Characterization of *Plasmodium falciparum* CTP:phosphocholine cytidylyltransferase, a prospective antimalarial drug target

Short Summary of PhD Thesis

Lívia Marton PharmD

Genome Metabolism and Repair Research Group
Institute of Enzymology
Research Centre for Natural Sciences
Hungarian Academy of Sciences

Doctoral School of Multidisciplinary Medical Science
Faculty of Medicine
University of Szeged

Supervisor: Beáta Vértessy PhD, DSc

Szeged

2017

LIST OF PUBLICATIONS

List of full papers directly related to the subject of the thesis:

I. Nagy GN, Marton L, Contet A, Ozohanics O, Ardelean L-M, Révész Á, *et al.* Composite Aromatic Boxes for Enzymatic Transformations of Quaternary Ammonium Substrates. Angew Chemie Int Ed. 2014;53: 13471–13476. doi:10.1002/anie.201408246b **IF: 11.261**

II. Marton L, Nagy GN, Ozohanics O, Lábas A, Krámos B, Oláh J, *et al.* Molecular Mechanism for the Thermo-Sensitive Phenotype of CHO-MT58 Cell Line Harbouring a Mutant CTP:Phosphocholine Cytidylyltransferase. PLoS One. 2015;10: e0129632. doi:10.1371/journal.pone.0129632 **IF: 3.057**

Cumulative impact factor of papers directly related to the thesis: 14.318

List of full papers indirectly related to the subject of the thesis:

III. Nagy GN, Marton L, Krámos B, Oláh J, Révész Á, Vékey K, *et al.* Evolutionary and mechanistic insights into substrate and product accommodation of CTP:phosphocholine cytidylyltransferase from Plasmodium falciparum. FEBS J. 2013;280: 3132–48. doi:10.1111/febs.12282 **IF: 3.986**

Cumulative impact factor of papers indirectly related to the thesis: 3.986

1. INTRODUCTION

Malaria is still one of the most serious vector-borne infectious diseases threatening approximately 2.75 billion people worldwide[1]. Among the parasite species infecting humans *Plasmodium falciparum* is the deadliest causing the majority of mortality related to malaria. The rapidly emerging drug resistance of parasites[2,3] urges the development of antimalarials with an alternative mechanism of action. Plasmodium parasites have a complex life cycle involving two hosts (mosquito stage in the vector Anopheles mosquitos, and liver and blood stage in the human host). Plasmodium parasites upon invasion of red blood cells produce large amount of membranes consisting mainly of phospholipids and within that of phosphatidylcholine (PC) and phosphatidylethanolamine. In Plasmodium the most abundant way of PC synthesis is the Kennedy pathway[4] consisting of three consecutive steps catalyzed by the enzymes choline kinase, CTP:phosphocholine cytidylyltransferase (CCT) and choline/ ethanolamine phosphotransferase. *In vitro* and *in vivo* antimalarial effects of bis-quaterner ammonium choline analogue compounds appointed the parasite Kennedy pathway as a potential target[5]. Thus *Plasmodium falciparum* CCT (*Pf*CCT) as the rate limiting enzyme of the pathway may be considered as a potential antimalarial drug target, which necessitates the characterization of the enzyme structure and function on molecular and cellular levels.

CCT (EC number: 2.7.7.15) is a key enzyme of the *de novo* PC biosynthesis catalyzing the enzymatic reaction of choline-phosphate (ChoP) and cytidine 5'-triphosphate (CTP) conversion to cytidine 5'-diphosphocholine (CDPCho) metabolic intermediate and pyrophosphate (PP_i). Most CCT enzymes form dimers in solution stabilized by the

[1]WHO. *World Malaria Report 2016*.
[2]Enjalbal, C. *et al*. *Anal. Chem.* **76,** 4515–21 (2004).
[3]Klein, E. Y. *Int. J. Antimicrob. Agents* **41,** 311–7 (2013).
[4]Sen, P., Vial, H. J. & Radulescu, O. *BMC Syst. Biol.* **7,** 123 (2013).
[5]Wengelnik, K. *et al*. *Science* **295,** 1311–4 (2002).

