (15%) stays in G2. Prolonged stalling of replication forks as a result of aphidicolin treatment results also in DNA damage and DNA damage checkpoint activation in human cells (Liu et al. 2003). A phosphorylated variant of H2AX histone plays central role in DNA damage signaling in eukaryotic cells sharing the SQ motif on C-tail (Georgoulis et al. 2017; Zhou and Elledge 2000) and we found that this posttranslational modification of histone H2A is induced in *Giardia* exposed to aphidicolin. We also demonstrated that this phosphorylation occurs naturally in *Giardia* cells, especially in stationary phase. As far as we know, this is the

first evidence of detection of H2A histone phosphorylation in *Giardia*. Together with observed cell cycle arrest upon aphidicolin treatment we believe that this is a strong indication for existence of functional DNA damage checkpoint in this parasite. Our inhibitory experiments (unpublished data) also indicate an existence of apical kinase from the family of phosphatidyl-inositol-3-kinase related kinases which is responsible for H2A histone phosphorylation.

Whereas DNA replication is stopped in aphidicolin-treated trophozoites, cell growth continues and trophozoites with G1 DNA content resemble cells in the G2 phase or trophozoites in ageing cultures. We described gradually increasing cell size that becomes even bigger then untreated G2 cells. We also found out significantly higher protein content in treated cells in comparison to untreated cells and a presence of median body in treated cells. Median body is a microtubular structure found in G2 cells which has a role in adhesive disc assembly (Tumova et al. 2007b). All these results show that aphidicolin causes dissociation of the nuclear and cytoplasmic cycles, a situation that has also been described for other inhibitors in mammalian cell lines (Campisi et al. 1996; Yeo et al. 2000).

In summary, inhibitors used for synchronizing cells usually target only a particular reaction or pathway in the complex cell cycle. It is therefore rather problematic to call such a population of cells synchronized, because these cells appear to be aligned solely according to a single characteristic, in this case to the DNA content. Thus, if aphidicolin is used for synchronization of *Giardia* trophozoites, this fact must be accounted for, and treatment with aphidicolin must be minimal, both concentration and time-wise.

5 CONCLUSIONS

I. The length of *Giardia* telomeres ranges from 0.5 to 2.5 kb and existence of an active telomerase enzyme synthesizing telomeric repeats in in this parasite was proved.

II. Incubation of cells with metronidazole causes phosphorylation of histone H2A in cell nuclei and fragmentation of DNA. Sublethal concentration affects the replication phase of the cell cycle and lethal concentration lead to rapid loose of adherence ability and cell death. In resistant lines about 40% of cells remain permanently positive for H2A in nuclei without any effects on the cell cycle progression. DNA damage which persists in these cells may contribute to accelerated mutagenesis and consequently to the development of resistance.

III. The overall morphology, condensation stages, and mitotic segregation of giardial chromosomes is similar to other model eukaryotes. Differently, the anaphase poleward segregation of sister chromatids are atypical and tend to generate lagging chromatids between daughter nuclei which could explain existence of aneuploidy in this parasite. On molecular level, we identified two putative members of the kleisin family thought to be responsible for condensin ring establishment.

IV. Treatment with aphidicolin leads to G1/S phase arrest and to phosphorylation of H2A histone. Thus, if aphidicolin is used for synchronization of *Giardia* trophozoites, DNA damage response activation must be accounted for, and treatment with aphidicolin should be minimal.

6 DECLARATION OF THE STUDENT PARTICIPATION ON PUBLICATIONS

I declare that Magdalena Uzlíková participated on preparation of all papers representing a basis of her PhD Thesis as follows: paper I. - 80% contribution (designed the experiments, performed telomere labeling, TRF analysis, Southern blotting and TRAP assay, wrote the manuscript); paper II. – 85% contribution (performed FACS, immunofluorescence microscopy, BrdU labeling assay, wrote the manuscript); paper III. – 30% contribution (performed telomere labeling and bioinformatics analyses, participated in writing the manuscript); paper IV. – 20% contribution (performed Western blotting and FACS).

Eva Nohýnková, PhD

PhD Thesis supervisor

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8 APPENDIX: PUBLICATIONS