Enzymes in Thymidylate Synthesis in *Ureaplasma parvum* as Medical Targets

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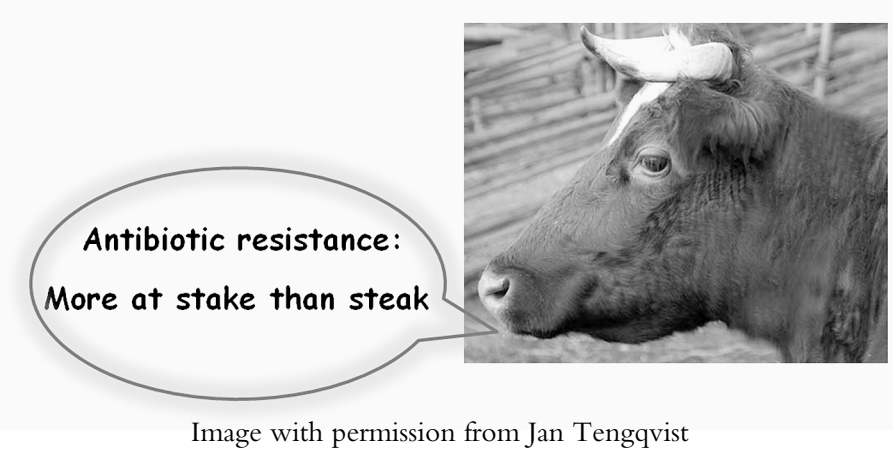

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Enzymes in Thymidylate Synthesis in *Ureaplasma parvum* as Medical Targets.

Abstract

The wall less bacterium *Ureaplasma parvum (Up)* is associated with ureathritis in adults and pneumonia in neonates. *Up* lack *de novo* nucleotide synthesis genes and has to import all DNA precursors. This thesis investigates known DNA biosynthesis pathways as targets for new antibiotics and concerns two enzymes in *Up* thymidylate synthesis; a thymidylate synthase (TS) and thymidine kinase (*Up*TK).

TS activity was detected in *Up*-extracts and UU572 DNA could rescue a TS mutant *E. coli*. UU572 appeared to be proteolytic cleaved and cell cycle regulated in *Up*. Codon modified UU572 was cloned for expression in *E. coli*. However, no protein expression could be detected. A codon optimized synthesized UU572 homolog; MPN358 from *Mycoplasma pneumonia* was expressed in *E. coli* and showed TS activity. Low sequence homology to existing TSs suggests that UU572 and its homologs, belong to a new class of TS enzymes, which may contribute to future antibiotic development in human and veterinary medicine.

Thirteen click chemistry-synthesized 3´-triazole thymidine analogs (**1-13**), using AZT as backbone, were evaluated with *Up*TK and hTK1. The bacterial TK exhibited a more open 3D structure than hTK1 explaining its substrate efficiency, while hTK1 seemed to have more closed structure as reflected by higher inhibition by the analogs. Docking models with **13** in TK1 structures revealed amino acid substitutions in the active site and most likely explain the different enzyme specificity. In addition, molecular docking could explain the 6-fold higher inhibition by the nucleoside analog 3´-azido-methyl-deoxythymidine (AZMT) with *Up*TK compared to hTK1. Nucleoside analogs have been used for fighting viruses with minimal side-effects. Why not use this strategy to control bacterial infections? The results presented in this thesis contribute towards attaining this goal.

Keywords: Thymidine kinase 1, Thymidylate synthase, Ureaplasma, nucleoside analogs, structure-activity relationship, Salvage pathway, AZMT, AZT

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To my family

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Wehelie Rahma, Lin Jay, Swedberg Göte, Eriksson Staffan and Wang Liya. (2009) Identification and characterization of *Ureaplasma parvum* thymidylate synthase. (*Manuscript*)

II Lin Jay, Roy Vincent, Wang Liya, You Li, Deville-Bonne Dominique, Agrofoglio Luigi, and Eriksson Staffan. (2009) Synthesis of 3´ triazole thymidine nucleoside analogs and their use as substrates for human and *Ureaplasma parvum* thymidine kinase for structure-activity investigations. (*Manuscript)*

Abbreviations

Enzymes/Proteins

(d)NDPK	(deoxy) ribonucleoside diphosphate kinase
(d)NMPK	(deoxy) ribonucleoside monophosphate kinase
dAK	deoxy adenosine kinase
*Dm*dNK	*Drosophila melanogaster* dNK
dNK	deoxyribonucleoside kinase
dCK	cytoplasmic deoxycytidine kinase
dGK	mitochondrial deoxyguanosine kinase
DHFR	Dihydrofolate reductase
hTK1	Cytosolic human thymidine kinase 1
HSV1-TK	*Herpes simplex* virus 1 TK
PRT	Phosphoribosyltransferases
RecA	Recombination protein A
RNR	RiboNucleotide Reductase
TS	Thymidylate Synthase
TK	Thymidine kinase
ThyA	Traditional thymidylate synthase
ThyX	Flavin-dependent Thymidylate synthase
TK2	Mitochondrial Thymidine Kinase 2
*Up*TK	*Ureaplasma parvum* TK

Nucleosides/Nucleotides

ATP	Adenosine triphosphate
dA	deoxyadenosine

dC	deoxycytidine
dG	deoxyguanosine
dN	deoxyribonucleoside
dNDP	deoxyribonucleoside diphosphate
dNMP	deoxyribonucleoside monophosphate
dT	deoxythymidine (Thymidine)
dTTP	deoxythymidine triphosphate
dU	deoxyuridine
$FADH/H_2$	Flavin adenine dinucleotide
$NADH/H_2$	Nicotinamide adenine dinucleotide
NMP	ribonucleoside monophosphates
NDP	ribonucleoside diphosphates

Nucleoside analogs

5-FdUMP	5-fluorodeoxyuridine monophosphate
5-FdUrd	5-Flouro deoxyuridine
AZMT	3´-azido-methyl-deoxythymidine
AZT	3´-azido-2´,3´dideoxythymidine
ddT	2´,3´-dideoxythymidine
NA	Nucleoside analog
AZMT	3´-Azido methyl thymidine
AZT	3´- Azido thymidine
dFdC	2',2'-difluorodeoxycytidine (Gemcitabine)
FIAU	2´-Flouro-arabinosyl-5-iodo-dU
FMAU	2´-Flouro-arabinosyl-5methyl-dU

