Salvage and *de novo* synthesis of nucleotides in *Trypanosoma brucei* and mammalian cells

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Table of contents

Abstract

All living cells are dependent on nucleic acids for their survival. The genetic information stored in DNA is translated into functional proteins via a messenger molecule, the ribonucleic acid (RNA). Since DNA and RNA can be considered as polymers of nucleotides (NTPs), balanced pools of NTPs are crucial to nucleic acid synthesis and repair. The *de novo* reduction of ribonucleoside diphosphates (NDPs) to deoxyribonucleoside diphosphates (dNDPs), the precursors for DNA synthesis, is catalyzed by the enzyme ribonucleotide reductase (RNR). In cycling cells the dominant form of mammalian RNR consists of two proteins called R1 and R2. A proteasome-mediated degradation completely deprives postmitotic cells of R2 protein. The nonproliferating cells use instead a p53 inducible small RNR subunit, called p53R2 to synthesize dNTPs for mitochondrial DNA replication and DNA repair. To address the ongoing controversy regarding the localization and subsequently function and regulation of RNR subunits, the subcellular localization of all the mammalian RNR subunits during the cell cycle and after DNA damage was followed as a part of this thesis. Irrespective of the employed methodology, only a cytosolic localization could be observed leading to a conclusion that the dNTPs are synthesized in the cytosol and transported into the nucleus or mitochondria for DNA synthesis and repair. Thus, our data do not support the suggestion that nuclear translocation is a new additional mechanism regulating ribonucleotide reduction in mammalian cells.

In an attempt to find a cure for African sleeping sickness, a lethal disease caused by a human pathogen, *Trypanosoma brucei*, nucleotide metabolism of the parasite was studied. The trypanosomes exhibit strikingly low CTP pools compared with mammalian cells and they also lack salvage of cytidine/cytosine making the parasite CTP synthetase a potential target for treatment of the disease. Following expression, purification and kinetic studies of the recombinant *T. brucei* CTP synthetase it was found that the enzyme has a higher *Km* value for UTP than the mammalian CTP synthetase. In combination with a lower UTP pool the high *Km* may account for the low CTP pool in trypanosomes. The activity of the trypanosome CTP synthetase was irreversibly inhibited by the glutamine analog acivicin, a drug extensively tested as an antitumor agent. Daily injections of acivicin to trypanosome-infected mice were sufficient to suppress the parasite infections. The drug was shown to be trypanocidal when added to cultured bloodstream *T. brucei* for four days at 1 μM concentration. Therefore, acivicin may qualify as a drug with “desirable” properties, *i.e.* cure within 7 days, according to the current Target Product Profiles of WHO and DNDi. Trypanosomes lack *de novo* purine biosynthesis and are therefore dependent on exogenous purines such as adenosine that is taken up from the blood by high-affinity transporters. We found that besides the cleavage-dependent pathway, where adenosine is converted to adenine by inosine-adenosine-guanosine-nucleoside hydrolase, *T. brucei* can also salvage adenosine by adenosine kinase (AK). The efficient adenosine transport combined with a high-affinity AK yields a strong salvage system in *T. brucei*, but on the other hand makes the parasites highly sensitive to adenosine analogs such as adenine arabinoside (Ara-A). The cleavage-resistant Ara-A was shown to be readily taken up by the parasites and phosphorylated by the *Tb*AK-dependent pathway, inhibiting trypanosome proliferation and survival by incorporation into nucleic acids and by affecting nucleotide levels in the parasite.

Publication list

The thesis is based on the following papers, which will be referred to by their roman numerals.

I Giovanna Pontarin, Artur Fijolek, Paola Pizzo, Paola Ferraro, Chiara Rampazzo, Tullio Pozzan , Lars Thelander, Peter Reichard and Vera Bianchi (2008) Ribonucleotide reduction is a cytosolic process in mammalian cells independently of DNA damage. *Proc. Natl. Acad. USA.* In Press.

II Artur Fijolek, Anders Hofer and Lars Thelander (2007) Expression, purification, characterization, and *in vivo* targeting of trypanosome CTP synthetase for treatment of African sleeping sickness. *J. Biol. Chem.* **282** (16), 11858-11865

III Munender Vodnala, Artur Fijolek, Reza Rofougaran, Marc Mosimann, Pascal Mäser and Anders Hofer (2008) Adenosine kinase mediates high affinity adenosine salvage in *Trypanosoma brucei*. *J. Biol. Chem.* **283** (9), 5380-5388

Introduction

Nucleotides

Nucleotides are molecules that are composed of a nitrogen-containing carbon ring coupled to a phosphorylated five-carbon sugar (ribose). They are a prerequisite for the existence of all known living organisms. Nucleotides can be divided into two groups according to structure. Cytidine (C), thymidine (T) and uridine mono/di/triphosphates (U) are pyrimidines because the nitrogen base of these molecules is a six-membered pyrimidine ring. Adenosine (A) and guanosine (G) mono/di/triphosphates are purines because they have an additional five-membered ring fused to the first ring (Fig. 1).

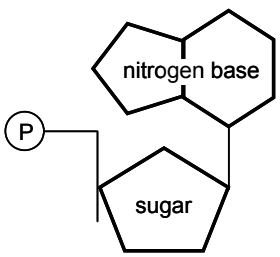


Fig. 1. A schematic view of a nucleotide with a purine base. P = phosphate.