conserved RYVD dimerization motif[6]. The catalytic domain of the enzyme adopts an α/β Rossmann fold followed by regulatory sequences including a membrane binding segment (M domain) and a phosphorylation region (P domain). A reversible membrane binding regulatory mechanism of the enzyme fine tunes the transition between the inactive cytosolic and the active membrane bound forms. This regulatory mechanism of CCT enables a feedback regulation of PC producing capacity through the Kennedy pathway.

De novo PC biosynthesis is essential for Plasmodium parasites shown by a gene disruption experiment in *P. berghei*[7]. Plasmodium genomes contain only a single annotated *cct* gene copy encoding duplicated CM segments, consisting of a cytidylyltransferase catalytic domain (C1/C2 domain) and a membrane binding domain (M1/M2 domain). The duplicated CM segments are linked with a Plasmodium specific segment comprising ca. 300 residues which together add up to a total length of 896 residues in case of *Pf*CCT. Beyond the fact of gene duplication there are further structural differences between the Plasmodium and mammalian CCTs which may make *Pf*CCT a suitable antimalarial drug target.

Due to *cct* gene duplication in Plasmodia, the respective full length enzyme is challenging to express and study *in vitro*. However cellular studies may be facilitated in the CHO-MT58 cell model, a Chinese Hamster Ovarian (CHO) cell line generated by chemical mutagenesis. It was described as a tool for the functional investigation of CCT, as the endogenous *cct* gene contains a point mutation resulting in a thermosensitive phenotype[8]. While at 37°C functional protein is formed albeit in smaller amount, at 40°C an accelerated rate of CCT degradation is observed causing drastically decreased PC levels, morphological changes and at last apoptosis. Although this cellular model has been extensively used to study the relation of CCT deficiency and apoptosis, still there are no direct *in vitro* enzyme studies to describe the molecular mechanism

[6]Lee, J., Johnson, J., Ding, Z., Paetzel, M. & Cornell, R. B. *J. Biol. Chem.* **284,** 33535–48 (2009).
[7]Déchamps, S., Shastri, S., Wengelnik, K. & Vial, H. J. Int. J. Parasitol. 40, 1347–65 (2010).
[8]Sweitzer, T. D. & Kent, C. *Arch. Biochem. Biophys.* **311,** 107–16 (1994).

causing this thermos-sensitive phenotype. As CHO-MT58 can be rescued either via exogenous PC supply, reverting the temperature to 37°C or heterologous CCT expression, this cell line has the potential for functional characterization of CCT orthologues in a cellular environment with conditional exclusion of the effect of the endogenous CCT.

2. AIMS

In the present PhD thesis I aimed to characterize the potent antimalarial drug target *Plasmodium falciparum* CTP:phosphocholine cytidylyltransferase *in vitro* and also in cellular environment. Accordingly, I focused to resolve the following questions:

1. What is the role of residues constituting the choline binding site of PfCCT in ligand binding and catalysis?
2. What is the exact molecular mechanism underlying the thermal instability of CCT from the thermosensitive CHO-MT58 cell line?
3. Can the heterologous expression of PfCCT rescue the mammalian CHO-MT58 cell line at the non-permissive temperature?
4. How can a well applicable test system be established for studying the structural differences of PfCCT and mammalian orthologues in a cellular environment?

3. MATERIALS AND METHODS

The *Pf*CCT$_{(528-795)}$ constructs and mutants used for *in vitro* studies were obtained using the previously described *Pf*CCT$_{(528-795)}$ (pET15b) catalytic domain construct lacking the lysine-rich Plasmodium specific loop (720-737). For site-directed mutagenesis the QuikChange method was applied. Primer synthesis and verification of the mutagenesis was performed by Eurofins MWG GmbH. For cellular experiments constructs containing the full length *Pf*CCT were obtained using a resynthesized cDNA with engineered restriction sites in the mammalian expression vector pIRES-puro-EGFP.