Other

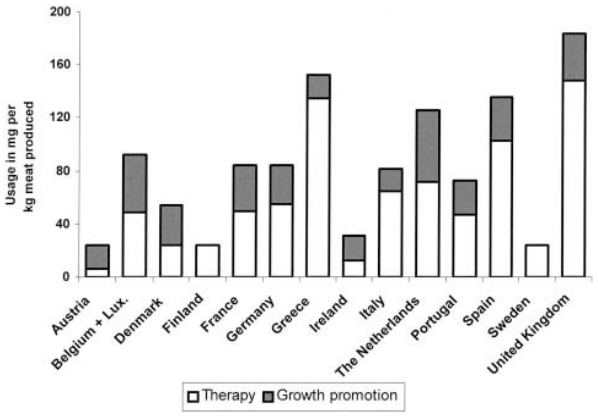
BPD	Bronchopulmonary dysplasia

1 Introduction

1.1 The need for new alternative antimicrobial agents

In order to survive, all living organisms strive to adapt to their environment. Thus, it should not be surprising to us that bacteria have shown a remarkable ability to endure and adapt to their environment, as they have done several eons before the human species appeared on Earth. The end results of this phenomena is that many bacteria have evolved resistance to almost all antibiotics used today and the problem is highlighted by the emergence of multiple-drug resistant strains-so-called "super-bugs" (Brown, 2006). Most classes of antibiotics were discovered during the 1940-1950s, and are directed against specific areas of bacterial physiology, mainly cell wall, DNA and protein biosynthesis. At that time, it seemed possible to eradicate most of the common infectious diseases from humanity. However, early signals of bacterial resistance and warning from the penicillin discoverer Sir Alexander Fleming confirmed that the bacterial eradication only was a dream. Chemical modification of existing antibiotics represents current antibiotic development. It is believed that widespread bacterial drug resistance is due to the limited choice of antibiotics and wide spread use of these drugs causing bacterial strain selection with resistance properties (Brown, 2006). What are the risk factors for the development of antibiotic resistance? Overuse of antibiotics in hospitalized patients, high availability of antibiotics without prescriptions, large amounts of antibiotics in agriculture industry, longer life expectancy with increased need of antibiotics in elderly. Modern food animal production is heavily depended on the use of massive amounts of antibiotics for animal disease control; Table 1 illustrates the use of antibiotics in production of one kilogram of meat in different EU-countries.

Table 1. *Estimated milligram of antimicrobial agents used in 1997 per kilogram produced meat in different countries of the EU (Source EMEA 1999).*



This favors the selection, spread and persistence of drug resistant bacteria from farm animals to humans and products of animal origin are traded worldwide. Thus, antimicrobial resistance in one country is a problem for all countries and difficulties in obtaining reliable information about drug consumption makes it more difficult to tackle resistance problems (Brown, 2006). This emerging drug resistance now poses a threat to both human and animal health and should be taken seriously. Development of new antibiotics is inevitable because we are moving towards an environment of ever-growing numbers of new infectious diseases. The net-result could be increased morbidity, mortality and community cost.

1.2 Bacteria in focus: *Ureaplasma parvum* (*Up*)

The smallest free living organisms known today are bacteria belonging to class of *Mollicutes* (*mollis* = soft and *cutis* = skin), which are widespread in nature and involving members from the genera *Mycoplasma* and *Ureaplasma*. Many species from these genera are both of human and veterinary medical importance (Razin *et al.*, 1998). These microbes are slow growing facultative anaerobes; many species need fatty acids and cholesterol for their growth, as well for osmotic and morphological stability. Fastidious growth requirements are usually met by complex medium containing yeast extract and serum, however sometimes these can be toxic for the organisms. It is therefore difficult to identify the right species for correct clinical treatments

for both humans and animals (Razin *et al.*, 1998). The bacterium *Ureaplasma urealyticum* (*Uu*) containing 14 serotypes, was found in the human ureogenital tract over 50 years ago (Robertson & Stemke, 1982; Shepard, 1956; Shepard, 1954). These are self replicating entities that grew at low pH with optimal growth at 6.0-6.5 and in presence of urea (Ford *et al.*, 1962). After prolonged debate, the serotypes were then divided into two groups; biovar 1 and biovar 2, because of different phenotypic markers such as antigentic types, polypeptide patterns, ribosomal RNA etc. They were later separated into two distinct species: *Ureaplasma parvum* (*Up*) (Serovars 1,3, 6 and 14) with smaller genomes and the rest of the serovars as *Ureaplasma urealyticum* (Kong & Gilbert, 2004; Robertson *et al.*, 2002).

Up is classified as an opportunistic human pathogen found in up to 80 % of all adults; it colonizes urogenital tract mucosa as well as the respiratory tract. Especially in neonates it is vertical transmitted from the mother (Pollack, 2001). *Up* is associated in a wide array of conditions affecting females and males, including non-gonococcal urethritis (NGU), infertility, prostatitis, spontaneous abortion, premature birth, meningitis and severe respiratory disease in newborns called bronchopulmonary dysplasia (BPD) (Schelonka & Waites, 2007; Waites *et al.*, 2005; Volgmann *et al.*, 2005; Pollack, 2001; Cassell *et al.*, 1993). Recent research in the medical field is concentrated on exploring its mechanisms for pathogenesis, especially its role as neonate pathogens. Lack of documentation for *Ureaplasma* eradication following completion of treatment and increased neonate sensitivity for side-effects has resulted in that no specific antibiotics against *Ureaplasma* is available today. However macrolides such as erythromycin is the most common choice of treatment, but with varying results (Waites *et al.*, 2005). Therefore there is a need for more specific anti-ureaplasma agent with less side-effects.

Up has a circular chromosome consisting of 750 kb and *Ureaplasma* like *Mycoplasma* have been exposed to degenerative evolution by significant loss of genome sequences (Razin *et al.*, 1998). Common properties are the lack of cell wall and are thereby resistance to most common antibiotics; evolved from gram positive bacteria (Glass *et al.*, 2000); high AT-ratio of ~70%; small genomes e.g. ~580 kb *Mycoplasma genitalium* (*Mg*); small genomes give rise to limited biochemical pathways and requires an host to obtain all nutrients. This class use stop-codon UGA as tryptophan, which is usually recognized as a stop codon. Therefore it is extremely difficult to express many *Mycoplasma* genes using *E. coli* as expression system. A way to avoid

this problem is to use an *E. coli* strain (ISM612) with modified tRNA inserting tryptophan into UGA codons. This is widely used for studying *Mycoplasma* proteins, however only <40% of the UGA codons are read, affecting the expression levels (Minion, 1998). However, this approach is still not able to compensate for the AT-ratio effect, resulting in low protein expression.