Nucleotides participate in numerous biological processes. They can be used as energy carriers, such as adenosine triphosphate (ATP), structural components of the phospholipids that make up our cell membranes or coenzymes with other molecules. Their essential role is to serve as building blocks for nucleic acids. The double stranded helix of deoxyribonucleic acid (DNA) is the source of genetic information in all living cells. In general, two strands of DNA are held together by hydrogen bonds between the nitrogen bases of C and G or T and A. Importantly, the DNA polymer consists of deoxyribonucleotides (Fig. 2) where the hydroxyl at the 2' position of the ribose of ribonucleic acid (RNA) is replaced by a hydrogen atom.

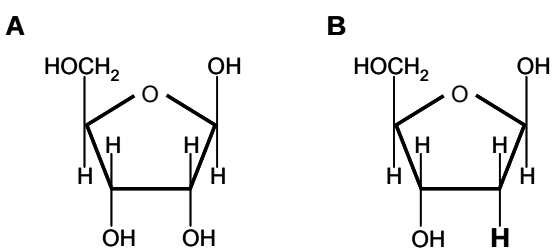


Fig. 2. The riboses of nucleotides. β-D-ribose in RNA (A) and β-D-deoxyribose in DNA (B).

Whereas the DNA molecule primarily serves as a repository of biological information, the single stranded RNA molecule (which uses U and not T) participates in the retrieval, translation and regulation of the information stored in genes. Messenger RNAs (mRNAs) are gene transcripts that serve as blueprints for protein synthesis by ribosomes, which act as protein making machines. The ribosomes themselves are complexes of proteins and ribosomal RNA (rRNA). There, at the site of the protein synthesis, three ribonucleotide bases of the mRNA (the codon) are paired with three matching bases (the anticodon) of a transfer RNA (tRNA) that carries an amino acid corresponding to the three codon bases of the mRNA. Through this codon to anticodon interaction, correct translation of genetic information into proteins is ensured. In addition, the recent discovery of non-coding RNAs has brought a new dimension to the regulation of gene expression, with small RNA molecules working together with specific proteins to degrade mRNA or induce changes in chromatin or the methylation state of DNA.

Because of their prominent role in nucleic acid synthesis, the pools of nucleotides must be tightly regulated. Elevated or unbalanced deoxyribonucleotide levels during replication can lead to breaks in DNA and mutagenesis (1). Furthermore, the lack of deoxyribonucleotide synthesis in terminally differentiated cells has recently been shown to result in mitochondrial DNA depletion. As a consequence, early lethality or severe mental and physiological disorders occur in patients suffering from this syndrome (2-5). Nucleic acid degradation is a frequent event in animals that generates free NTPs and dNTPs. It occurs through the turnover of RNA, digestion of nucleic acids present in food, cell death and DNA repair. If a cell has sufficient pools of nucleotides, the oligonucleotides from digested nucleic acids will be degraded to free nitrogen bases and ribose-1-phosphates. The process is reversible, and the cells can therefore rescue the nitrogen bases and nucleosides and use them if the need arises. This reutilization of degraded nucleic acids is called the salvage pathway of nucleotides. Cells can also synthesize nucleotides *de novo*. In this pathway, the nucleotides are synthesized from low molecular weight precursors, such as 5'-phospho-α-D-ribosyl-1-pyrophosphate (PRPP) and amino acids (6).

Ribonucleotide reductase

The rate limiting step in the synthesis of DNA precursors in the *de novo* pathway is the reduction of ribonucleotides to deoxyribonucleotides by the ribonucleotide reductase (RNR) enzyme. Using free radical chemistry, the enzyme catalyzes replacement of the 2' hydroxyl group of the ribose with hydrogen. The classical view of the mammalian RNR is a tetramer of two large (90 kDa) R1 proteins and two small (45 kDa) R2 proteins. The Fe-O-Fe center resides in the small R2 subunit, which generates and stabilizes a tyrosyl free radical. Upon binding of the substrate to the active site of the large R1 subunit, the radical is transferred from a tyrosine residue in the R2 subunit to the active site, which results in reduction of the ribonucleotides (7). The oxidized form of the RNR is then regenerated by reduction of two cysteines in the active center by the coenzyme thioredoxin to perform another round of

reducing reactions (8). RNR activity is tightly regulated through allosteric effector interactions to yield the correct balance of deoxyribonucleotides. Furthermore, the R1/R2 complex is only active during the S-phase of the cell cycle, during which cells are replicating DNA. Unlike the R1 protein, which remains constant throughout the cell cycle, the small R2 subunit is degraded in late mitosis. It was therefore unclear how non-dividing cells in terminally differentiated tissues, such as muscle, supply dNTPs for DNA repair and mitochondrial DNA (mtDNA) synthesis. The answer came with the discovery of a novel p53-inducible RNR subunit, p53R2. Although this protein is present at 30-fold lower levels than R2 protein in S-phase cells (9), p53R2 subunit is constantly expressed, even in non-dividing cells. It was subsequently shown that p53R2 protein forms an active RNR complex with R1 (10). This protein escapes cell cycle-dependent degradation because, unlike the R2 subunit, it lacks a KEN box - a specific triple amino acid sequence recognized by the Cdh1-APC ubiquitin ligase complex that targets the R2 subunit for proteolysis via ubiquitination (11). Since expression of p53R2 protein is induced by p53, it was suggested that the main function of the active R1/p53R2 complex was to supply dNTPs for DNA repair in non-dividing cells. Recently, however, several studies have shown that patients suffering from mtDNA depletion have mutations in the gene encoding p53R2 (2-5).

