Targets and strategies for drug development against human African sleeping sickness

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Abstract

*Trypanosoma brucei* is a causative agent of African sleeping sickness. It is an extracellular parasite which circulates in the blood, lymph and eventually invades the central nervous system. There is a great need for new medicines against the disease and specific properties of nucleoside kinases in the pathogen can be exploited as targets for chemotherapy.

*T. brucei* contains a gene where two thymidine kinase sequences are fused into a single open reading frame. These types of tandem thymidine kinases were found only in different types of parasites, which made us to believe that it might be beneficial for them. Each thymidine kinase sequence in these tandem enzymes are here referred to as a domain. By cloning and expressing each domain from *T. brucei* separately, we found that domain 1 was inactive and domain 2 was as active as the full-length enzyme. *T. brucei* thymidine kinase phosphorylated the pyrimidine nucleosides thymidine and deoxyuridine and to some extent purine nucleosides like deoxyinosine and deoxyguanosine. Human thymidine kinase increases the affinity to its substrates when it forms oligomers. Similarly, the *T. brucei* two thymidine kinase sequences, which can be viewed as a pseudodimer, had a higher affinity to its substrates than domain 2 alone.

*T. brucei* lacks *de novo* purine biosynthesis and it is therefore dependent on salvaging the required purine nucleotides for RNA and DNA synthesis from the host. Purine salvage is considered as a target for drug development. It has been shown that in the presence of deoxyadenosine in the growth medium, the parasites accumulate high levels of dATP and the extensive phosphorylation of deoxyadenosine leads to depleted ATP pools. Initially, we wondered if deoxyadenosine could be used as a drug against *T. brucei*. However, we found that *T. brucei* is partially protected against deoxyadenosine because it was cleaved by the enzyme methylthioadenosine phosphorylase (MTAP) to adenine and ribose-1-phosphate. At higher concentration of deoxyadenosine,
the formed adenine was not efficiently salvaged into ATP and started to inhibit MTAP instead. The deoxyadenosine was then instead phosphorylated by adenosine kinase leading to accumulation of dATP. The MTAP reaction makes deoxyadenosine itself useless as a drug and instead we focused on finding analogues of deoxyadenosine or adenosine that were cleavage-resistant and at the same time good substrates of \textit{T. brucei} adenosine kinase. Our best hit was then 9-(2-deoxy-2-fluoro-ß-D-arabinofuranosyl) adenine (FANA-A). An additional advantage of FANA-A as a drug was that it was taken up by the P1 nucleoside transporter family, which makes it useful also against multidrug resistant parasites that often have lost the P2 transporter function and take up their purines solely by the P1 transporter. In parallel with our study of nucleoside metabolism in \textit{T. brucei}, we also have a collaboration project where we screen essential oils from plants which are used in traditional medicine. If the essential oils are active against the trypanosomes, we further analyze the different components in the oils to identify new drugs against African sleeping sickness. One such compound identified from the plant \textit{Smyrnium olusatrum} is isofuranodiene, which inhibited \textit{T. brucei} proliferation with an IC$_{50}$ value of 3 \(\mu\)M.
This thesis is based on the following papers:


III. Ranjbarian F, Vodnala M, Alzahrani KH, de Koning HP, Hofer A. 9-(2-deoxy-2-fluoro-ß-D-arabinofuranosyl) adenine as a therapeutic agent against trypanosoma brucei (manuscript submitted).


1. The first two authors contributed equally.
Papers not included in this thesis:


# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADA</td>
<td>Adenosine deaminase</td>
</tr>
<tr>
<td>AK</td>
<td>Adenosine kinase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>Ara-A</td>
<td>Arabinofuranosyl adenine</td>
</tr>
<tr>
<td>CATT</td>
<td>Card Agglutination Test</td>
</tr>
<tr>
<td>CDA</td>
<td>Cytidine deaminase</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral Spinal Fluid</td>
</tr>
<tr>
<td>DFMO</td>
<td>Difluoromethylornithine</td>
</tr>
<tr>
<td>FANA-A</td>
<td>9-(2-deoxy-2-fluoro-D-arabinofuranosyl) adenine</td>
</tr>
<tr>
<td>GMP</td>
<td>Guanosine monophosphate</td>
</tr>
<tr>
<td>IAG-NH</td>
<td>Inosine-adenosine-guanosine-nucleoside hydrolase</td>
</tr>
<tr>
<td>IMP</td>
<td>Inosine monophosphate</td>
</tr>
<tr>
<td>MTAP</td>
<td>Methylthioadenosine phosphorylase</td>
</tr>
<tr>
<td>NECT</td>
<td>Nifrutimox-eflornithine combination therapy</td>
</tr>
<tr>
<td>PRPP</td>
<td>Phosphoribosyl pyrophosphate</td>
</tr>
<tr>
<td>PRTase</td>
<td>Phosphoribosyltransferase</td>
</tr>
<tr>
<td>Tb</td>
<td>Trypanosoma brucei</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>UMP</td>
<td>Uridine monophosphate</td>
</tr>
<tr>
<td>UPRT</td>
<td>Uracil phosphoribosyl transferase</td>
</tr>
<tr>
<td>VSG</td>
<td>Variable Surface Glycoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
Aim of the thesis

Developing new therapy against African sleeping sickness by targeting the nucleotide metabolism of *Trypanosoma brucei* and characterizing the enzymes involved in the pyrimidine and purine salvage pathways.

