CHARLES UNIVERSITY

FIRST FACULTY OF MEDICINE

Institute of Immunology and Microbiology

Ph.D. study program: Parasitology

Udržování integrity chromosomů na modelu *Giardia intestinalis*

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

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Summary of the Ph.D. Thesis

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1 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.

2 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. Sub-lethal 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.

3 Introduction

Infection caused by *Giardia intestinalis* is the most common protozoan infection of the human intestine with worldwide distribution. The life cycle is direct and infection of host is provided by fecal-oral route (Adam 2001). Clinical manifestation of the infection includes watery diarrhea, epigastric pain, nausea and vomiting. Chronic infections may lead to malabsorbtion and weight loss and consequently to growth slowdown and cognitive impairment in children in developing countries (Certad et al. 2017). Beside its medical importance, it is evolutionary distant protist used as an attractive model organism for studies of minimal requirements for cellular processes (Morrison et al. 2007). In present study we focused on mechanisms which enable *Giardia*, similarly to other eukaryotes, safely transmit genetic material from mother to daughter cells. These mechanisms are complex and include number of processes having a role on different levels of organization of the cell.

Solving the end replication problem and consequent telomere shortening is one of such processes. In *Giardia*, sequence of telomeric pentanucleotide TAGGG is known for long time (Adam et al. 1991), however telomeric repeats were only visualized on chromosomes separated by PFGE and their length was predicted not to exceed 1 kb (Le Blancq et al. 1991). Observation of telomeres on condensed chromosome has never been done since the first visualization of metaphase chromosomes by classic cytogenetic techniques was carried out by Tumova et al. (2007b). The enzyme telomerase, which is responsible for prolongation of telomeric repeats on 3´end of DNA, consists of a reverse transcriptase part (TERT) and an RNA template. *Giardia* encodes the telomerase catalytic subunit TERT, which has been shown to be sequence-divergent (Malik et al. 2000). These differences led to speculations about alternative telomere maintenance by retrotransposons in this parasite (Arkhipova and Morrison 2001; Mason et al. 2016).

Recognition of damaged DNA is a crucial event in each cell life. If a double strand break occurs on DNA, majority of eukaryotes mark this DNA lesion with phosphorylated form of H2AX histone variant. In *Giardia*, this phosphorylation was firstly observed in cells incubated with synchronization agent, aphidicolin (Hofstetrova et al. 2010), lately also when cells were irradiated with UV-C light (Einarsson et al. 2015). Also metronidazole, the most commonly used drug for treatment of giardiasis, has been suggested to act as a DNA damaging agent in this parasite. Generally, metronidazole forms toxic intermediates within cells of anaerobes and microaerophiles that are presumed to cause DNA damage (Edwards 1993). Also recent studies demonstrated that metronidazole treatment of *Giardia* cells induces DNA damage *in vivo*, as shown in experiments where incubation of cells with different concentration of this drug led to positive signals on TUNEL assay (Bagchi et al. 2012) or to fragmentation of DNA visualized on agarose gel (Ghosh et al. 2009). Nevertheless, the role of damaged DNA in the overall cytotoxic effect in reaction to metronidazole in *Giardia* has not been resolved. For *Trichomonas vaginalis* the authors express doubts about DNA damage being the main cytotoxic factor. They come with an idea that DNA damage may be secondary to the drug´s direct attack on specific protein(s) (Leitsch et al. 2009).

The process of condensation of DNA into metaphase chromosomes is another essential process for transmission of genetic material from mother to daughter cell. In *Giardia*, metaphase chromosomes were firstly observed by Tumova et al. (2007a) using

cytogenetic methods. This binucleated parasite has haploid set of five chromosomes (Morrison et al. 2007) and the current consensus assumes that each nucleus is diploid and the whole cell is tetraploid (Bernander et al. 2001). The total chromosome number in the karyotype and the ploidy of *Giardia* however remain controversial because different chromosome sets has been described (Tumova et al. 2007a). On molecular level, *Giardia* has all four nucleosome core histones (H2A, H2B, H3, H4) and lacks the linker histone H1 gene (Morrison et al. 2007; Wu et al. 2000; Yee et al. 2007). Proteins involved in chromosome condensation and sister chromatid cohesion are either absent or have diverged markedly (Eme et al. 2011; Malik et al. 2007). Detailed structural organization of *Giardia* chromosome is missing due to the fact that *Giardia* chromosomes are very small and therefore difficult to study using standard techniques.