The controversy regarding the p53R2 does not stop there. The original reports on p53R2 suggest that this protein is transported into the nucleus in response to DNA damage (12,13). Considering that R1 subunit is necessary to form an active RNR complex, the proposed nuclear translocation of p53R2 protein would have major implications on RNR regulation. The idea of nuclear translocation of RNR subunits is not new, as it was proposed a long time ago by veer Reddy and Pardee (14). The authors claimed that RNR subunits formed a 'replitase' complex in the nucleus together with polymerases and other proteins during the replication of DNA. Later findings by Engström and Rozell, however, showed that mammalian R1 and R2 proteins localized in the cytosol (15,16), which has cast doubt on the replitase model. Following the suggestions that p53R2 protein localizes to the nucleus, recent reports claim that R1 and R2 subunits also undergo nuclear translocation in response to DNA damage (17). To clarify this matter, we have readdressed the question of RNR subcellular localization. Using different techniques, we set out to study the localization patterns of R1, R2 and p53R2 subunits in cycling and quiescent cells both in the presence and absence of DNA damage. The results of the first paper in this thesis are intended to clarify RNR localization patterns and provide insight into the regulation of RNR activity and function of p53R2.

African sleeping sickness

Because RNR plays a central role in DNA synthesis, it has been considered as an attractive therapeutic target. For example, the RNR of *Herpes simplex* virus has been successfully targeted by peptidomimetic drugs (18). Our lab has studied the human parasite *Trypanosoma brucei* RNR (19,20). These

protozoans are the causative agent of African sleeping sickness, a fatal disease that affects the rural areas of sub-Saharan Africa.

Trypanosomes are transmitted to humans through bites from the blood sucking tsetse fly (Glossina sp.), which is their second host. Once inside the mammalian host, the extracellular parasites change from their non-dividing metacyclic form into long, slender trypomastigots, at which point they proliferate in the blood and lymphatic system (21). At this stage in their life cycle, the parasites are covered by a variable surface glycoprotein (VSG) coat that enables them to escape the human immune system. As the immune system produces antibodies specific to the trypanosomes, a new clone with a totally different set of surface proteins will arise. In this way, the trypanosome population remains resistant to the attack of antibodies. At this stage of the infection, the disease symptoms are rather unspecific, including fever, headache, muscle ache and joint pain. The infection is therefore often misdiagnosed as another disease, such as malaria. If untreated, the parasites eventually invade the central nervous system (CNS) and cause encephalopathy. A reverse of the sleep-wake cycle (for which the name of the disease was coined) is followed by mental and coordination disorders, coma and finally death.

There are currently no more than four drugs available for the treatment of African sleeping sickness. Pentamidine and suramine are only effective during the first stage of the disease, since they cannot penetrate the blood-brain barrier. Difluoromethylornithine (DFMO) is one of the two drugs that are able to pass into the cerebrospinal fluid. Unfortunately, DFMO is only active against one of the two trypanosomal subspecies that cause the disease, *Trypanosoma brucei gambiense*. The other subspecies, *T. b. rhodesiense*, causes an acute form of the sickness that can be lethal within weeks to a few months. The only way to cure patients suffering from *T. b. rhodesiense* CNS infections is to administer an old arsenical derivate, Melarsoprol. Unfortunately, this compound is very toxic to patients. One of the side effects associated with this drug is the induction of encephalopathy with a fatal outcome in up to 10% of patients. Moreover, resistance to Melarsoprol is rising, with up to 30% treatment failures reported by several areas in central Africa (22,23).

CTP synthetase

The limited number of available drugs has prompted a great demand for new and better treatments. The studies by Hofer *et al.* on trypanosomal RNR were conducted with the hope of developing new and highly specific drugs against the parasite. Beside the differences between the mammalian and trypanosomal enzyme in respect to allosteric regulation, the authors found that trypanosomes have very low CTP pools as compared to those found in mammalian cells (19,20). By adding tritiated uracil to the growth medium of cultured trypanosomes and measuring the amount of label incorporated into UTP and CTP pools, the authors showed that there was a rapid accumulation of label in the UTP but not the CTP pool. More specifically, at least 95% of the

label was incorporated into nucleic acids and only a small amount was found in cytidine and uridine, the first two degradation products of CTP. Thus, the low CTP pool is due to slow *de novo* synthesis and not rapid degradation (24). Moreover, it was shown that salvaging of cytidine and cytosine did not occur, meaning that the trypanosomes could only synthesize CTP *de novo*. The conversion of UTP to CTP is catalyzed by an amidotransferase, the CTP synthetase (CTPS) (Fig. 3). This enzyme was originally identified in *E. coli* by Lieberman in 1956. He showed that the partially purified enzyme utilizes ATP, nitrogen and magnesium to synthesize CTP from UTP (25). Later studies have revealed that glutamine serves as a nitrogen donor, which is necessary for catalysis of the reaction, and that GTP is a positive allosteric effector for the enzyme (26,27).

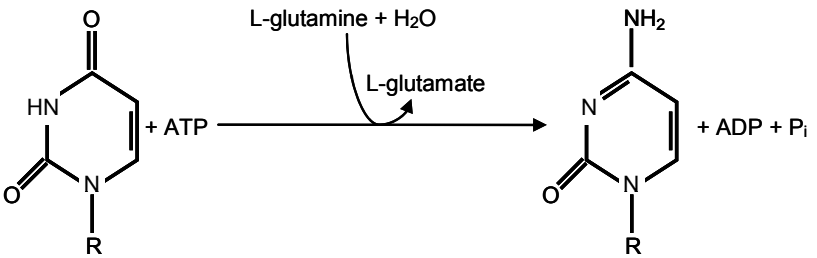
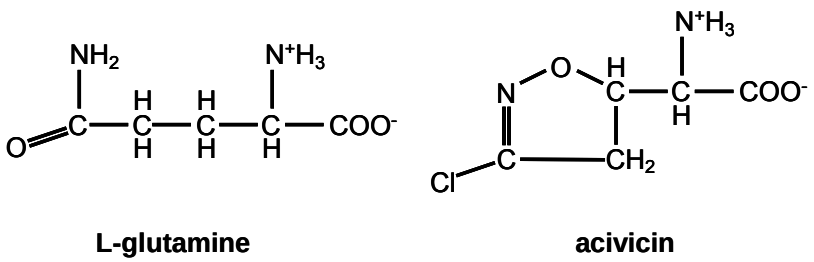


Fig. 3. The reaction catalyzed by CTP synthetase.