Introduction

African trypanosomiasis affects the life of people living in 36 countries in sub-Saharan Africa. African trypanosomiasis is an infectious disease in humans and animals. The disease is caused by protozoan parasites of the genus *Trypanosoma* that live and multiply extracellularly in the bloodstream and lymph of the mammalian hosts. They are able to evade the antibody response through antigenic variation. Trypanosomes are proliferating in the mammalian bloodstream as long slender forms, which can be replaced by the non-proliferating short stumpy form when the number of parasites increases (1). The short stumpy form is adapted to be transmitted by the bite of an infected tsetse fly (*Glossina* sp), in which the parasite go through several life cycle stages before it can be transmitted to the next mammalian host. African animal trypanosomiasis or nagana disease is mainly caused by *T. congolense*, *T. vivax* and *T. brucei*. The animal disease Nagana is different from human African sleeping sickness. Affected animals have enlarged lymph nodes, severe anemia, weight loss and develop visceral and mucosal hemorrhages. Human African sleeping sickness is caused by *T. brucei rhodesiense* and *T. brucei gambiense* that give acute and chronic forms of the disease, respectively. The chronic form accounts for more than 98% of reported cases. The disease has two stages. First the parasites are restricted to circulate in the blood and tissue fluids. This is also called the haemolymphatic stage. In the second stage, the parasites cross the blood-brain barrier and invade the central nervous system. During the second stage, the patient can present a variety of neurological symptoms
such as alteration of circadian sleep/awake pattern and personality changes. In the absence of treatment, the disease progresses and the patient goes into coma and finally dies. Before the second stage, the person can be infected for months or years without major symptoms. When the symptoms become prominent, the patient is already in the advanced stage of the disease where the parasites have infected the central nervous system (2). *T. b. gambiense* is transmitted mostly through a human-fly-human cycle but it may also follow an animal-fly-human cycle, whereas in *T. b. rhodesiense*, animal reservoirs play an important role in the transmission cycle (3).

Based on information from the World Health Organization (WHO) on human African trypanosomiasis, about 70 million people are estimated to be at different levels of risk in Africa. The number of new human African trypanosomiasis cases reported between 2000 and 2012 dropped by 73%. Transmission of the disease seems to have decreased in spite of the continuous displacement of population, war and poverty, which are important factors that ease the transmission. People in over 1.5 million square kilometer remains at risk of contracting the disease (4). With WHO support, it became possible to screen the population at risk of the disease by using immunological and parasitological test. The card agglutination tests (CATT) developed in 1978 (5), is used for screening the affected population by *T. b. gambiense*. The diagnosis proceeds by systematic stage determination and assessing the cerebrospinal fluid (CSF) to check for the presence of parasites. The presence of parasites in the CSF is a sign that the disease has progressed to the second stage.

**Trypanosoma brucei**

**History of human African sleeping sickness**

The history of human African trypanosomiasis(6) dates back to the 18th century during the slave trade. In 1734, the English naval surgeon John Atkins published the first medical report on African sleeping sickness. He
described only neurological symptoms of the late stage of the disease. In 1803, an English physician reported some signs of swollen lymph and gradually the number of reports on sleeping sickness increased but the cause of the disease remained unknown for a long time. It was not until the very end of the 19th century that parasites for the first time were found in the cerebrospinal fluid of a patient with sleeping sickness and David Bruce described the pathogen behind African trypanosomiasis in 1896. At that time, they thought it was transmitted by tsetse flies mechanically but later it was found to be a cyclic transmission with specific life cycle stages in the fly. In 1916, Paul Ehrlich with the help of a chemist team and the German pharmaceutical company Bayer developed the first effective drug for treatment of human African trypanosomiasis. The compound, Bayer 205 (later named as suramin), is still in use for the therapy of early stage *T. b. rhodesiense* infections.