4 Aims of the thesis

The main objectives were:

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

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

III. To describe structural organization of *Giardia* chromosomes

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

5 Summary and conclusions

This thesis summarizes results of four publications in peer-reviewed journals. These are focused on telomere and telomerase biology in *Giardia* (I.), the effect of the most commonly used drug for treatment, metronidazole, on the cell cycle and DNA of *Giardia* cells (II.), structural organization of *Giardia* chromosomes (III.) and influence of synchronization agent aphidicolin on *Giardia* cells (IV.).

I. In the first study we presented characteristics of telomeres and telomerase. Using fluorescence in situ hybridization, we localized telomeres during all stages of the trophozoite cell cycle and demonstrated differences in the observed number of telomeric foci, indicating telomere clustering. We determined the length of *Giardia* telomeres using terminal restriction fragment analysis and showed that the length ranges from 0.5 to 2.5kb. We used BAL-31 digestion experiment and did not find any long interstitial telomeric sequences in the genome. Furthermore, despite the absence of the specific T motif in the telomerase catalytic subunit, we proved presence of an active telomerase enzyme synthesizing telomeric repeats using TRAP assay. Telomerase localization in nuclei was determined by the expression of

recombinant GiTERT. Except for the *Giardia*-type TAGGG telomeric repeat, we also observed other repeat variants, TAAGG and TAAGGG to be synthesized in vitro. Our partial findings support the view that the chromosomal termini in *Giardia* are maintained in a conservative manner that is common to other eukaryotes.

II. The second article describes the effects of metronidazole (MTZ) treatment on DNA and cell cycle progression in MTZ-sensitive and *in vitro* -derived MTZ-resistant cell lines. We detected phosphorylated form of histone H2A in treated cells. We performed electrophoresis of genomic DNA isolated from cells treated with increasing concentration of MTZ and observed DNA fragmentation. Both these results prove MTZ to be DNA damaging agent. The flow cytometry analysis and a bromo-deoxy-uridine (BrdU) labeling assay showed that the sub-lethal drug concentration affects the replication phase of the cell cycle. In contrary, cells incubated with lethal drug concentration exhibited unchanged DNA profile, only about 50% of cells were positive for γH2A and these cells lost ability to attach to the surface after few hours of incubation. These results suggest that the early reaction of cells to lethal concentration of MTZ is not primarily initiated by the reaction to DNA damage but rather by the immediate interaction of MTZ with biomolecules where activated MTZ is generated. 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. These results indicate that DNA damage caused by MTZ treatment persists in these cells and accelerated mutagenesis caused by MTZ-induced DNA damage may therefore be a new factor contributing to the development of natural resistance.

III. The third paper describes in great detail the overall morphology, condensation stages, and mitotic segregation of *Giardia* chromosomes. For this purpose we used light microscopy, high-resolution field emission scanning electron microscopy, and in situ hybridization. *Giardia* chromosomes lack primary and secondary constrictions, thus preventing their classification based on the position of the centromere. The anaphase poleward segregation of sister chromatids is atypical in orientation and tends 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. We also examined effects of the DNA polymerase inhibitor 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. This phosphorylation is involved in a signaling pathway triggered as a reaction to double stranded DNA breaks. 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 List of publications (including abstracts)

I. Uzlíková M, Fulnečková J, Weisz F, Sýkorová E, Nohýnková E, Tůmová P. (2017). Characterization of telomeres and telomerase from the single-celled eukaryote *Giardia intestinalis*. Mol Biochem Parasitol. 211:31-38. doi: 10.1016/j.molbiopara.2016.09.003.