The binding of ATP and UTP by the bacterial and mammalian CTPS was shown to induce tetramerization (28,29). A single subunit of this homotetrameric complex consists of two domains, called the synthetase domain and the glutamine amidotransferase domain (GAT), which are connected by a helical interdomain linker. The reaction presumably begins at the synthetase domain where the bound UTP is activated by Mg-ATP-dependent phosphorylation at the 4-position. The activation of UTP is suggested to induce conformational changes that promote glutamine binding and reaction with a cysteine residue in the GAT domain (30). From structural studies of *E. coli* CTPS, it was proposed that the ammonia generated at the GAT site is then transported through a tubular passage to the synthetase domain. There, it displaces the 4-phosphate group which leads to the formation of CTP. The proposed GTP binding site resides near the GAT active site. The binding of GTP most likely stabilizes the interaction of glutamine and the active site of the glutaminase domain.

Like other glutamine amidotransferases, the CTPS is inhibited by glutamine analogs. Originally isolated from *Streptomyces sviceus*, the antimetabolite acivicin (Fig. 4) irreversibly inhibits glutamine amidotransferases by alkylating the active site cysteine (31-33). The drug was extensively studied and tested as a potential anticancer chemotherapeutic agent, but, due to the ability of cancer cells to salvage purines and pyrimidines, it never made a break through into widespread clinical use (34). Trypanosomes lack this ability, meaning that acivicin has great potential as a drug against African sleeping sickness. Besides the fact that the toxicological properties of the drug are already known, it has other advantages. For example, acivicin can pass

through the blood-brain barrier and therefore may be active against CNS infections. In addition, it can be administrated orally, which would likely not require a lengthy hospital stay. The addition of acivicin to the growth medium of cultured trypanosomes resulted in lowered GTP and CTP pools, inhibiting the growth of the parasites (24).



L-glutamine **acivicin**

Fig. 4. Structures of L-glutamine and its analog, acivicin.

Since *T. brucei* lacks the ability to salvage cytidine and cytosine (Fig. 5), addition of these pyrimidines and the purine guanine could rescue only the GTP pool, leaving the CTP pool unchanged (24).

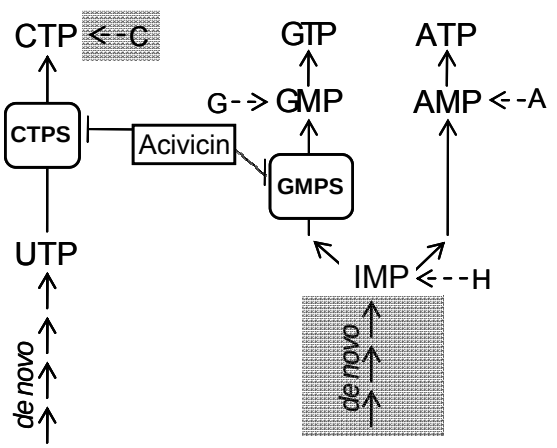


Fig. 5. The main targets of acivicin in nucleotide biosynthesis pathways. CTPS = CTP synthetase, GMPS = GMP synthase, A = adenine or adenosine, C = cytidine or cytosine, G = guanine or guanosine, H = hypoxanthine. The salvage pathways of purine and pyrimidine bases are marked by the dotted arrows. Unlike mammalian cells, trypanosomes lack the ability to salvage C and synthesize purines *de novo* (marked here by shaded boxes).

Based on these findings, it became clear that the trypanosomal CTPS may constitute a target for the treatment of the African sleeping sickness. Therefore, in the second paper of this thesis, we have cloned, expressed and studied the properties of the *T. brucei* CTP synthetase. We are also developing a treatment for the disease by targeting the trypanosomal enzyme by acivicin.

Adenosine kinase

The inability of *T. brucei* to salvage cytidine and cytosine is not the only characteristic of the trypanosomal nucleotide metabolism that sets it apart from mammalian cells. Unlike humans, the parasites are not able to synthesize purines *de novo*. Instead, they depend exclusively on purines salvaged from blood of their human host (35). To be able to compete for purines with the mammalian cells, trypanosomes have high affinity adenosine transporters that efficiently transport and concentrate adenosine inside the parasites (36,37). Until now, it was believed that the conversion of adenosine to AMP was achieved by inosine-adenosine-guanosine-nucleoside hydrolase (IAG-NH)-dependent cleavage and phosphoribosylation of the resulting adenine (35). The *T. brucei* genome, however, contains two adenosine kinase (AK) genes that directly phosphorylate adenosine to AMP (38). Indeed, when expression of AK is silenced by RNAi, the parasites are less sensitive to the action of an adenosine analog, cordycepin (3'-deoxyadenosine) (39). This drug is incorporated into RNA, thereby inhibiting transcription. It must, however, first be phosphorylated by AK to gain activity. Upon administration to *T. brucei*-infected mice together with deoxycoformycin, which protects the cordycepin against deamination by deoxyadenosine deaminase, a complete cure was achieved (40). These findings strongly suggest that the *T. brucei* AK is directly involved in salvaging of adenosine. We decided to investigate whether this is indeed the case by studying purified recombinant *T. brucei* AK. Furthermore, by administering adenosine analogs to tissue cultured trypanosomes and mammalian cells, we investigated the role of AK in the activation of these prodrugs.