Genome sequencing and other observations have surprisingly revealed that *Mollicutes* in general exhibit limit capacity to synthesize DNA and RNA precursors (Vasconcelos *et al.*, 2005; Glass *et al.*, 2000). Almost all *Mollicutes* are unable to synthesize the DNA building blocks from scratch, therefore species like *Ureaplasma parvum* has to import all DNA precursors or have other converting mechanisms (Glass *et al.*, 2000; Mitchell & Finch, 1977). In addition, *Ureaplasma* also has the unique ability to harvest 95% of all energy by using urease to hydrolyze urea to ammonium ion production (Razin *et al.*, 1998; Smith *et al.*, 1993).

1.3 Overview of DNA precursor biosynthesis

A balanced supply of the four DNA building blocks (figure 1A) i.e. the deoxyribonucleoside triphosphates (dNTPs), is necessary to maintain and transmit the genetic information to the next generation. This rule involve also viruses, however some have a RNA- rather than DNA as genetic material. DNA precursors are synthesized by two pathways: the *de novo* or the salvage pathways (figure 1B).

The *de novo* synthesis uses different enzymes for numerous reactions with small molecules like amino acids, phosphoribosylpyrophosphate (PRPP), CO_2, ATP and NH_3 to build up ribonucleoside monophosphates (NMPs). These NMPs are further phosphorylated to ribonucleoside diphosphates (NDPs), which can be reduced to deoxyribonucleoside diphosphates (dNDPs) by the key enzyme ribonucleotide reductase (RNR), which uses an extraordinary protein radical for this catalytic process. Because RNR is rigorous allosterically and cell cycle regulated, its role as a regulator in the *de novo* synthesis is vital and conserved in most organisms (Nordlund & Reichard, 2006).

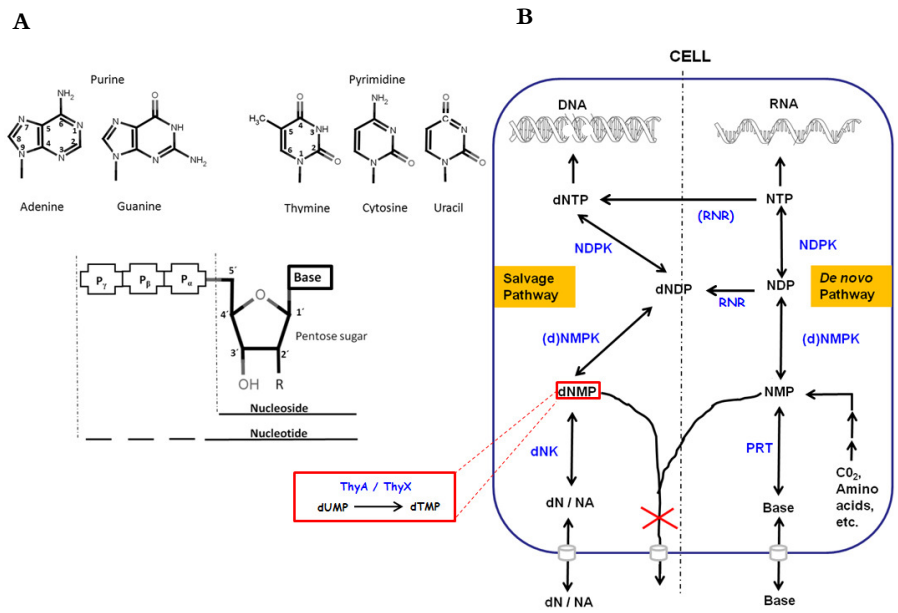


Figure 1. A) Components of nucleoside, consisting of a base (either a purine or pyrimidine), sugar and phosphate-group/s for nucleotides. ***B***) Schematic routes for nucleotide synthesis. The salvage nucleotide synthesis pathway on the left panel and *de novo* nucleotide synthesis on the right panel. Enzymes are in blue and other reactants are in black. RNR has also been observed in NTP to dNTP conversation (Nordlund & Reichard, 2006) and recently a new thymidylate synthase named ThyX was discovered (red box) (Myllykallio *et al.*, 2002).

Uracil, a non-methylated precursor of thymine is not a component of DNA, therefore an additional step are required for converting uracil to thymine. The synthesis of dTMP from dUMP is catalyzed by thymidylate synthase (ThyA), which is an important enzyme conserved in many organisms (Costi *et al.*, 2005; Costi, 1998). Recently a novel thymidylate synthase, ThyX was discovered (Myllykallio *et al.*, 2002), however, no gene coding for ThyA or ThyX was detected in the *Up* genome (Glass *et al.*, 2000). Previous studies revealed that phosphorylated deoxyuridine (dU) could be further converted to dTTP and incorporated into *Up* DNA indicating a TS activity (Carnrot *et al.*, 2003). This activity will be further investigated in paper I.

The alternative pathway; the salvage synthesis, occurs in all cells complementing the *de novo* synthesis and recycles deoxyribonucleosides from degraded DNA. The first phosphorylation of the deoxynucleosides

(dNs) is performed by deoxynucleoside kinases (dNKs). This is often the rate-limiting step in this pathway (Arner & Eriksson, 1995), trapping the negative charged nucleoside within the cell. Our group showed that *Up* phosphorylated all natural dexonucleosides (Wang *et al.*, 2001). Later characterization and structural crystallization revealed two enzymes; a thymidine kinase and a deoxyadensine kinase responsible for these catalytic events (Welin *et al.*, 2007; Welin *et al.*, 2004; Carnrot *et al.*, 2003). Just as in the *de novo* pathway, (deoxy)ribonucleoside monophosphate kinases, (d)NMPKs and ribonucleoside diphosphate kinases (NDPKs) phosphorylate the last two steps giving the dNTP is used for DNA replication and repair (Arner & Eriksson, 1995). However no homolog of NDPKs is found in the *Up* genome (Glass *et al.*, 2000). Recently, it was observed that *Up* ribonucleoside monophosphate kinases (NMPKs) exhibited diphosphate kinase activities, which strongly indicated that these enzymes could substitute the NDPK function in vivo (Wang, 2007).