*Trypanosoma brucei* life cycle

The infection of the mammalian host starts when the infected fly bites and introduces the growth-arrested metacyclic trypomastigote to the mammalian bloodstream. The metacyclic trypomastigote transforms into a long slender form and establishes a bloodstream infection. The long slender forms penetrate the blood vessels and enter extravascular tissue including the central nervous system. The long slender form can change into short stumpy forms, which are cell cycle arrested and pre-adapted for survival in tsetse flies. When a tsetse fly bites an infected host, the parasite is taken up along with the blood meal into the midgut where the short stumpy form differentiates into a procyclic trypomastigote, which resumes cell division. The procyclic trypomastigote moves into the proventriculus and the salivary gland. In the proventriculus, procyclic trypomastigotes restructure to long and short epimastigotes. The short ones attach to epithelial cells to generate metacyclic trypomastigotes that are free in the salivary gland (7).
Disease control and vector control

The surveillance and treatment have a powerful effect in human African trypanosomiasis control. One health concept important for the control of African trypanosomiasis is that it has been shown that parasites causing human and animal African trypanosomiasis can coexist in the same tsetse flies (8). It has been shown in the case of T. b. gambiense that transmission rates are high, and vector control is required. In fact, vector control increases the sustainability of medical intervention. Different strategies used for vector control are aerial spraying insecticides, the sterile insect technique and paratransgenic approaches with genetically modified symbiotic bacteria that produce trypanocides that inhibit trypanosome survival, development and maturation (9, 10).

Treatment of the disease

Vaccine development against T. brucei, is prevented by the existence of variable surface glycoproteins (VSGs) covering the body of the parasite. The T brucei genome contains more than 1000 VSG sequences, but only one is expressed at a time. The VSG proteins cover the whole cell membrane with the purpose of making the parasites undetectable by immune system (11). These proteins are varied in the amino acid sequence and attached sugars but they have a conserved structure. The immune defense will develop antibodies against the particular VSG variant expressed, but the ability of the parasite to continuously switch to new variants of the glycoprotein makes that the immune system is always one step behind. Such a regulatory system helps the parasite to survive for a sufficient time for their transmission to the next host. Clinical drugs are available. However, these drugs suffer from high toxicity and low efficacy dependent on stage of the disease and subspecies causing the infection. The drugs used against human African trypanosomiasis are pentamidine, suramine, melarsoprol and difluoromethylornithine (DFMO) (Table 1).
Melarsoprol has been the most universal and effective drug against all forms of African sleeping sickness. It also effective against the second stage of the disease and is active against *T. b. rhodesiense*, which is generally more difficult to treat than infections caused by *T. b. gambiense*. It is an arsénical compound with severe side effects. Suramine and pentamidine are effective at curing the first stage of the disease when the parasites are restricted to the blood and lymph, but are not useful against the second stage because they do not pass the blood-brain barrier. Pentamidine is less toxic than suramin and is therefore the preferred drug against *T. b. gambiense*, whereas suramin is generally used against first stage *T. b. rhodesiense* infections. DFMO is effective against the second stage of *T. b. gambiense* infections. However, it is less active against *T. b. rhodesiense* and very expensive to synthesize. Nifurtimox is a drug used for treatment of American trypanosomiasis (Chagas disease), but for *T. brucei* it is not very effective as a single agent. However, the combination of nifurtimox and DFMO (NECT) will be safer, cheaper and more easy to administrate than DFMO itself (12). Decreased drug uptake has emerged as the most common cause of drug resistance. Some of the drugs are becoming ineffective due to the loss of transporters which are involved in drug uptake. An ideal drug against sleeping sickness needs to meet some criteria; it should be active against both subspecies and be effective against the two stages of the disease. It should also have low or no toxicity, being orally available and not too expensive to synthesize. In addition, a better understanding of drug uptake by the parasites helps to find ways to avoid drug resistance. One of the strategies for developing new drugs against African sleeping sickness is to test drugs, which are approved or in clinical use for the treatment of other diseases in order to identify less toxic drugs and to limit the high cost of clinical trials.
<table>
<thead>
<tr>
<th>Drugs</th>
<th>Drug efficacy</th>
</tr>
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<tbody>
<tr>
<td>Pentamidine</td>
<td>First stage / <em>T. b. gambiense</em></td>
</tr>
<tr>
<td>Suramine</td>
<td>First stage / both subspecies</td>
</tr>
<tr>
<td>Merlarsoprol</td>
<td>Both stages / both subspecies (very toxic)</td>
</tr>
<tr>
<td>Eflornithine (DFMO)</td>
<td>Both stages / <em>T. b. gambiense</em></td>
</tr>
<tr>
<td>DFMO+Nifurtimox (NEC)</td>
<td>Both stages / <em>T. b. gambiense</em></td>
</tr>
</tbody>
</table>

Table 1. Chemotherapy against human African sleeping sickness.

**Purine and pyrimidine metabolism in *Trypanosoma brucei***

**Purine metabolism**

In general, purine nucleotides are synthesized via *de novo* and salvage pathways. The *de novo* pathway utilizes simple compounds to synthesize purine nucleotides. IMP is synthesized from amino acids, CO$_2$, tetrahydrofolate derivatives and PRPP (Figure 1).