The His-tagged *Pf*CCT$_{(528-795)}$ catalytic domain construct used as a wild type (WT) reference in *in vitro* studies and its mutants were expressed using the BL21 (DE3) Rosetta *E. coli* expression system and were purified by Ni-NTA affinity chromatography in 20 mM HEPES, pH 7.5 buffer, containing 100 mM NaCl. Samples for MS analysis were further purified by size-exclusion chromatography (gel filtration) using a GE Healthcare ÄKTA system with a Superose12 column.

Steady-state activity measurements were performed using a continuous coupled pyrophosphatase enzyme assay, which employs pyrophosphatase and purine nucleoside phosphorylase auxiliary enzymes and 7-methyl-6-thioguanosine substrate for colorimetric phosphate detection at 360 nm. The reaction was performed at 20°C in a Specord 200 spectrophotometer. In the heat inactivation assay, protein samples were incubated for 15 min at various temperatures (10-60°C) followed by the immediate use in enzyme activity assay at 20°C. Kinetic data were fitted with Michaelis–Menten and competitive substrate inhibition equations using OriginPro 8.6.

Isothermal titration calorimetric (ITC) measurements were performed on a MicroCal-ITC 200 calorimeter (Malvern) at 20°C. In the mass spectrometric study of protein complexes, a commercial Waters QTOF Premier instrument equipped with electrospray ionization source was used in the positive ion mode. Mass spectra were obtained under native conditions.

The homodimer homology models of *Pf*CCT$_{(528-795)}$ were constructed based on the catalytic domain structure of *Rattus norvegicus* CCT (*Rn*CCT) (PDB: 4MVC)[9] using the MODELLER 9.14 software. Model data are made available in the Protein Model Database (PMDB) under the accession numbers PM0079950 (*Pf*CCT$_{(528-795)}$ WT) and PM0079951 (*Pf*CCT$_{(528-795)}$ R681H). Molecular dynamics (MD) simulations were carried out for both enzyme variant models using the same computational protocol, namely CHARMM program and CHARMM27 force field was applied using the self-consistent GBSW implicit solvent model.

The CHO-K1 and CHO-MT58 cell lines were maintained in F-12 medium supplemented with 10% FBS and 1% Penicillin-Streptomycin at 37°C or 40°C in a humidified 5% CO_2 atmosphere. In case of microscopic experiments the incubation temperature of transiently transfected CHO-K1 and CHO-MT58 cells grown in 6-well plates was shifted to 40°C post-transfection. After 10 days the transfected and non-transfected cells serving as controls were inspected by fluorescence microscopy. In case of fluorescence activated cell sorting (FACS) analysis the transiently transfected CHO-K1 and CHO-MT58 cells were collected 72 hours post-transfection. The GFP positivity (%) was detected by FACS Attune® Acoustic Focusing Cytometer, Blue/Violet. Intact cells were gated based on the forward scatter (FSC) and side scatter (SSC) parameters while dead cells were excluded based on propidium iodide (PI) positivity.

[9]Lee, J., Taneva, S. G., Holland, B. W., Tieleman, D. P. & Cornell, R. B. *J. Biol. Chem.* **289,** 1742–55 (2014).

4. RESULTS

4.1. *In vitro* analysis of the different amino acid residues building up the *Pf*CCT choline binding site