Results

Paper I

Ribonucleotide reduction takes place in the cytosol of mammalian cells independent of the cell cycle phase and DNA damage.

In this paper, we studied the subcellular localization of the R1, R2 and p53R2 proteins in the presence and absence of DNA damage. We used cell fractionation as the first approach. Four different procedures were applied to separate the nuclei from cytosol. Specific antibodies against R1, R2, p53R2, HDAC1 (histone deacetylase 1, a nuclear marker) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase, a cytosolic marker) were used to determine the localization of the RNR subunits by Western blotting. We concluded that all three subunits consistently resided in the cytosol irrespective of the cell cycle phase or DNA damage and despite the induction of p53R2 protein. The same results were achieved with a second method, in which the studied cells were fixed and subsequently stained with specific antibodies. The monoclonal R1 antibody exclusively stained the cytosol. This was also the case when two different monoclonal antibodies against R2 were used. However, the commercial antipeptide polyclonal antibody against

p53R2, which has been used in publications from other laboratories, stained the nuclei. Western blot analyses revealed that different commercial polyclonal antibodies against p53R2 protein were quite unspecific. We therefore purified one of the polyclonal antibodies by affinity chromatography using pure his-tagged recombinant human p53R2 coupled to a CNBr-activated Sepharose resin. The purified antibody indicated cytosolic localization of p53R2 subunit, even in the case of DNA damage. On the other hand, antibodies in the flow-through fraction stained the nuclei of the cells. This strongly suggests that data from other laboratories may be explained by unspecific staining by p53R2 antibodies. The evidence for cytosolic localization was further strengthened when we transiently transfected mammalian cells with fluorescently tagged proteins. A cDNA encoding green fluorescent protein was ligated to the 3´end of mouse R1 cDNA in the Okayama-Berg vector. The cDNA encoding mouse p53R2 was, in contrast, ligated to the 3´end of DsRed in the pDsRed-Monomer-C1 vector. Both vectors contained the SV40 origin, resulting in high expression of the tagged proteins upon transfection of T antigen-containing COS-7 cells. This approach made it relatively easy to detect the fluorescent signals. Both proteins localized to the cytosol even when the cells were subjected to the DNA damage inducing agent adriamycin. To minimize the risk of artifacts associated with protein overexpression, we transiently transfected NIH 3T3 cells, which do not contain the T antigen. In this case, a UV pulse was used instead of adriamycin to elicit DNA damage since the nuclear fluorescence of adriamycin interferes with DsRed emission. As was shown previously, R1 and p53R2 protein localized to the cytosol irrespective of DNA damage.

Unlike yeast, mammalian cells do not increase their dNTP pools in response to DNA damage (9,11). A direct supply of dNTPs at the site of DNA repair by the p53R2 protein residing in the nucleus could compensate for this shortcoming. However, since we have not observed translocation of the R1 subunit into the nucleus, which would be necessary for formation of an active RNR complex, this model is difficult to accept. We have demonstrated cytosolic localization of p53R2, R1 and R2 proteins, which supports our previous model of cytosolic dNTP production. Since dNTPs easily diffuse through nuclear pores, cytosolic ribonucleotide reduction should not impede DNA repair polymerase activity. In addition to its role in DNA repair, our results also suggest an important function of p53R2 protein in the supply of dNTPs for mitochondrial polymerases. A nuclear activity of the p53R2/R1 complex would certainly make it difficult to explain efficient mtDNA synthesis. The cytosolic localization of these three subunits also speaks against the suggestion that the RNR activity is controlled by nuclear translocation. Instead, our data support the earlier findings (41) that transcriptional regulation, allosteric interactions and cell cycle-specific degradation of R2 subunit modulate RNR activity (42).

Paper II

Expression, purification, characterization and *in vivo* targeting of trypanosome CTP synthetase for treatment of African sleeping sickness.

Aiming to develop a treatment for African sleeping sickness we have expressed, purified and studied the kinetic properties of a recombinant *T. brucei* CTPS. Expressing an active His_6-TEV protease site tagged *T. brucei* CTPS proved quite difficult due to the formation of inclusion bodies. To minimize the formation of inclusion bodies, we grew BL21(DE3)pLysS bacteria without IPTG induction at 15°C in Terrific Broth medium. Since IPTG induction was omitted, the expression of recombinant *T. brucei* CTPS was low and an efficient purification procedure was necessary to maximize the yield of the trypanosomal enzyme. A Talon Metal Affinity resin was used to selectively bind the His-tagged CTPS. A His-tagged TEV protease was used to specifically cleave off the His-tag of the eluted *T. brucei* CTPS. The proteins were subsequently loaded onto a fresh Talon resin-containing column. Since the His-tag was removed, recombinant CTPS was collected in the flow-through fraction and most impurities, together with the His-tagged TEV protease, remained bound to the resin. As a last polishing step, anion exchange chromatography was applied, resulting in a homogenous *T. brucei* CTPS solution. This strategy permitted purification of a sufficient amount of recombinant *T. brucei* CTPS to conduct studies of its kinetic properties.