![Diagram](Image)

*Figure 1. The right side shows *de novo* biosynthesis of purines from PRPP to IMP in mammalian cells, and the left side shows the main salvage pathways of purine formation in mammalian cells and *T. brucei*.*
In contrast to mammalian cells, *T. brucei* lacks *de novo* purine biosynthesis and the parasites are therefore completely dependent on purine uptake from the host and use the salvage pathway for synthesizing the required purine nucleotides (13). The salvage pathway is a type of recycling process, which reutilizes the compounds from endogenous or exogenous degradation in order to form purine nucleotides for RNA and DNA synthesis. Due to the large phylogenetic distance of the host and parasite, there are some differences in the enzymes of the purine salvage pathways, which can be targeted by developing new chemotherapy against parasites. The parasite has evolved very efficient transporters to take up purine nucleobases or nucleosides (14). It is enough with just one purine source (*e.g.* adenosine or hypoxanthine) for the trypanosomes because they have all the enzymes needed to catalyze the interconversion of AMP, IMP and GMP in both directions (15). In that way, *T. brucei* can use all the physiological purine bases and nucleosides to make nucleotides. The purine salvage enzymes are nucleoside hydrolases/phosphorylases, purine phosphoribosyltransferases (PRTases) and adenosine kinase. Adenosine kinase phosphorylates adenosine and to some extent deoxyadenosine (16). Adenosine and hypoxanthine are the two major purine sources in human blood. The level of adenosine is 2 µM while the level of hypoxanthine is between 0.6 and 2 µM (17, 18). Adenosine is salvaged in *T. brucei* through different ways. Adenosine, as well as inosine and guanosine, is cleaved by IAG-NH (*I*inosine, *A*denosine, *G*uanosine- *n*ucleoside hydrolase) into adenine and ribose (19). Adenosine (and deoxyadenosine) can also be cleaved by the methylthioadenosine phosphorylase (MTAP) into adenine and ribose-1-phosphate (20). The base from the cleavage pathways is then salvaged through a PRTase (APRTase in the case of adenine). The cleavage–dependent salvage of adenosine has an unfavorable Km (*Km* = 15 µM in IAG-NH (19) and *Km* = 23 µM in MTAP (21)) in comparison to the concentration in blood. *T. brucei*, adenosine kinase has much higher affinity than *Tb*IAG-NH and *Tb*MTAP with a *Km* in the nanomolar range (0.041µM) (16). When the level of adenosine is critical for the parasites in the host, the
trypanosomes may become dependent on the high-affinity \( TbAK \) pathway to synthesize purine nucleotides. Moreover, the high affinity pathway is conserving energy. One ATP is required to synthesize AMP from adenosine whereas cleavage-dependent AMP synthesis requires phosphoribosylpyrophosphate (PRPP) that is made from ribose and 3 ATP.

**Purine nucleoside, nucleobase transport**

In order to survive in an environment with poor purine contents, the parasites have developed very efficient transporters to take up purines from the host blood. Unlike human cells that use facilitated diffusion to take up purines, the trypanosomes have active transporters. Another difference is that the nucleoside transporters in mammalian cells have broader substrate specificities which transport both purines and pyrimidines, and this is not the case for the trypanosomes.