Rational drug design ultimately requires an in-depth knowledge on the architecture and interactions of the active site of the potential antimalarial target *Pf*CCT. Analysis of *Pf*CCT catalytic domain homology model structure showed that the choline binding subsite is a partially hydrophobic pocket that accommodates the quaternary ammonium moiety of the substrate CDPCho. The strictly conserved residues include D623 and Y741 that provide electrostatic interactions; and Y714 and W692, as cation-π interactors. To investigate the role of these residues in catalysis and in the structural integrity of the choline subsite, a series of point mutants were designed in the *Pf*CCT$_{(528-795)}$ catalytic domain construct by modulating the character of charged (D623N, Y741F) and aromatic (W692F, W692Y and Y714F) residues or fully abolishing the aromatic character (W692A). Indirect effects of the designed residue alterations on the quaternary enzyme structure of the different protein constructs was studied by electrospray ionization mass spectrometry and verified that the mutants oligomerization state did not differ significantly from that of the wild type. Results of enzyme kinetic investigations indicate that the substitution of charged to non-charged residues yielded serious perturbation effects on catalytic efficiency, which is caused mainly by the altered choline coordination as presented by $K_{M,ChoP}$ values (Table 1).

Table 1. Kinetic parameters of *Pf*CCT$_{(528-795)}$ and its point mutants perturbing either cation-π or charged interactions. ND: not determined due to low enzymatic activity.

Enzyme	k_{cat} s^{-1}	$K_{M,CTP}$ µM	$K_{M,ChoP}$ mM	rel. $k_{cat}/K_{M,CTP}$	rel. $k_{cat}/K_{M,ChoP}$
WT	1.45 ± 0.05	168 ± 17	1.8 ± 1.1	1	1
Y714F	0.21 ± 0.01	575 ± 60	10.2 ±1.1	0.042	0.026
W692F	0.13 ± 0.03	890 ± 380	7.5 ± 2.4	0.018	0.022
W692Y	0.03 ± 0.003	191 ± 64	1.3 ± 0.2	0.017	0.029
D623N	0.006 ± 0.001	457± 191	13.1 ±3.1	0.0015	0.00057
Y741F	0.019 ± 0.002	786 ±175	8.5 ± 2.0	0.0028	0.0028
W692A	$6 \cdot 10^{-4}$	ND	ND	ND	ND

In contrast, when the aromatic character of residues involved in cation-π interactions was partially conserved, i.e. only the electrostatics, ring size and quadrupole moment of the aromatic ring was modulated, the mutation was found to be somewhat compatible with catalysis. Based on the kinetic analysis of the mutants the charged or cation-π type interaction is distinguishable and the charged interactors have a key role in the binding of the choline moiety (Figure 1). To assess the full contribution of the tryptophan residue present at position 692 facing the quaternary ammonium moiety of CDPCho and also stabilizing the active site by forming interactions with helix αB and loop L5, alanine mutagenesis was used as an alternative investigation approach. Elimination of the aromatic character of W692 disrupted the catalytic function.

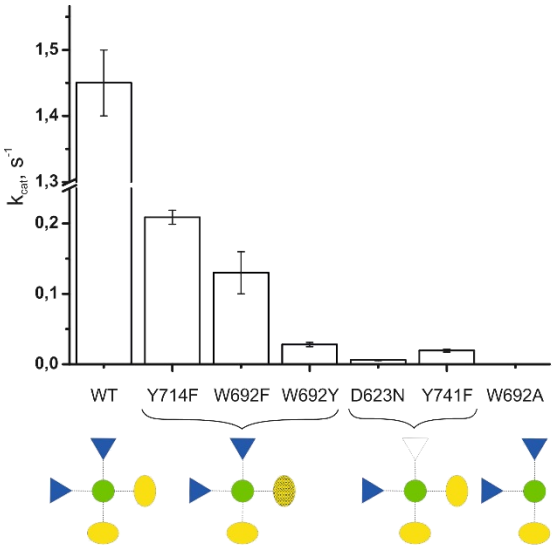

Figure 1. Consequences of *Pf*CCT mutations on enzyme catalytic turnover (k_{cat}). Colored schematic representation shows the engineered modification of residues providing cation-π interactions (yellow ellipse) and charged interactions (blue triangle) to the quaternary ammonium ligand moiety (green circle).