Most organisms have both DNA precursor synthesis pathways, however bacteria like *Ureaplasma parvum* (*Up*) has no gene for RNR (Glass *et al.*, 2000) and therefore has to rely on salvage enzymes to maintain balanced dNTP pools for replication and repair (Carnrot *et al.*, 2003; Wang *et al.*, 2001). Free purine and pyrimidine bases recycling are also part of the salvage pathway, but will not be further described here.

1.4 Thymidylate synthase (TS)

Thymidylate synthase (ThyA), an enzyme with catalytic and regulatory functions, converts dUMP by reductive methylation to a DNA precursor dTMP. ThyA was considered the only enzyme responsible for *de novo* dTMP formation, however comparative genomics revealed that a large number of microbial genomes apparently lack several pathways for *de novo* and salvage synthesis of dTMP (Myllykallio *et al.*, 2002). In 1989 a gene of unknown function was implicated in the thymidine metabolism of *Dictyostelium discoideum* by genetic complementation tests (Dynes, 1989). Myllokallio *et al.*, (2002) later proved experimentally that these enzymes possess a flavin-dependent thymidylate synthase activity both *in vivo* and *in vitro,* and was designated ThyX. Although both ThyA and ThyX are N5, N10-methylenetetrahydrofolate (CH_2H_4folate) dependant enzymes, they differ regarding their reductive mechanisms. These enzymes likely have different evolutionary origins, since both enzyme classes lack sequence and

structural similarities to each other (figure 2) (Mathews *et al.*, 2003; Myllykallio *et al.*, 2003; Murzin, 2002; Myllykallio *et al.*, 2002).

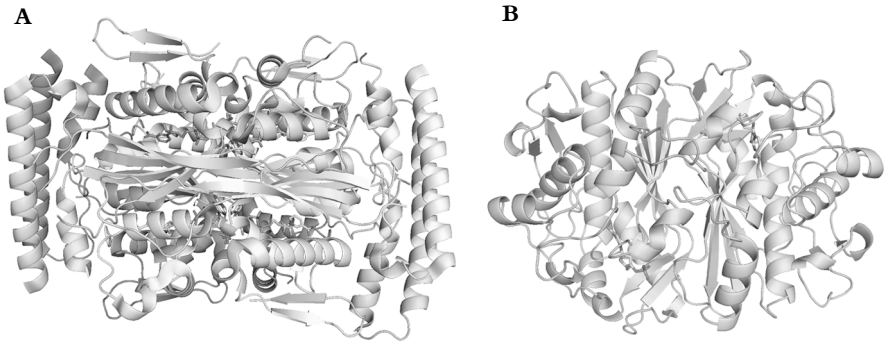


Figure 2. **A**) Homotetramer of *Mycobacteria tuberculosis* ThyX (*Mt*ThyX) (2GQ2) **B**) Dimer of *E. coli* ThyA

1.4.1 Traditional thymidylate synthase (ThyA)

The classical thymidylate synthase (ThyA) is active as a dimer and catalyze the *de novo* production of dTMP *in vivo*. The enzymatic step of dTMP synthesis from dUMP is conserved in most living organisms. ThyA uses N5, N10-methylenetetrahydrofolate as one-carbon donor and reducing agent resulting in dTMP and dihydrofolate (H_2folate) production (figures 3-4), (Costi *et al.*, 2005; Costi, 1998).

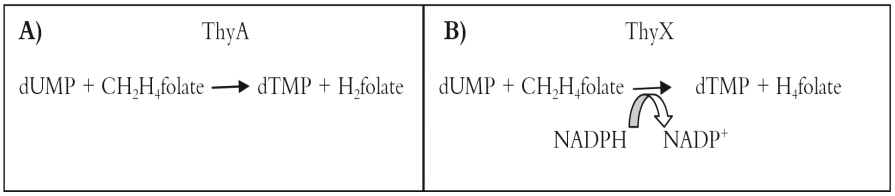


Figure 3. Schematic reaction mechanisms of different thymidylate synthases **A**) ThyA and **B**) ThyX.

Reduced folates are essential for many biological functions, H_2folate is therefore rapidly reduced by NADPH to tetrahydrofolate (H_4folate) in reaction by dehydrofolate reductase (DHFR) (Myllykallio *et al.*, 2003; Costi, 1998).

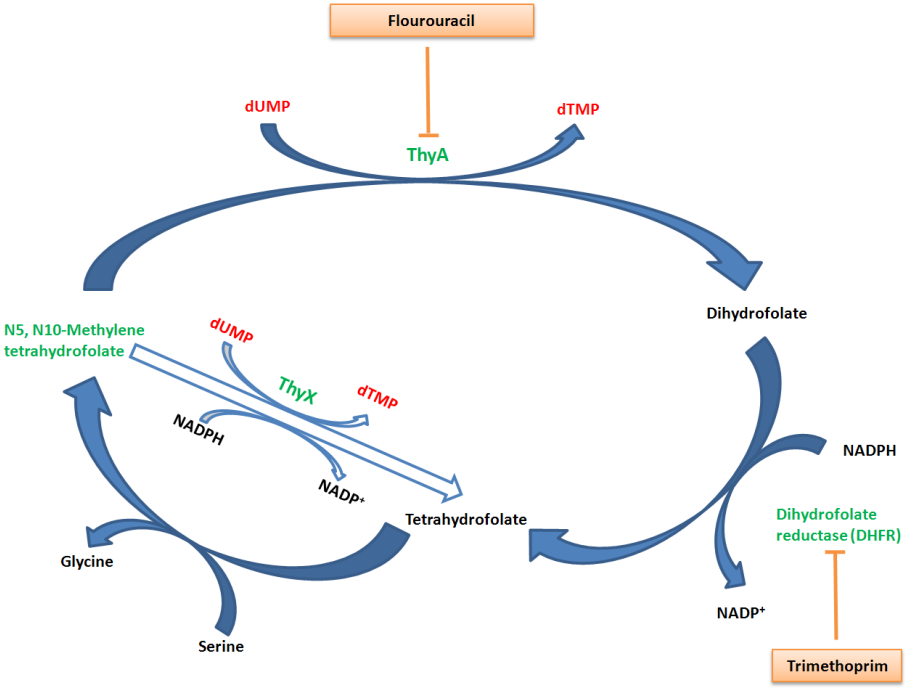


Figure 4. Schematic representation of dTMP synthesis in the dUMP-folate cycle involving ThyA or ThyX (enzymes are in green). Although both produce dTMP, each enzyme uses a different reductive mechanism.