The rate of uptake of adenosine is greater than the other purines. There are two main distinct adenosine transporters in \( T. brucei \), P1 and P2 (22). The P1 transporter is responsible for oxopurine nucleosides uptake (inosine and guanosine) but can also transfer adenosine. The P2 transporter has a good affinity for aminopurines, adenosine and the nucleobase adenine. It has been shown that the P2 transporter interacts with the N1 and 6-amino groups of purines like adenosine and tubercidine but it does not interact with oxopurines (23). The oxopurine nucleobases are taken up by other transporters. The high affinity purine nucleobase transporters are H1, H2, H3 and H4. The H2 and H3 transporters are present in \( T. brucei \) bloodstream forms while the H1 and H4 transporters are present in procyclic forms (24). Sensitivity and drug resistance in parasites are highly dependent upon the level of drug uptake. Melarsoprol and pentamidine are two of the main drugs, which are used for the treatment of African sleeping sickness in humans. Cross-resistance to these drugs was shown already 60 years ago for African sleeping sickness. Drug resistance typically appears when genetic changes
(mutation, deletion or amplification) alter uptake, drug metabolism, drug target interaction or efflux. It has been shown that melarsoprol resistance was linked to the absence of the P2 aminopurine transporter activity (22). In fact, the cross resistance to melarsoprol and pentamidine is due to that the two drugs are imported through the same transporters. The physiologic substrates for the P2 transporter, adenosine and adenine, are able to compete out melarsoprol to protect the parasite from the lysis effect of melarsoprol in vitro. The gene encoding the P2 transporter is TbAT1. TbAT1 gene deletion and loss of function point mutations were described in drug resistant strains generated in the lab, and the same point mutations were found in a patient infected with melarsoprol-resistant T. brucei gambiense (25). Analyzing the P2 transporter in T. brucei showed a high affinity for several trypanocidal diaminidines including pentamidine (26). The uptake of pentamidine was partially blocked by adenosine. The sensitivity to pentamidine decreased two-fold in TbAT1 gene knock out trypanosomes compared to wild type, while resistance to other diaminidines like diminazene was stronger. This indicates the presence of another transporter, which is insensitive to adenosine and can take up pentamidine. However, the later discovery of the other transporter for the uptake of pentamidine and melarsoprol represents a gap in the understanding of drug resistance associated with the uptake of drugs (27). It has previously been shown that it is not easy to induce resistance to purine analogues in T. brucei when the uptake is mediated through multiple transporters (28). It was therefore surprising that pentamidine and melarsoprol are taken up by two types of transporters, and yet the trypanosomes could still become resistant by downregulating the transport of the drugs.
Trypanosoma brucei is capable to make pyrimidines by de novo synthesis from glutamine and aspartate as well as salvaging pyrimidine nucleosides or nucleobases, which are transported from the host environment or growth medium by specific transporters (Figure 2). The parasite makes uridine monophosphate (UMP), which the cell obtains through de-novo biosynthesis or phosphoribosylation of uracil in salvage synthesis. From UMP, the cells can make all the required pyrimidines. The parasites lack uridine kinase to phosphorylate uridine to UMP, instead uridine or deoxyuridine are cleaved by uridine phosphorylase to uracil. Uracil can be taken up through the TbU1 and TbU3 transporters in procyclic and bloodstream forms of trypanosomes, respectively (29-31). Uracil phosphoribosyl transferase (UPRT) converts uracil to UMP and further phosphorylation by nucleotide kinases makes it to form uridine diphosphate (UDP) and uridine triphosphate (UTP). In T. brucei, the synthesis of cytidine nucleotides depends on the production of UMP. T. brucei cannot take up cytosine or cytidine. Therefore, it needs to be made by the de novo pathway through CTP synthetase, which makes CTP from UTP as a precursor for RNA synthesis. T. brucei possess de novo dNTP synthesis via ribonucleotide reductase, which can make dNDPs from the corresponding NDPs but the parasites have limited supplies of CDP (and CTP) for biosynthesis of dCDP. This is compensated for by having a ribonucleotide reductase with high affinity for CDP and by lacking dCMP deaminase, an enzyme that is present in most other eukaryotes that participates in a pathway which converts dCTP to dTTP. This may limit dTTP biosynthesis but the parasites are able to compensate for this problem by acquiring dTTP via thymidine kinase-mediated salvage synthesis. Uptake of pyrimidine nucleobases or nucleosides are less efficient and requires higher concentration than purines. For example, the P1 purine transporters can only transport thymidine with low affinity (31). T. brucei encodes three pyrimidine salvage enzymes: UPRT, thymidine kinase (TK), and uridine phosphorylase.
(UPP), and in addition cytidine deaminase (CDA) which converts deoxycytidine to deoxyuridine. One of the key regulatory steps in the salvage pathway is phosphorylation of nucleosides and deoxynucleosides, which is an alternative to \textit{de novo} synthesis of DNA precursors. The phosphorylation is dependent on nucleoside- and deoxynucleoside kinases. The reaction catalyzed by nucleoside- and deoxynucleoside kinases is irreversible and the first phosphorylation is generally the rate-limiting step. In human cells, the phosphorylation of pyrimidine nucleosides and deoxynucleosides at the 5' position is catalyzed by two cytosolic enzymes, thymidine kinase 1 (TK1) and deoxycytidine kinase (dCK), and by one mitochondrial enzyme, thymidine kinase 2 (TK2). All the three enzymes have distinct but overlapping substrate specificities. In addition, there is another mitochondrial enzyme, deoxyguanosine kinase (dGK) which only phosphorylate purine deoxynucleosides (Table 2).

<table>
<thead>
<tr>
<th>Name</th>
<th>Natural substrates</th>
<th>Sub-cellular location</th>
</tr>
</thead>
<tbody>
<tr>
<td>hTK1</td>
<td>dT, dU</td>
<td>Cytosol</td>
</tr>
<tr>
<td>hdCK</td>
<td>dC, dG, dA</td>
<td>Cytosol</td>
</tr>
<tr>
<td>hTK2</td>
<td>dT, dU, dC</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>hdGK</td>
<td>dG, dA</td>
<td>Mitochondria</td>
</tr>
</tbody>
</table>