4.2. Molecular mechanism for the thermosensitive phenotype of CHO-MT58 cell line harboring a mutant CCT

The inducible CCT knock-down cell line CHO-MT58 contains a single point mutation at amino acid position 140 (R140H) causing the thermosensitive phenotype. As this amino acid is part of the ^{140}RYVD^{143} motif, which was shown to have prime importance in dimer stabilization in *Rn*CCT[10], conservation pattern of the motif was analyzed by comparing two hundred CCT sequences from different evolutionary clusters. The previously proposed high degree of conservation was verified; the first amino acid of the motif has a conserved basic character (R/K), while the second position is featured with aromatic residues (Y/W) with RYVD being the most frequently occurring combination. To visualize inter-chain interaction of formed *Pf*CCT$_{(528\text{-}795)}$ dimers, a homology model was built based on the *Rn*CCT crystal structure. Inter-subunit polar interactions of the ^{681}RWVD^{684} motif corresponding to the cognate motif of ^{140}RYVD^{143} in the rat sequence were studied thoroughly. In total, thirteen polar inter-subunit interactions identified in the *Pf*CCT second catalytic domain structural model contribute to dimer stability. *In silico* modelling of the R681H mutation showed that half of the interactions are lost, which indicated a possibility for decreased stability of the dimer.

The functionality of the *Pf*CCT$_{(528\text{-}795)}$ R681H mutant enzyme, generated for *in vitro* studies, was not altered substantially as proved by k_{cat} and K_M values of Michaelis-Menten titrations performed at 20°C. These results are in good agreement with findings on the CHO-MT58 cell line, which was shown to have a WT phenotype when cultured at 37°C. For assessment of the temperature dependence of protein stability and functionality enzyme samples were incubated at different temperatures form 4°C to 60°C for 15 minutes then their enzyme activity was measured at 20°C. The R681H variant has lost its activity between 20°C and 30°C, while similar thermal inactivation of the wild type enzyme could be observed between 40°C and 55°C. This marked

[10]Lee, J., Johnson, J., Ding, Z., Paetzel, M. & Cornell, R. B. *J. Biol. Chem.* **284,** 33535–48 (2009).

difference occurred in kinetic stability at the physiological temperature range, which can be paralleled with the temperature sensitivity of the CHO-MT58 cell line.

The proposed role of R681 in dimerization and the drastically impaired thermal stability of the R681H enzyme variant suggested that the mutation might perturb the oligomerization of *Pf*CCT$_{(528\ 795)}$ R681H. ESI-MS analysis showed that while reasonable amount of dimer was present in the WT enzyme, dimer:monomer ratios were ca. 20 times lower in case of the mutant enzyme indicating a perturbed dimer formation. To provide further experimental evidence for the underlying mechanism of the thermosensitivity of *Pf*CCT$_{(528\ 795)}$ R681H, molecular dynamics simulations were performed on the homology models of the WT and mutant enzymes. The average inter-chain interaction energy of the mutant enzyme decreased by 25% compared to the WT. To illustrate that the impaired interaction of the R681H variant monomers can be observed particularly at the ^{681}RWVD^{684} conserved motif two representative inter-chain distances were followed during the MD simulations. The distance d(R/H681, CA - H679', O) is the shortest inter-monomer distance, while d(I680, CA I680', N) is an atomic distance presumably unaffected by the mutation. The characteristic deviation of d(R/H681, CA - H679', O) between the R681H variant and the WT argued for severely perturbed local contacts (Figure 2).

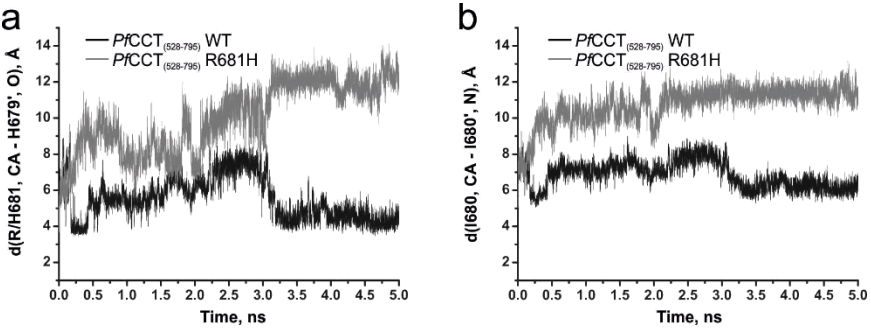

Figure 2. Variation of characteristic inter-chain interaction distances during the course of MD simulations of *Pf*CCT$_{(528\text{-}795)}$ WT and R681H.