DHFR is tightly coupled to ThyA, so if the ThyA gene is observed then there is also a DHFR gene. Both enzymes have been used widely as drug targets for development of antimicrobial and anticancer agents. For example bacterial DHFR is specifically inhibited by trimethoprim. The anticancer pro-drug 5-flourouracil (5-FU), exerts its toxic activity in the deoxyribonucleotide form (5-FdUMP); binds to ThyA and acts as a suicide inhibitor (figure 4), (Costi *et al.*, 2005; Costi, 1998)

1.4.2 Newly discovered thymidylate synthase

ThyX; a flavin-dependent thymidylate synthase, was first characterized by Myllykallio *et al.*, (2002). ThyX genes are found in many pathogenic bacteria and several double stranded DNA viruses (Graziani *et al.*, 2006; Zhong *et al.*, 2006; Griffin *et al.*, 2005; Sampathkumar *et al.*, 2005; Graziani *et al.*, 2004; Myllykallio *et al.*, 2003; Myllykallio *et al.*, 2002). Its activity relies on NADH/NADPH oxidation and uses tetrahydrofolate (CH_2H_4folate) as one-carbon donor and as reducing agent pyrimidine nucleotide called flavin adenine dinucleotide ($FADH/H_2$) to form dTMP

and tetrahydrofolate, (figure 3B) (Ulmer *et al.*, 2008; Gattis & Palfey, 2005; Griffin *et al.*, 2005; Graziani *et al.*, 2004; Leduc *et al.*, 2004; Myllykallio *et al.*, 2002). ThyX contains the motifs -T/RHRX$_{7-8}$S- and the majority of ThyX proteins are composed of around 200-250 amino acids. However homologues of *Chlamydia* (*Ct*ThyX) and *Thermoplasma* species are larger, containing two fused ThyX domains in the same protein suggesting duplication events have taken place several times independently (Sampathkumar *et al.*, 2006; Griffin *et al.*, 2005; Mathews *et al.*, 2003).

1.5 Thymidine kinase 1, thymidine kinase 2 and other deoxynucleoside kinases

The dNKs are found in a wide range of species with different quantities and are enzymes catalyzing the first step in the salvage pathway. In mammals, there are four dNKs with overlapping substrate specificities for the four DNA precursors: cytosolic thymidine kinase 1 (TK1) phosphorylates thymidine (dT) and dexoxyuridine (dU); cytoplasmic deoxycytidine kinase (dCK) phosphorylates deoxycytidine (dC), deoxyadenosine (dA) and deoxyguanosine (dG); mitochondrial deoxyguanosine kinase (dGK) phosphorylates dA and dG; and mitochondrial TK2 (TK2) phosphorylating both dT and dC (Eriksson *et al.*, 2002). Many nucleoside triphosphates have the ability to act as phosphate donors, whereas the universal biological currency ATP is accepted by all dNKs. dNKs use a general phosphorylation mechanism illustrated in figure 5.

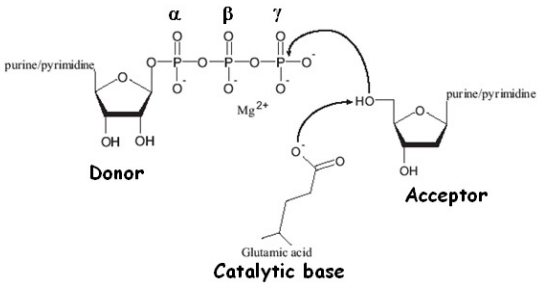


Figure 5. Common catalytic reaction mechanisms for phosphate group transfer by dNKs. Modified illustration from (Eriksson *et al.*, 2002).

Usually a glutamatic acid or aspartic acid act as base and starts the reaction by deprotonation of hydroxyl group of the sugar. The activated 5´-OH performs a nucleophillic attack on the γ-phosphate of ATP (donor) enabling the transfer of the phosphate group to a nucleoside (acceptor) and dNMP

and ADP are produced (Eriksson *et al.*, 2002; Johansson *et al.*, 2001). Their activities are regulated by feedback inhibitors, the end-product triphosphate of the preferred nucleoside, which binds to the active site as bisubstrates, occupying both acceptor and donor site of the specific dNK in question, see figure 9A-B (Birringer *et al.*, 2005; Kosinska *et al.*, 2005; Welin *et al.*, 2005; Welin *et al.*, 2004; Carnrot *et al.*, 2003; Eriksson *et al.*, 2002). For example: TK2 phosphorylates dT and the dTTP subsequently formed at later stage will then act as a feedback inhibitor (Eriksson *et al.*, 2002; Arner & Eriksson, 1995).

Structural studies of dCK, dGK, *Drosophila melanogaster* dNK (*Dm*dNK) and *Herpes simplex* virus 1 TK (*HSV1*-TK) revealed similar 3D-folds, and TK2 is also believed to exhibit a similar fold. These kinases are considered as one superfamily, the dNKs (Sandrini *et al.*, 2006; Sandrini & Piskur, 2005). The TK1 family shares little sequence similarity and the first structural studies of hTK1 and *Up*TK (Birringer *et al.*, 2005; Welin *et al.*, 2004) reveled a different structure than the dNK family, indicating a different evolution origin forming another superfamily (Sandrini *et al.*, 2006; Sandrini & Piskur, 2005). However, both superfamilies share a common phosphate-binding loop motif (P-loop). Extensive studies have been done with all mention kinases the last few decades with the aim of understanding their role as precursor-supplier for repair, maintenance and DNA replication.

1.6 Properties of the TK1 family

The TK1 family is widely distributed in most organisms, in addition, also exist in some viruses. The TK1-like enzyme is strictly pyrimidine nucleoside specific and catalyzes the irreversible phosphorylation of dT to dTMP and uses ATP as phosphate donor and feedback inhibited by dTTP (Eriksson *et al.*, 2002). In the following section, members of the TK1 family from bacterial and human origin will be discussed.