\textbf{Table 2. Human deoxynucleoside kinases.}

In \textit{T. brucei}, TK phosphorylates thymidine and deoxyuridine to form dTMP and dUMP, respectively (32, 33). There are some specific properties of nucleoside- and deoxynucleoside kinases in pathogens, which are interesting from a point of view for drug development. \textit{Tb}TK has been involved in the metabolic activation of some deoxynucleoside analogs as antiviral or anticancer agents. Acyclovir and other nucleoside analogs used against herpes virus are activated by the viral TK but not recognized by the host deoxynucleoside kinases. It has been shown that \textit{Tb}TK is essential for \textit{in vitro} growth and for infectivity \textit{in vivo} as well (34). Data shows that \textit{Tb}TK activity plays a key role in the synthesis of
dTMP even in cells grown in a pyrimidine-free environment. This was surprising because *T. brucei* has the enzyme thymidylate synthetase which converts dUMP to dTMP. However, a study with *TbTK* knockout cells showed that in the absence of external pyrimidines, the cells accumulate TK substrates like Thd and dUrd, and will deplete the dTTP pools, suggesting the presence of 5’-nucleotidases that catalyze the conversion of deoxynucleotides into deoxynucleosides. In humans, the function of TK can be compensated for by dCMP deaminase (DCTD) which is able to increase the supply of dUMP that can be used by thymidylate synthase to make dTMP. *T. brucei* lacks DCTD, which is in support of the importance of *TbTK*. It has been shown *in vitro* for human TK1 that low temperature and enzyme concentration in the presence of ATP favor an activation process of the enzyme. The incubation of the protein with ATP induces its transition to a more active form with higher substrate affinity and a conformational change of the enzyme from a homodimer to homotetramer structure. The two structures have different properties. The tetramer binds to thymidine with 20 times higher affinity than the dimer. It has been proposed that the ability to change between these two conformations, dimers and tetramers, are involved in cell cycle regulation of TK activity (35).
Figure 2. *T. brucei* pyrimidine synthesis pathways.

**Drug development**

Developing a new drug is a complex process. One of the most important steps in drug discovery is target identification and validation. After initial target identification, you need to find a molecule, which is suitable to that specific target and can be an acceptable drug. The selected candidate will be analyzed in preclinical tests and if successful go into clinical evaluation. A common drug target-based approach is to identify a protein in the parasite which is essential and is different from the host proteins. In *T. brucei*, the purine salvage pathway offers this opportunity for metabolic drug targeting utilizing an efficient transport system. Individual enzymes in the *T. brucei* salvage synthesis are non-essential because there are several pathways involved, but from a drug discovery interest, they are still important. These enzymes have a role in the activation of purine nucleobase- or nucleoside analogues, which only become toxic when they are converted to nucleotide analogues. The nucleoside analogues need to be phosphorylated inside the cells to form the corresponding nucleotides. The stability of the analogues against degradation by enzymes present in the bloodstream and in the
parasites, is also important to consider for the development of nucleoside–
based therapeutics against African sleeping sickness and might serve as a
starting point for drug discovery. These analogues should pass the blood-brain
barrier via purine transporters (36). In *T. brucei*, the main cause of drug
resistance is changes in specific transporters. Resistance develops easily,
especially when the drug is taken up by a single non-essential transporter
protein. The challenge here is to find an analogue that is taken up by multiple
transporters to decrease the risk of emerging drug resistance. Other
approaches for developing new drugs are high throughput screening as well as
searching for naturally occurring compounds. Here we have focused on *in vitro*
antitrypanosomal screening of traditional medicinal plants used previously to
treat various microbial and non-microbial diseases. This kind of study can lead
to the identification of new therapeutic agents.
Summary of the study

The main idea behind the project was to develop new therapy against African sleeping sickness by targeting the nucleotide metabolism of *T. brucei* and characterizing enzymes involved in pyrimidine and purine salvage pathways. This includes:

- Characterizing the *TbTK* enzyme of the pyrimidine salvage pathway to find a difference with the host hTK1 as a target for drug development (work 1).
- Studying *T. brucei* purine salvage enzymes. Trypanosomes lack *de novo* purine biosynthesis and need to compensate for that by unusually efficient salvage. We already knew that *TbAK* is a very efficient enzyme for adenosine and deoxyadenosine salvage. Here we characterized *TbMTAP* another metabolic enzyme for adenosine and deoxyadenosine salvage in trypanosomes.
- Screening purine nucleoside or deoxynucleoside analogues which are activated by *TbAK* and able to kill the parasites. In addition, the analogues need to be resistant to cleavage and taken up by the *T. brucei* P1 nucleoside transporter. The third work describes the identification of an adenosine analogue that meets all these criteria.
- Screening biological compounds or traditional plant medicines for new antitrypanosomal agents. In this work, we analyzed the antitrypanosomal activity of essential oils and purified compounds from the plant *Smyrnium olsustratum*. 
1. *Trypanosoma brucei* thymidine kinase is a tandem protein consisting of two homologous parts, which together enable efficient substrate binding