We found that the presence of ATP or UTP alone was sufficient to induce tetramer formation. More importantly, we have discovered that the parasitic enzyme has twice the K_m for UTP compared to its mammalian counterpart (0,16 mM and 0,07 mM, respectively). Considering that the UTP concentration in trypanosomes is only 0,26 mM compared to 0,6 mM in mammalian cells (43), the higher K_m may account for the low CTP levels in *T. brucei*. The parasitic enzyme was clearly inhibited by acivicin with a K_i for the initial binding equal to 2,3 μM, which is approximately the same as that of the mammalian CTPS (44). Still, Hofer *et al.* showed that growth of trypanosomes is 80% inhibited following 15 hours of incubation with 1 μM acivicin (24). A 14-fold higher concentration of the drug is required to achieve the same inhibitory effect on mouse L1210 cells (45). This distinction can be explained by the lack of cytidine and cytosine salvaging in trypanosomes, which solely rely on CTPS for CTP synthesis. Moreover, we observed that the inhibition is more pronounced when the enzyme is preincubated with UTP, ATP and GTP. This result supports the suggestion from structural studies of the *E. coli* enzyme that binding of UTP induces conformational changes that promote glutamine binding (30). Because of the irreversible nature of inhibition by acivicin, we were interested in evaluating whether the drug promoted direct killing of tissue cultured trypanosomes or if it was trypanostatic. Indeed, trypanosomes were no longer able to recover after they were incubated with 1 μM acivicin for at least four days. Furthermore, we clearly observed a trypanocidal effect during the incubation with a dramatic decrease in parasite number. Previous phase I clinical trials showed that plasma acivicin concentrations above 5 μM are required to cause CNS toxicity in humans (46,47). Encouraged by the *in vitro* data, we set out to test if we could cure trypanosome infected C57Bl mice. We

were able to show that the infections of mice treated with daily intraperitoneal injections of acivicin (5-10 mg/kg) were suppressed; the trypanosome level decreased below the detection limit (0,1 x 10^6 trypanosomes/ml) during the course of the treatment. Unfortunately, the parasites have thus far always reemerged after completion of the treatment. Our measurements of acivicin plasma levels show that the drug concentration rapidly decreased after the injections, dropping from approximately 50 μM to only 1 μM after three hours. The fast excretion most likely explains the reoccurring parasitemia. A more frequent administration or a constant infusion therapy is needed to reach constant acivicin plasma levels. Our recent *in vivo* experiments (not published here) support our hypothesis. When infected mice received acivicin injections every four hours, the reoccurrence of parasitemia was delayed by several days. According to WHO, a drug with 'desirable' properties should be able to cure an infection within seven days to minimize the costs and inconvenience of the treatment. Our data are certainly encouraging, and we hope to achieve a complete cure using improved administration strategies.

Paper III

Adenosine kinase mediates high affinity adenosine salvage in *Trypanosoma brucei*

In the last paper of this thesis, we have cloned, expressed and characterized the *T. brucei* AK (*Tb*AK) in an attempt to understand its role in purine biosynthesis by parasites. We found that the purified recombinant enzyme was able to phosphorylate adenosine. The K_m of *Tb*AK for adenosine was between 0,04-0,08 μM, and the preferred sources of phosphates were ATP and GTP. Since ATP is present at higher concentrations, it most likely is the major phosphate donor *in vivo* (20). Contrary to other AKs of closely related protozoan species, such as *Leishmania donovani*, the *Tb*AK exhibits substrate inhibition - similar to the mammalian enzyme (48,49) **-** suggesting independent development of this characteristic. The concentration of adenosine in the blood of the mammalian hosts has been difficult to demonstrate unequivocally with reported values of 2 μM or less (50). Considering that the K_m of IAG-NH for the binding of adenosine is as high as 15 μM (51), *Tb*AK is expected to play a major role in adenosine salvage. *TbAK* activity is probably even more critical in cases of high parasitemia, when the soaring demand for adenosine decreases the purine concentration even further. Moreover, the conversion of adenosine to AMP by *TbAK* requires only one ATP molecule compared to the three molecules utilized in the cleavage-dependent pathway**,** making the salvage of adenosine more energetically efficient. Cordycepin and other adenosine analogs, such as adenine arabinoside (Ara-A) and fludarabine (F-Ara-A), however, are also phosphorylated by *Tb*AK. Ara-A was found to be cytotoxic to tissue cultured trypanosomes in an AK-dependent manner, since *Tb*AK knock-down trypanosomes were approximately 10-times less sensitive to the drug. This was also true for F-Ara-A, but the sensitivity did not decrease as much as Ara-A in the knockdown cells (approximately 3-times). This suggests that F-Ara-A is only partially dependent on *Tb*AK. Despite the fact that *TbAK* has a much

higher K_m for Ara-A compared to adenosine (230 μM and 0,04-0,08 μM, respectively), the trypanosomes were still able to accumulate the phosphorylated form of the analog. Measurements of the NTP pools in mammalian cells and *T. brucei* incubated in media containing increasing levels of Ara-A revealed that the salvage of Ara-A was in fact very efficient in the parasites. Contrary to the mammalian cells, the Ara-ATP became one of the major nucleotides in *T. brucei* already at 20 μM Ara-A in the incubation medium. The same tendency was observed when cell growth was studied. The IC_{50} for mouse Balb/3T3 cells was about 25-times higher than for *T. brucei*. The human WS1 fibroblast cell line salvaged Ara-A to an even lesser extent, and no susceptibility was observed at the tested concentrations. Salvage of Ara-A resulted in a general decrease of ribonucleotide levels, with the exception of GTP. This may be due to impaired activity of the enzymes that participate in nucleotide biosynthesis or general energy depletion, since phosphorylation of Ara-A requires ATP. Interestingly, the levels of deoxyribonucleotides increased. The accumulation of dNTPs can be explained by the decreased incorporation of tritiated thymidine and the products of tritiated uracil (UTP, CTP, dTTP and dCTP) into the nucleic acids. This is suggestive of impaired DNA and RNA synthesis, a probable cause for the growth inhibition of *T. brucei*.