1.6.1 Bacterial TKs

Ongoing microbial genome sequencing projects have revealed that many bacterial species posses TK1 genes, but there are also many lacking them. The first biochemical characterized bacterial TK was from *Escherichia coli* (*E. coli*), where it is the only one present (Karlstrom, 1970). From a phylogenetic point of view TK1-like enzymes can be arranged into two separate groups; Group 1 containing gram positive bacteria and eukaryotic

TK1s, while group 2 is categorized as TKs from gram negative bacteria (Sandrini *et al.*, 2006). Several conserved amino acids and motifs in gram negative TKs are different e.g. serine substitutions in the P-loop, cystein residues in the magnesium binding motif and loss of an amino acid in one of the zink motifs upstream of the lasso loop (figure 6).

Figure 6. Multiple sequence alignment of different TK sequences from gram positive bacteria *Ureaplasma parvum* (*Up*) (gi|13358159), *Bacillus anthracis* (*Ba*), human (gi|339719), *Vaccinia virus* (*Vv*) (gi|47088414) and gram negative bacterium *Escherichia coli*. (*E.coli*) (gi|16129199). The P-loop, Mg-binding, Zn-binding, KEN motifs and *Up*TK Cys183 are boxed. Triangles indicates residues forming main chain hydrogen bonds with the nucleoside, "*"identical residues in that column in all sequences in the alignment column, ":" conserved substitution and "." semi-conserved substitution.

The starting methionine is always located nine residues upstream of the P-loop in group 2, while there is variation in group 1. The implication of these changes remains unclear because no structure is available for group 2 TK1. However, it is expected that this group share the same overall fold, but many differences are probably found in the subunit-subunit interactions and kinetic parameters (Sandrini *et al.*, 2006).

TKs from *Ureaplasma parvum* (*Up*TK) and *Bacillus anthracis* (*Ba*TK) have been cloned, purified and characterized (Carnrot *et al.*, 2006; Carnrot *et al.*, 2003). *Up*TK shares only 29 % sequence identity with hTK1 and 44 % with *Ba*TK. They all share similar enzymatic properties with hTK1 despite the amino acid differences, e.g. they only phosphorylate dT and dU of the natural substrates. All are feedback inhibited by the end-product dTTP, but there are some properties of *Up*TK that are different, which involves sensitivity to salts and detergents (Carnrot *et al.*, 2003). hTK1 showed positive cooperativity with ATP (Munch-Petersen *et al.*, 1993), while *Up*TK demonstrated classical Michaelis-Mentan kinetics (Carnrot *et al.*, 2003). This might indicate that the subunit-subunit interactions in the tetramer are different than in hTK1. Despite similar substrate specificities between the TK1 enzymes, they exhibit different phosphorylation efficiencies and inhibitory properties, which can be seen especially with certain nucleoside analogs. In this thesis we further explore substrate and inhibitory properties of dT analogs with 3´-substitutions to achieve a better understanding of the structure function relationship of this enzyme family (Kosinska *et al.*, 2007; Carnrot *et al.*, 2006; Kosinska *et al.*, 2005).

1.6.2 Human thymidine kinase 1 (hTK1)

The hTK1 gene is located on chromosome 17 (Petty *et al.*, 1996) encoding a polypeptide of 234 amino acids representing a molecular weight of 25,5 kDa (Munch-Petersen *et al.*, 1991). It accepts only the natural nucleosides dT and dU as substrates. hTK1 uses all nucleoside triphosphates as donors except the feedback inhibitor dTTP, and ATP and dATP are the favored donors (Eriksson *et al.*, 2002). TK deficient mice are viable, but have increased mutational rates, abnormal immune system and develop a fatal kidney disease for unknown reason (Dobrovolsky *et al.*, 2003). TK1 activity is virtually absent in resting cells, but the expression level is highest in late G1, early S-phase cells and then drops to almost undetectable levels at the end of mitosis (Sherley & Kelly, 1988). This cell cycle regulation was not seen with the other human dNKs. It was showed that the degradation of hTK1 occurs through an ubiquitin ligase complex APC/C-Cdh1 targeting hTK1 by recognizing a KEN-box located within the last thirty residues of the C-terminal (Ke & Chang, 2004). This motif is absent in bacteria and viral-TK sequences explaining their shorter C-terminals (figure 6). Besides this posttranslational regulation, hTK1 can interchange between its dimeric and tetrameric forms representing low and high catalytic forms. This dimer/tetramer interchange in hTK1 is most likely a fine-tuning mechanism

to provide a balanced supply of dTTP for DNA replication (Li *et al.*, 2004; Munch-Petersen *et al.*, 1995; Munch-Petersen *et al.*, 1993). TK1 can phosphorylate several nucleoside analogs and the modification accepted are as follows: small modifications replacing the methyl group, such as halogens, ethyl, and no group at 5-position of the base are good substrates, however bulkier or more polar substitutions drastically decreases the activity (Al-Madhoun *et al.*, 2004; Johansson & Eriksson, 1996; Munch-Petersen *et al.*, 1991).

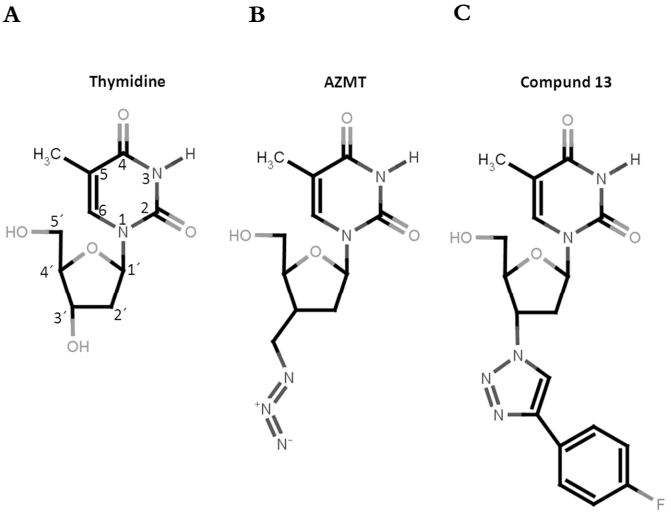


Figure 7. **A)** The structure composition of dT and 1-6 refers to the numbering position of the base and 1´-5´refers to the atom numbering of the sugar. Substitutions are done at 3´-position of the dT-sugar, replacing the existing OH-group e.g. dT analogs tested with TK1 involving **B)** AZMT and **C)** Compound **13** with 3´triazole-phenyl moiety.