Impact Factor: 2.158 (2018)

Abstract. The ends of linear chromosomes, telomeres, are most commonly maintained by the enzyme telomerase. Our study presents the characteristics of telomeres and telomerase from the single-celled parasitic eukaryote *Giardia intestinalis*. Using fluorescence in situ hybridization, we localized telomeres during all stages of the trophozoite cell cycle and demonstrated differences in the observed number of telomeric foci, indicating telomere clustering. The length of *Giardia* telomeres was determined in different cell lines derived from WB clinical isolate using terminal restriction fragment analysis and ranged from 0.5 to 2.5kb; moreover, a BAL-31 digestion experiment did not reveal any long interstitial telomeric sequences in the genome. Despite the absence of the specific T motif in the telomerase catalytic subunit, the presence of an active telomerase enzyme synthesizing telomeric repeats in *Giardia* was proved by a Telomere repeat amplification protocol assay, and its localization in nuclei was determined by the expression of recombinant GiTERT. Except for the *Giardia*-type TAGGG telomeric repeat, *Giardia* telomerase was proved to synthesize in vitro also other repeat variants, TAAGG and TAAGGG. In summary, despite its unusual characteristics, including a structurally divergent but active telomerase, unique terminal sequences and relatively short telomeres, the present data support the view that the chromosomal termini in *Giardia* are maintained in a conservative manner that is common to other eukaryotes.

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

Impact Factor: 2.158 (2018)

Abstract. 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 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 flow cytometry analysis and a bromo-deoxy-uridine (BrdU) labeling assay showed that the sub-lethal drug concentration

affects the replication phase of the cell cycle. Cells incubated with lethal drug concentration exhibit unchanged DNA profile, only about 50% of cells are positive for γH2A and lose an ability to attach to a surface after few hours of incubation. It is likely that the early reaction of cells to lethal concentration of MTZ is not primarily initiated by the reaction to DNA damage but rather by the immediate interaction of MTZ with biomolecules where activated MTZ is generated. 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 a new factor contributing to the development of natural resistance.

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

Impact Factor: 3.530 (2018)

Abstract. During mitotic prophase, chromosomes of the pathogenic unicellular eukaryote *Giardia intestinalis* condense in each of the cell's two nuclei. In this study, *Giardia* chromosomes were investigated using light microscopy, high-resolution field emission scanning electron microscopy, and in situ hybridization. For the first time, we describe the overall morphology, condensation stages, and mitotic segregation of these chromosomes. Despite the absence of several genes involved in the cohesion and condensation pathways in the *Giardia* genome, we observed chromatin organization similar to those found in eukaryotes, i.e., 10-nm nucleosomal fibrils, 30-nm fibrils coiled to chromomeres or in parallel arrangements, and closely aligned sister chromatids. DNA molecules of *Giardia* terminate with telomeric repeats that we visualized on each of the four chromatid endings of metaphase chromosomes. *Giardia* chromosomes lack primary and secondary constrictions, thus preventing their classification based on the position of the centromere. The anaphase poleward segregation of sister chromatids is atypical in orientation and tends to generate lagging chromatids between daughter nuclei. In the *Giardia* genome database, we identified two putative members of the kleisin family thought to be responsible for condensin ring establishment. Thus far, *Giardia* chromosomes (300 nm to 1.5 µm) are the smallest chromosomes that were analyzed at the ultrastructural level. This study complements the existing molecular and sequencing data on *Giardia* chromosomes with cytological and ultrastructural information.

IV. Hofštetrová K, **Uzlíková M**, Tůmová P, Troell K, Svärd SG, Nohýnková E. (2010). *Giardia intestinalis*: aphidicolin influence on the trophozoite cell cycle. Exp Parasitol. 124(2):159-66. doi: 10.1016/j.exppara.2009.09.004.