4.3. Heterologous expression of *Pf*CCT rescues CHO cells deficient in PC biosynthesis Kennedy pathway

Previously it has been shown that CHO-MT58 cells cultured at 40°C undergo apoptosis due to PC defieciency[11], which was reported to be restored by heterologous expression of *Rn*CCT reducing the onset of apoptosis[12]. My aim was to test whether heterologous expression of CCT from *P. falciparum* can also rescue CHO-MT58 at 40°C despite the structural and functional differences compared to rat CCT. Transient transfection microscopy experiments showed that heterologous expression of *Pf*CCT$_{(1-896)}$, the full length functional enzyme could rescue CHO-MT58 cells compared to the non-transfected control.

To design a relatively simple model system for testing the efficiency of the rescue appropriate controls are needed. To verify that for the rescue the cytidylyltransferase activity is responsible modified *Pf*CCT constructs were chosen as the basis for comparison. For a full length *Pf*CCT construct harboring thermosensitizing point mutations corresponding to the R140H mutation in the endogenous CCT of CHO-MT58 the R/H mutation was introduced in both catalytic segments. Another point mutant construct targeting the conserved HxGH motif of the cytidylyltransferase enzyme family was created to gain a presumably catalytically deficient *Pf*CCT. To investigate the effect of the mutation *in vitro*, the point mutant construct *Pf*CCT$_{(528-795)}$ H630N was generated. Kinetic analysis of the mutant revealed a severe, three orders of magnitude activity decrease as compared to the original constitutively active *Pf*CCT$_{(528-795)}$ used as control. Thus, it is apparent that the asparagine replacement of the first histidine in HxGH signature sequence in both catalytic domains will yield a catalytically deficient form of *Pf*CCT that corresponds to an inactive enzyme phenotype.

For the quantitative characterization of the rescue potential of the protist *Pf*CCT in the mammalian cell line CHO-MT58 I aimed to design a well applicable test system.

---

[11]Cui, Z. *et al. J. Biol. Chem.* **271,** 14668–71 (1996).

[12]Sweitzer, T. D. & Kent, C. *Arch. Biochem. Biophys.* **311,** 107–16 (1994).

Transiently transfected cells were incubated at 37°C for 24 h then the culturing temperature was shifted to 40°C. Samples were prepared for FACS analysis 72 h post-transfection. The rescue potential of the different *Pf*CCT constructs was calculated based on the proportion of PI negative i.e. live cells referred to the corresponding control experiments. As reported by the results, transfection of the inactive *Pf*CCT construct provided only 1.6% rescue potential to CHO-MT58 cells. A somewhat higher percentage of cells (4.7%) was rescued by the transfection of the thermosensitive mutant while 13.6% of cells expressing the fully functional *Pf*CCT$_{(1\text{-}896)}$ were able to escape apoptosis (Figure 3). Although the percentage of rescued cells is compromised due to low transfection efficiency (typically 5-15%), the almost 9-fold increase in the rescue potential is remarkable and demonstrates that catalytically functional and structurally intact form of the additional *Pf*CCT is required to rescue CHO-MT58 cells lacking a functional endogenous CCT at 40°C.