We have shown that four parasites including *T. brucei* contain genes where two or four thymidine kinase sequences are fused into a single open reading frame. Each TK sequence is here referred to as a domain, and in the case of *TbTK*, the protein is divided into two domains. These kinds of tandem TKs were found in four species from three phylogenetically unrelated parasite families, but not found in any non-parasitic species among the top 20,000 hits from GenBank. It may therefore represent an adaptive trait for the parasites. Phylogenetic analysis showed that the two domains of *TbTK* are most likely the result of a duplication. The same was true for the TKs of two of the other parasites *A. suum* with two domains and *T. congolense* with four domains. Whereas, in *T. vaginalis* TK each domain was distinct from each other, which means that two genes are fused together rather than having one gene that is copied. The DNA sequence analysis of *TbTK* showed that the two domains have exchanged genetic material in recent time, as evident by a stretch of 89 nearly identical base pairs in both domains. This region encodes for an ATP/GTP binding site. Full length *TbTK* and each of its domains were separately expressed and characterized. Domain 1 was inactive whereas domain 2 had a similar activity as the full-length protein. The inactive domain 1 lacks three-conserved amino acid residues, which are important for catalysis or substrate recognition based on the human TK1 crystal structure. Of particular importance is Glu-98 (human numbering). This residue plays an important role in all known deoxynucleoside kinases by abstracting a proton from the 5'-OH group of the deoxynucleoside substrate, to enable its nucleophilic attack on the Y-phosphate of ATP (37). Proteins from the TK1 family are generally dimers or tetramers. The tetramer form has higher affinity to the substrate compared to the dimer. *TbTK* domain 2 was mainly monomeric and had a 5
times reduction in affinity for its main substrates, thymidine and deoxyuridine, as compared to the full-length protein. However, in the presence of domain 1, it can be considered as pseudo-dimer showing an enhanced binding affinity to the substrates.

From a point of interest for developing nucleoside analogs as a drug against African sleeping sickness, TbTK was less discriminative to purines than the human TK1. The enzyme phosphorylated the pyrimidine nucleosides, thymidine and deoxyuridine, and to some extent the purine nucleosides deoxyinosine and deoxyguanosine. It has therefore broader substrate specificity than human TK1, indicating that the catalytic site of the protein has more room in the active site. This is interesting for drug discovery, indicating that the enzyme could be able to accept pyrimidine analogues with bulkier substituents by making them looking something in between purines and pyrimidines. These kinds of analogues would perhaps be transported by purine transporters, but still being phosphorylated by TbTK.

Figure 3. Comparison of TbTK and human TK1. A, schematic picture of human TK1 and TbTK B, oligomerization of TK proteins increases substrate affinity
2. *Trypanosoma brucei* methylthioadenosine phosphorylase protects the parasite from the antitrypanosomal effect of deoxyadenosine: implications for the pharmacology of adenosine metabolites

A previous study showed that *T. brucei* accumulates high level of dATP in the presence of 1mM dAdo (38). The accumulation of high levels of dATP is dependent on adenosine kinase and causes the parasites to die within a few hours (16). In this paper, we have shown that *T. brucei* treated with 1mM dAdo accumulates higher dATP levels than mammalian cells, but only if the concentration of dAdo is high. At lower concentrations of dAdo, *T. brucei* methylthioadenosine phosphorylase (*TbMTAP*) protected the parasites from the antitrypanosomal effect of dAdo. *TbMTAP* will cleave dAdo to adenine for ATP synthesis, and deoxyribose 1-phosphate. One of the reaction products, adenine, inhibited the enzyme, which can explain why the high concentration of dAdo is toxic for parasites. At the presence of adenine as a *TbMTAP* inhibitor in the culture medium, growth inhibition in *T. brucei* bloodstream forms was enhanced (Figure 4).

Human cells are protected against dAdo by adenosine deaminase, whereas this enzyme could not be found in *T. brucei* (39). In this paper, we showed that *TbMTAP* is instead having this role in *T. brucei*. This is important to know for drug discovery. In order for adenosine or dAdo analogues to be efficient against *T. brucei*, it is not enough that they are phosphorylated by *TbAK*. They also need to be resistant to *TbMTAP* and other cleavage enzymes in *T. brucei*. 
Figure 4. Metabolic pathways of dAdo in *Trypanosoma brucei*. At high concentrations of dAdo, *TbAK* efficiently catalyzes the first step in the phosphorylation of dAdo to dATP. *TbMTAP* is then inhibited by feedback inhibition of its enzymatic reaction product, adenine. At lower concentrations of dAdo, the adenine formed by the *TbMTAP* reaction does not reach concentrations high enough to inhibit the enzyme, and the parasites are then protected from dAdo toxicity. *APRT* is using the adenine for ATP synthesis, and it is only when this activity is saturated by too high levels of adenine that the *TbMTAP*-mediated protection fails.
3. 9- (2-Deoxy-2 Fluro-ß-D-arabinofuranosyl) adenine as a therapeutic agent against *Trypanosoma brucei*