Concluding remarks

From the data presented in the first paper of this thesis, we conclude that RNR activity is not regulated by shuttling of the subunits between the cellular compartments. The relative contribution of p53R2 to DNA repair compared to mtDNA synthesis is difficult to assess. An investigation of mutation rates in cells lacking p53R2 would likely shed more light on p53R2 function and its involvement in DNA repair. Also, a study of dNTP translocation and its subsequent interactions with DNA polymerases would significantly contribute to our understanding of how non-dividing mammalian cells supply deoxyribonucleotides for DNA repair and mtDNA synthesis. Unfortunately, no suitable fluorescent tags are currently available to study intracellular localization of the dNTPs *in vivo*.

As mentioned in the introduction, African sleeping sickness causes a great deal of suffering in the affected countries. Of the available drugs, DFMO presents the best treatment option because of its relatively small side effects and ability to penetrate the blood-brain barrier. Unfortunately, this treatment requires lengthy infusions in hospital settings, and it is only effective against *T. b. gambiense*. These disadvantages are even more worrying, considering the recent reports on the spread of DFMO-resistant *T. b. rhodesiense* in *T. b. gambiense* endemic areas (52,53). We have shown that trypanosomal CTPS is a potential therapeutic target for treatment of this disease. Although sequence comparisons between the *T. brucei* and the mammalian enzyme show high homology in the catalytic domains of the protein, kinetic data imply structural differences between these proteins. The structures of the *Thermus thermophilus* (54), *E. coli* (30) and human CTPS (PDB ID 2VO1 and 2VKT)

are known, but the structure of the *T. brucei* CTPS has not been described. Preliminary crystals of the trypanosomal CTPS have been acquired, and hopefully the full structure will be obtained in the near future. Knowledge of the structure of this protein would help us to design a more specific *T. brucei* CTPS inhibitor.

Similar to acivicin, Ara-A can pass through the blood-brain barrier. As mentioned before, this is crucial for the successful treatment of late stage trypanosomiasis. Furthermore, Ara-A has been thoroughly tested in clinical trials, which have exposed its pharmacological and toxicological properties. Our preliminary experiments on *T. brucei*-infected mice are encouraging, but, as in the case of acivicin, the reoccurrence of parasitemia after the treatment schedule necessitates optimization of drug administration.

Considering the disadvantages of existing drugs, new medicines are in high demand. Our studies of nucleotide metabolism show that we can use the differences between the mammalian cells and trypanosomes to our advantage. The *T. brucei* CTPS and AK are certainly attractive drug targets; they offer new therapeutic possibilities for the treatment of African sleeping sickness.

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References

1. Reichard, P. (1988) *Annu. Rev. Biochem.* **57**, 349-374
2. Bornstein, B., Area, E., Flanigan, K. M., Ganesh, J., Jayakar, P., Swoboda, K. J., Coku, J., Naini, A., Shanske, S., Tanji, K., Hirano, M., and DiMauro, S. (2008) *Neuromuscul. Disord.* **18**(6), 453-459
3. Spinazzola, A., and Zeviani, M. (2005) *Gene* **354**, 162-168
4. Leanza, L., Ferraro, P., Reichard, P., and Bianchi, V. (2008) *J. Biol. Chem.* **283**(24), 16437-16445
5. Bourdon, A., Minai, L., Serre, V., Jais, J. P., Sarzi, E., Aubert, S., Chretien, D., de Lonlay, P., Paquis-Flucklinger, V., Arakawa, H., Nakamura, Y., Munnich, A., and Rotig, A. (2007) *Nat. Genet.* **39**(6), 776-780
6. Mathews, C. K., Van Holde, K. E., and Ahern, K. G. (2000) *Biochemistry*, 3. Ed., Benjamin Cummings, San Francisco, Calif.
7. Jordan, A., and Reichard, P. (1998) *Annu. Rev. Biochem.* **67**, 71-98
8. Holmgren, A., and Bjornstedt, M. (1995) *Methods Enzymol.* **252**, 199-208
9. Hakansson, P., Hofer, A., and Thelander, L. (2006) *J. Biol. Chem.* **281**(12), 7834-7841
10. Guittet, O., Hakansson, P., Voevodskaya, N., Fridd, S., Graslund, A., Arakawa, H., Nakamura, Y., and Thelander, L. (2001) *J. Biol. Chem.* **276**(44), 40647-40651
11. Chabes, A., Georgieva, B., Domkin, V., Zhao, X., Rothstein, R., and Thelander, L. (2003) *Cell* **112**(3), 391-401
12. Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y., and Nakamura, Y. (2000) *Nature* **404**(6773), 42-49
13. Yamaguchi, T., Matsuda, K., Sagiya, Y., Iwadate, M., Fujino, M. A., Nakamura, Y., and Arakawa, H. (2001) *Cancer Res.* **61**(22), 8256-8262
14. Prem veer Reddy, G., and Pardee, A. B. (1980) *Proc. Natl. Acad. Sci. USA* **77**(6), 3312-3316
15. Engstrom, Y., and Rozell, B. (1988) *Embo J.* **7**(6), 1615-1620
16. Engstrom, Y., Rozell, B., Hansson, H. A., Stemme, S., and Thelander, L. (1984) *Embo J.* **3**(4), 863-867
17. Xue, L., Zhou, B., Liu, X., Qiu, W., Jin, Z., and Yen, Y. (2003) *Cancer Res.* **63**(5), 980-986
18. Liuzzi, M., Deziel, R., Moss, N., Beaulieu, P., Bonneau, A. M., Bousquet, C., Chafouleas, J. G., Garneau, M., Jaramillo, J., Krogsrud, R. L., Lagacé, L., McCollum, R. S., Nawoot, S., and Guindon, Y. (1994) *Nature* **372**(6507), 695-698
19. Hofer, A., Schmidt, P. P., Graslund, A., and Thelander, L. (1997) *Proc. Natl. Acad. Sci. U S A* **94**(13), 6959-6964
20. Hofer, A., Ekanem, J. T., and Thelander, L. (1998) *J. Biol. Chem.* **273**(51), 34098-34104
21. Matthews, K. R. (2005) *J. Cell. Sci.* **118**(Pt 2), 283-290
22. Brun, R., Schumacher, R., Schmid, C., Kunz, C., and Burri, C. (2001) *Trop. Med. Int. Health.* **6**(11), 906-914
23. WHO. (1998) *WHO Technical Report Series* **881**
24. Hofer, A., Steverding, D., Chabes, A., Brun, R., and Thelander, L. (2001) *Proc. Natl. Acad. Sci. U S A* **98**(11), 6412-6416
25. Lieberman, I. (1956) *J. Biol. Chem.* **222**(2), 765-775
26. Hurlbert, R. B., and Kammen, H. B. (1960) *J. Biol. Chem.* **235**(2), 443-449