Impact Factor: 1.719 (2018)

Abstract. This study is a thorough examination of the effects of the DNA polymerase inhibitor aphidicolin on the nuclear cycle and cell cycle progression characteristics, as well as their reversibility, in *Giardia intestinalis*. *Giardia* trophozoites are arrested in the G1/S-junction after aphidicolin treatment according to their DNA content. However, cell growth continues and trophozoites arrested with aphidicolin resemble cells in the G2 phase and trophozoites in ageing cultures. Extensive treatment with aphidicolin causes side effects and we detected positive signals for phosphorylated histone H2A, which, in mammalian cells, is involved in a signaling pathway triggered as a reaction to double stranded DNA breaks. These results suggest that aphidicolin causes dissociation of the nuclear and cytoplasmic cycles, a phenomenon that has also been described for other inhibitors in mammalian cell lines. Thus, if aphidicolin is used for synchronization of *Giardia* trophozoites, this fact must be accounted for, and treatment with aphidicolin must be minimal.

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- Support of the Charles University in Prague, grant No. SVV 260 152, SVV-260 026 and PRVOUK P24

8 Curriculum vitae

Education

2016 – present	Ph.D. study in Parasitology The First Faculty of Medicine, Institute of Immunology and Microbiology, Charles University, Prague Ph.D. Thesis: Maintenance of chromosomes integrity in *Giardia intestinalis* as a model organism
2007 - 2016	Ph.D. study in Parasitology Department of Infectious and Tropical Diseases, The First Faculty of Medicine, Charles University and Na Bulovce Hospital, Prague Ph.D. Thesis: Maintenance of chromosomes integrity in *Giardia intestinalis* as a model organism
2002 – 2007	MSc. in Parasitology Department of Parasitology, Faculty of Science, Charles University, Prague Supervisor: Prof. RNDr. Jaroslav Flegr, CSc. Diploma Thesis: Fylogenetická analýza rodu *Blastocystis*

Stays abroad

2011 Research stay at University of California Berkeley, Department of Molecular and Cell Biology, 345 Life Sciences Addition, UCB, Berkeley, California, 94720-3200, USA
Laboratory of Professor Zacheus Cande
Duration: 2 months

2009 Research stay at Uppsala University, Biomedicinska Centrum BMC, Department of Cell and Molecular Biology, Husarg. 3, 751 24, Uppsala, Sweden
Laboratory of Professor Staffan G. Svärd
Duration: 3 months

Conference presentations

2012 oral presentation "Efekt metronidazolu na DNA a buněčný cyklus giardií"
42. Jírovcovy protozoologické dny, 7. - 11.5. 2012, Kletečná u Humpolce, Czech Republic

2010 oral presentation "Hrátky s aktivací G1 checkpointu buněčného cyklu u *Giardia intestinalis*"
40. Jírovcovy protozoologické dny, 3. - 7.5. 2010, Ledeč nad Sázavou, Kouty, Czech Republic

2009 poster „DNA damage response in *Giardia* - the effects of UV-C radiation on the cell cycle progression ”
III International Giardia and Cryptosporidium Conference, 11. - 16.10., Orvieto, Italy

2008 poster "Phosphorylation of H2A histone in *Giardia intestinalis* cells-does *Giardia* share the same DNA damage response with other Eukaryotes?"
Xth European Multicolloquium of Parasitology, 24. - 29.8., Paris, France

Work experience

2019 – present Analyst, Synlab Czech s.r.o., Prague
responsibilities: laboratory diagnostics of parasites, molecular diagnostics of pathogens

2016 – 2018 maternity leave

2014 – 2015 Inspector for tissues and cells, State Institute for Drug Control, Prague
responsibilities: inspections at healthcare facilities working with human tissues and cells

2012 – 2013 Quality Inspector, Institute of Applied Biotechnologies, Prague
responsibilities: quality control of CE IVD diagnostics kits

9 Full list of publications

Uzlíková M, Fulnečková J, Weisz F, Sýkorová E, Nohýnková E, Tůmová P. (2017). Characterization of telomeres and telomerase from the single-celled eukaryote Giardia intestinalis. Mol Biochem Parasitol. 211:31-38. doi: 10.1016/j.molbiopara.2016.09.003.

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