In the study, above (work 2), we showed the importance of *Tb*MTAP for drug discovery because *Tb*AK substrate analogues need to be resistant to cleavage by *Tb*MTAP to be effective against *T. brucei*. Known antitrypanosomal nucleoside analogues such as tubercidine and cordycepin have successfully been used to cure infected mice, and they are all *Tb*MTAP cleavage resistant and good substrates of *Tb*AK. However, they also have the limitation that they are taken up by the P2 purine nucleoside transporter. It has been reported that treatment failures with melarsoprol against second stage sleeping sickness can be due to the loss of function of the P2 purine transporter, which is implicated in melarsoprol uptake (40). A reason why the parasites get easily resistant to treatment is that the P2 purine transporter is encoded by a single gene. In contrast, the P1 purine transporter can be encoded by multiple genes, which are spread out on different parts of the genome. It is very unlikely that the parasite can accumulate loss of function mutations on all these genes, making the appearance of drug resistance less likely. Our main goal here is to find an adenosine- or dAdo analogue, which is taken up by the P1 transporter (or by both transporters) at the same time as it is a good substrate of *Tb*AK and resistant to the enzyme activity of *Tb*MTAP and other cleavage enzymes. Based on these criteria, we started to look for adenosine or dAdo analogues. The selected compounds were first tested in enzyme assay with *Tb*MTAP and *Tb*AK. We have previously shown that Ara-A is a substrate of *Tb*AK that is not cleaved by *Tb*MTAP (16). It is also a good antitrypanosomal drug with an IC$_{50}$ of 0.071 µM against *T. brucei* proliferation (16). Ara-A is characterized by having an OH group in the upward direction at the 2’ position of the ribose, *i.e.* the opposite direction as compared to adenosine. Our idea was to find analogues with other substituents instead of the upward 2’-OH group of Ara-A and see if these analogues also are *Tb*MTAP-cleavage resistant (Figure 5). From these analogues, two compounds were selected: FANA-A that is
fluorinated in the 2’ upwards direction and 2’-C-methyladenosine that have a methyl group at this position. Both analogues were resistant to cleavage by purified TbMTAP and by T. brucei cell extracts. FANA-A was an efficient inhibitor of T. brucei proliferation with an IC₅₀ of 0.0028 µM. TbAK phosphorylated FANA-A efficiently and the enzyme activity with TbAK was much higher than with human AK with this substrate. A transport assay showed that FANA-A can compete with the purine uptake of both transporters, and experiments on knockout T. brucei parasites lacking the P2 transporter showed that they were equally sensitive to the parent T. brucei strain. This indicates that the P1 transporter is enough to give full sensitivity to the drug although both transporters are most likely involved in the uptake. FANA-A could cure T. brucei infected mice in combination with the adenosine deaminase inhibitor deoxycoformycin (dCF). However, the affinity of human adenosine deaminase to adenosine and dAdo is much lower than the efficiency of T. brucei purine transporters P1 and P2. Nevertheless, we must consider the stability of the compound in blood. If we incubated dAdo with T. brucei growth medium (containing 10% mammalian serum) in the absence of dCF, dAdo was completely converted to deoxyinosine after 48 h. FANA-A was also deaminated under these conditions. A major challenge here is to improve FANA-A to overcome the adenosine deaminase-mediated reaction. Indeed, co-administration of FANA-A and dCF as adenosine deaminase inhibitor increases the growth inhibition sensitivity of parasites. However, the long-term inhibition of an important metabolic enzyme of the host, such as adenosine deaminase, may have toxic side-effects. It has been reported that dCF shows a teratogenic effect in pregnant mice, which makes it uncertain if the drug can be used as chemotherapy during pregnancy (41). The best alternative might be to search for a new derivative of FANA-A that is deamination resistant and therefore can be used as a single agent in chemotherapy.
Figure 5. Ara-A and modified analogues of Ara-A
4. An overlooked horticultural crop, *Smyrnium olusatrum*, as a potential source of compounds effective against African trypanosomiasis

Essential oils are concentrated extracts taken from plant tissues such as roots, seeds, leaves or flowers. Each plant tissue contains its own mix of active compounds. Essential oils are widely used as traditional medicines for treatment of microbial, viral and non-infectious diseases. Analyzing the composition of essential oils will reveal the chemical profile of the plant. By separating the chemical constituents, it is subsequently possible to find out which of them is responsible for the desired effect. It has previously been shown that essential oils can be used as an alternative treatment against antibiotic resistant bacterial infections (42) and some of them also have antitrypanosomal activity (43). In the current work, the antitrypanosomal activity of essential oils obtained from different parts of *Smyrnium olusatrum* was assayed. The fruit part had the highest antitrypanosomal activity with an IC$_{50}$ value of 1.97 µg/ml. Isofuranodiene as one of the major component of the fruits, was the main inhibitor with an IC$_{50}$ value of 3 µM (0.65 µg/ml). To investigate why the fruits were more active in comparison to the other parts of the plant, the other major components of the fruit were tested to check if they could affect the activity of isofuranodiene. $\beta$-acetoxyfuranoeudesm-4(15)-ene is present in large amounts in fruits and increased the sensitivity of isofuranodiene. This work represents the first report of the antitrypanosomal activity of *S. olusatrum* and isofuranodiene as an active compound, which is very hydrophobic and easily can be absorbed by membranes.
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