27. Chakraborty, K. P., and Hurlbert, R. B. (1961) *Biochim. Biophys. Acta* **47**, 607-609
28. Long, C. W., Levitzki, A., and Koshland, D. E., Jr. (1970) *J. Biol. Chem.* **245**(1), 80-87
29. McPartland, R. P., and Weinfeld, H. (1976) *J. Biol. Chem.* **251**(14), 4372-4378
30. Endrizzi, J. A., Kim, H., Anderson, P. M., and Baldwin, E. P. (2004) *Biochemistry* **43**(21), 6447-6463
31. Hanka, L. J., and Dietz, A. (1973) *Antimicrob. Agents. Chemother.* **3**(3), 425-431
32. Cooney, D. A., Jayaram, H. N., Ryan, J. A., and Bono, V. H. (1974) *Cancer Chemotherapy Reports. Part 1* **58**(6), 793-802
33. Tso, J. Y., Bower, S. G., and Zalkin, H. (1980) *J. Biol. Chem.* **255**(14), 6734-6738
34. Fischer, P. H., Pamukcu, R., Bittner, G., and Willson, J. K. (1984) *Cancer Res.* **44**(8), 3355-3359
35. el Kouni, M. H. (2003) *Pharmacology & Therapeutics* **99**(3), 283-309
36. Carter, N. S., and Fairlamb, A. H. (1993) *Nature* **361**(6408), 173-176
37. de Koning, H. P., Watson, C. J., and Jarvis, S. M. (1998) *J. Biol. Chem.* **273**(16), 9486-9494
38. Vodnala, M., Fijolek, A., Rofougaran, R., Mosimann, M., Maser, P., and Hofer, A. (2008) *J. Biol. Chem.* **283**(9), 5380-5388
39. Luscher, A., Onal, P., Schweingruber, A. M., and Maser, P. (2007) *Antimicrob. Agents Chemother.* **51**(11), 3895-3901
40. Rottenberg, M. E., Masocha, W., Ferella, M., Petitto-Assis, F., Goto, H., Kristensson, K., McCaffrey, R., and Wigzell, H. (2005) *J. Infect. Dis.* **192**(9), 1658-1665
41. Nordlund, P., and Reichard, P. (2006) *Annu. Rev. Biochem.* **75**, 681-706
42. Chabes, A. L., Pfleger, C. M., Kirschner, M. W., and Thelander, L. (2003) *Proc. Natl. Acad. Sci. U S A* **100**(7), 3925-3929
43. Savage, C. R., and Weinfeld, H. (1970) *J. Biol. Chem.* **245**(10), 2529-2535
44. Neil, G. L., Berger, A. E., McPartland, R. P., Grindey, G. B., and Bloch, A. (1979) *Cancer Res.* **39**(3), 852-856
45. Neil, G. L., Berger, A. E., Bhuyan, B. K., Blowers, C. L., and Kuentzel, S. L. (1978) *Adv. Enzyme Regul.* **17**, 375-398
46. Earhart, R. H., Koeller, J. M., Davis, T. E., Borden, E. C., McGovren, J. P., Davis, H. L., and Tormey, D. C. (1983) *Cancer Treat. Rep.* **67**(7-8), 683-692
47. Weiss, G. R., McGovren, J. P., Schade, D., and Kufe, D. W. (1982) *Cancer Res.* **42**(9), 3892-3895
48. Palella, T. D., Andres, C. M., and Fox, I. H. (1980) *J. Biol. Chem.* **255**(11), 5264-5269
49. Datta, A. K., Bhaumik, D., and Chatterjee, R. (1987) *J. Biol. Chem.* **262**(12), 5515-5521
50. Slowiaczek, P., and Tattersall, M. H. N. (1982) *Analytical Biochemistry* **125**(1), 6-12
51. Parkin, D. W. (1996) *J. Biol. Chem.* **271**(36), 21713-21719
52. Berrang-Ford, L., Berke, O., Abdelrahman, L., Waltner-Toews, D., and McDermott, J. (2006) *Emerg. Infect. Dis.* **12**(5), 813-820
53. Welburn, S. C., Coleman, P. G., Maudlin, I., Fevre, E. M., Odiit, M., and Eisler, M. C. (2006) *Trends. Parasitol.* **22**(3), 123-128

54. Goto, M., Omi, R., Nakagawa, N., Miyahara, I., and Hirotsu, K. (2004) *Structure* **12**(8), 1413-1423