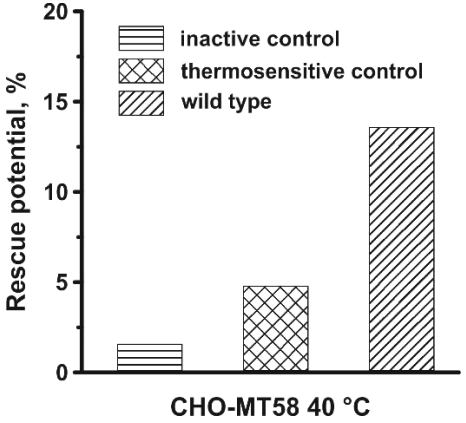

Figure 3. Rescue potential of different *Pf*CCT constructs in CHO-MT58 cells at 40°C.

5. DISCUSSION

Kinetic and ligand binding characterization performed here uncover novel insights into the involvement of key active site residues in CCT mechanism of action. The present study showed that during the CCT catalytic mechanism enzymatic function at the choline binding site is governed by a combination of cation-π and charged interactions forming a composite aromatic box. However, in many examples, the quaternary ammonium ion is recognized by the so called aromatic box, where three or four aromatic rings are positioned to face the bound charged moiety of the ligand and thus ensure exceptionally strong ligand binding. By contrast in composite aromatic boxes besides cation-π interactions provided by aromatic rings electrostatic or charged interactions have also a prominent role in ligand binding and catalysis. The dissimilar behavior of the point mutant catalytic domain constructs proves the different aromatic or electrostatic character of the active site residue interactions.

Upon investigation of the molecular mechanism underlying the thermosensitive phenotype of CHO-MT58 I found that formation of an intact dimer is essential for the enzymatic function of *Pf*CCT. This finding is in good agreement with the preferential dimer assembly of the evolutionarily related cytidylyltransferases involving CCT, CTP:glycerol-3-phosphate cytidylyltransferase (GCT) and CTP:ethanolamine phosphate cytidylyltransferase (ECT). A recently identified splice variant of the murine ECT (Pcyt2γ) lacking the C-terminal CT domain and being completely inactive indicated also that both cytidylyltransferase domains are required for activity.

While the most reliable description of protein function can be derived from studies of the entire protein so far it was challenging to investigate the full length *Pf*CCT. Noteworthy, to my best knowledge this is the first time that functional investigation of the full length *Pf*CCT subjected to domain duplication is presented. It is demonstrated here that the heterologous expression of *Pf*CCT$_{(1\text{-}896)}$ can prevent apoptosis of CHO-MT58 cells at 40°C. I verified that the enzymatic activity of *Pf*CCT is responsible for this effect as transfection with cDNA of catalytically deficient *Pf*CCT yielded considerably lower population of live cells at 40°C than the WT. Considering

the devastating effect of the absence of a functional CCT on several organisms and cell lines, the herein discussed inducible CCT deficient cell-based assay is an advantageous approach for studying CCT functionality with the conditional exclusion of the endogenous background. The designed test system can also be applied to study the selectivity of potential antimalarials targeting CCT.

6. THESES OF THE PHD DISSERTATION

Theses of my PhD dissertation can be summarized as follows:

1. *Pf*CCT active site residues D623 and Y741 provide charged interactions to the quaternary ammonium moiety of CDPCho and have decisive role in catalysis.
2. The partial conservation of the aromatic character of cation-π interactors W692 and Y714 is somewhat compatible with catalysis, while W692 has also a significant role in the structural stability of the active site.
3. The R681H mutation of the conserved motif RWVD in *P. falciparum* does not directly compromise the enzyme activity. Instead, it induces reduced thermal stability which leads to the inactivation of the enzyme.
4. *In vitro* and *in silico* results together show attenuation of dimer interactions induced by the R/H mutation, thus maintaining intact dimer interactions is critical for enzymatic activity of *Pf*CCT.
5. I demonstrated that the heterologous expression of *Pf*CCT can decrease the onset of apoptosis in CHO-MT58 at 40°C. The importance of the functionality of the expressed enzyme is verified by the lack of rescue of cells transfected with an inactive *Pf*CCT construct.
6. By FACS analysis of the cells transfected with different *Pf*CCT constructs, a rescue potential can be defined as a quantitative measure of the functionality of the enzyme.