Substitutions at the 3-position of the base have adequate activities (Al-Madhoun *et al.*, 2004). Fluctuating activities are seen with 3´ substitutions in comparison to dT, e.g. 50% for AZT, 40% for ddT (2´,3´-dideoxythymidine) and bulkier substitutions, and 15% for AZMT (3´-azido-methyl-dT) (figure 7B) (Al-Madhoun *et al.*, 2004; Johansson & Eriksson, 1996). 2´-Flouro-arabinosyl-5-iodo-dU (FIAU) and 2´-Flouro-arabinosyl-5methyl-dU (FMAU) are the only exceptions of arabinosyl sugars that are accepted by TK1 (Al-Madhoun *et al.*, 2004). The 3´-position substitutions are further explored with new NAs (figure 7B-C) and taken to the next level in this thesis to further understand activity-structure relationship between TK1 from human and bacterial origin.

1.6.3 The 3D structures of TK1 family

The first structural studies of hTK1 enzymes and bacterial *Up*TK (Birringer *et al.*, 2005; Kosinska *et al.*, 2005; Welin *et al.*, 2004) with the feedback inhibitor dTTP or dT (Kosinska *et al.*, 2005) revealed that the active site was positions between two domains, the lasso domain covering the substrate/dTTP base that is buried deep in a hydrophobic pocket. The Zn^{2+}-ion is believed to have only a structural role, which provides stability to the whole structure (Segura-Pena *et al.*, 2007b; Birringer *et al.*, 2005; Welin *et al.*, 2004). All interactions with the base and sugar occurred with main chain hydrogen bonds in the Zink-lasso domain and the second α/β-domain (figure 8).

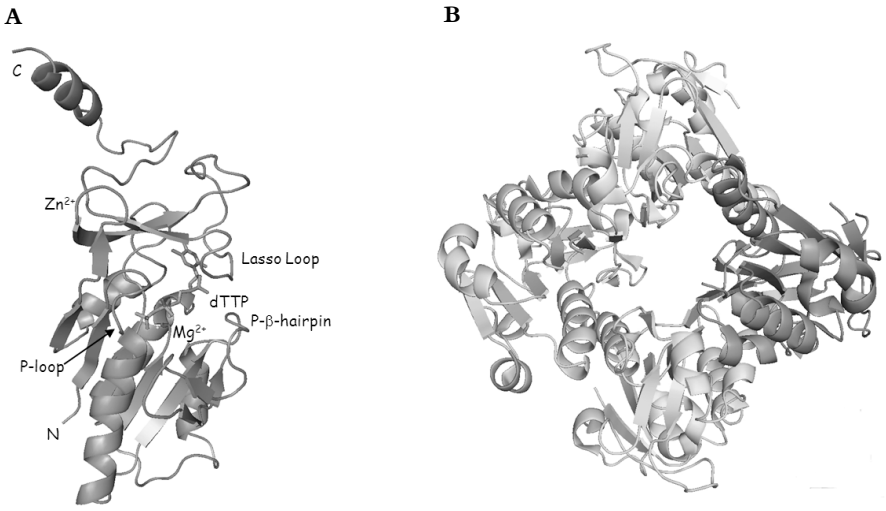


Figure 8. **A**) A monomer of *Up*TK with dTTP (2U3Z). **B**) A Tetramer of hTK1 (1W4R).

The later domain contained six central parallel β-sheets surrounded by one long α-helix and three shorter helicase on the other side. Further investigations have shown that this domain is more similar to a domain in the RecA proteins (Welin *et al.*, 2004). There is a conserved phosphate binding motif (P-loop), G/SXMXXGKS/T as a turn connecting β1 to α1. The methionine of the motif is assumed to constrain the 5´-hydroxyl of dT, forcing it to the catalytic base (Segura-Pena *et al.*, 2007b). A β-hairpin connects β2 with β3, both secondary structures together with the conserved magnesium binding motif DEXQF with bound Mg^{2+} ion, also contained the catalytic glutamic acid and all are participating in the phosphate transfer from ATP to 5´OH-dT (figure 6 and figure 8).

During the catalytic event the subunits of the enzymes remain largely unchanged; however two flexible regions, lasso loop and P-β hairpin undergo conformational changes upon acceptor (dT) and donor (ATP) binding. Bound dT provides binding partners for the residues of the lasso loop, so that it becomes visible in the structure, while the binding of ATP is to the P-β hairpin folding. dTTP has also the ability to organize both the lasso loop and part of the P-β hairpin (Kosinska *et al.*, 2007; Segura-Pena *et al.*, 2007a; Segura-Pena *et al.*, 2007b; Birringer *et al.*, 2005; Kosinska *et al.*, 2005; Welin *et al.*, 2004). Currently no TK1-like structures with its natural substrates dT and ATP bound simultaneously are known. However ATP analogs with dT bound to TK1 revealed that the flexible regions remain the same as with the bound dTTP. Differences in the quaternary structure was observed, where the tetramer form of TK1 display a more open state when the ATP site between the monomers is occupied (Kosinska *et al.*, 2007; Segura-Pena *et al.*, 2007a; Segura-Pena *et al.*, 2007b).

1.7 Medical interests in the salvage enzymes

All organisms, including viruses have to reproduce their genomes for survival and many studies have revealed the importance of functioning deoxyribonucleoside salvage enzymes. Humans with deficiencies in mitochondrial dNKs (TK2/dGK) suffer from multiple-system failure (Eriksson & Wang, 2008; Wang *et al.*, 2005; Saada *et al.*, 2001). These enzymes are also important from a medical point of view and many drugs are nucleoside analogs (NA) targeting cancers and viral diseases. NA performs their effects after activation by cellular dNKs from pro-forms to triphosphate forms. These NAs mimic building blocks of DNA by impairing the metabolism and structure of nucleic acid. A typical example is the pro-drug AZT (Zidovudine®), which is activated by cellular TK1 and blocking this replication by chain termination (Sperling, 1998). Another example is the pro-drug Acyclovir (Zovirax®), which is highly selective for *Herpes* virus treatment with almost no side-effects (Sheffield *et al.*, 2003). Some dNKs can be used as a diagnostic marker; e.g. there is an increased TK1 level in sera of cancer patients indicating rapidly growing tumor cells (Chen *et al.*, 2008; Ondrej & Lubos, 2008; Li *et al.*, 2005). Other application of dNKs is suicide gene therapy, which aims to increase the selectivity of pro-drugs for tumor cells (Niculescu-Duvaz & Springer, 2005). The approach is based on that tumor cells are transfected by a specific drug-activating enzyme, accompanied by supply of the prodrug.

The transfected cells will then be wiped out by the cytotoxic effects of activated prodrugs. However, delivery of the genes and control of gene expression are currently problems to be solved and is still under development. Cerapro® (Ark Therapeutics) is an example of suicide gene therapy now in phase III trails, based on Herpes TK in an adenoviral vector for treatment of brain cancer (malignant glioma).

Fighting viruses and tumor cells with nucleoside analogs has been very successful, so why not use this principle to fight bacteria? This is especially with the increase in multi-resistant bacteria. Studies with NA used in treatment of cancers and viral infections were tested with some pathogenic bacteria. It was concluded that these NAs are activated by bacterial dNKs in a species-specific manner, e.g. adult mice were infected with a fatal dose of pathogenic *Streptococcus pyogenes* and afterwards received the NA gemcitabine ((2',2'-difluorodeoxycytidine (dFdC)), preventing it to develop into a systematic infection (Sandrini *et al.*, 2007a; Sandrini *et al.*, 2007b). Thus, nucleoside analogs have a potential to be employed as antibiotics combating emerging multi-resistant bacteria.

1.8 Computational biochemistry

Most drug companies use computational methods as a first approach in compound screening through molecular docking. Docking refers to a computational method optimizing the orientation between two biomolecules (protein and ligand) within a binding site by energy calculations (posing). This approach is often followed by ranking (categorizing estimated free energy) and scoring (rough measure of the fit of a ligand into active site) (Kitchen *et al.*, 2004). Additional data (e.g. biochemical, mutational, etc.) may improve the performance and provide more correct biological explanations. In this thesis we use the molecular modeling and drug docking software Arguslab in combination with biochemical data to explain possible sequence-structure-function relationship with 3´-substituted dT analogs with the thymidine kinase structures from both bacterial and human origin. This approach is applied in paper 2, however a more extended application is illustrated in the 2.2.3.

Arguslab 4.0 is distributed freely for the windows platform by Planaria software and it has become an appreciated molecular docking package mainly because of its user-friendly interface (Thompson, 2004; Mark A. Thompson). Its internal docking engine, Argusdock, approximates an exhaustive search method with similarities to other docking tools such as DOCK and GLIDE. In addition, Arguslab also has a genetic algorithm (GA)

as an option, which is another search technique to find exact or approximate solutions to optimization and search problems. However, this is a modified GA, called a Lamarckian genetic algorithm (LGA) handling both local and global searches (Thompson, 2004; Morris *et al.*, 1998). Flexible ligand docking is also possible where the ligand is described as a torsion tree, and grids are constructed overlying the chosen binding site. The lowest energy poses are retained and a final set of poses undergoes coarse minimization, re-clustering and ranking (Thompson, 2004; Mark A. Thompson). Compounds were drawn with the program Marvin sketch from the Marvin beans package (http://www.chemaxon.com/) and Smiles Translator to produce the pdb-files. Docking and superimposition results were visualised with the PyMOL graphic system (DeLano Scientific).

2 Present investigations

2.1 Aim

The work done in this thesis aims to understand two bacterial enzymes responsible for thymidylate synthesis in the *Ureaplasma parvum*. The enzymes include a thymidine kinase (TK) and a newly discovered enzyme with putative TS activity. Synthesized 3´ substituted thymidine analogs were tested with recombinant *Up*TK and human TK1 in combination molecular docking for explaining important differences in enzyme activity. Cloned UU572 rescues a thymidine auxotrophic phenotype of a TS deficient *E. coli* phenotype strain. The low sequence homology to existing TSs suggests that *Ureaplasma* UU572 belongs to a new thymidylate synthase family. This information may provide a platform for synthesis of new anti-bacterial agents.

2.2 Results and discussion

Papers I-II in the appendix describes the methods used in more detail and will therefore not be described in this section. The last subchapter 2.2.3 regarding "Computational docking analysis: AZMT docked in *Up*TK and hTK1" will be presented in more detail.

2.2.1 Identification and characterization of *Ureaplasma parvum* thymidylate synthase (Paper I)

Ureaplasma parvum (*Up*) is associated with ureathritis in adults and chronic lung disease diseases in neonates. *Up* lack *de novo* nucleotide synthesis genes and has to import all DNA/RNA precursors. The dTTP production relies on thymidine kinase, but also on an unknown thymidylate synthase (TS), which is neither a *thyA* nor a *thyX* homolog. UU572, coding for a 67.3 kDa protein, rescued the thymidine auxotrophic phenotype of *E. coli* (ISM612) grown on M9 minimum medium. TS activity was detected in extracts prepared from *Up* and the enzyme was partially purified. It catalyzed dUMP to dTMP formation when mixed with 5,10-methylenetetrahydrofolate (CH_2THF), which acted as methyl donor. Homologs to UU572 were also identified in the genomes of *Mycoplasma pneumonia* (*Mpn*) and *Mycoplasma genitalium* (*Mg*). Similar to thymidine kinase, a cell cycle regulated enzyme, time dependent expression of *Ureaplasma* TS activity was observed. Extracts prepared from thyA mutant ISM612 carrying UU572 plasmid was eluted from exclusion chromatography in fractions, corresponding to higher molecular weight than *E. coli* ThyA. Antibodies against the UU572 protein recognized a clear ~35 kDa protein in *Up* extracts, suggesting a proteolytic cleavage.

The UU572 gene was cloned with two mutated (UGA to UGG) codons, preventing protein truncation when using *E. coli* as protein expression system. We tried to express the recombinant UU572 by using the pET system with either N-terminal or C-terminal histidine tags in combination with several bacteria hosts. However, no UU572 protein expression was detected. We suspect that the problem was due to the codon usage (76% A+T) and high hydrophobic amino acid composition (>50%) of the UU572 protein. This combination made expression in *E. coli* systems not possible and may explain why UU572 failed to complement the TS knocked mutant *E. coli* (x2913) strain. To overcome this problem we chose to express the UU572 homolog MPN358 after codon optimization. It was also cloned into several expression vectors and expressed in several *E. coli* hosts. Detectable expression was only observed with pET32b and the Origami (DE3) host strain. Expression optimization resulted in a soluble protein and purified MPN358 fusion protein showed TS activity. The results from this study showed that some *Mollicute* proteins are very difficult to express and study. However, codon optimization is essential to be able to express these types of proteins in *E. coli*.

The low sequence homology to either ThyX or ThyA suggested that *Ureaplasma* TS (UU572) and its homologs, belong to a new thymidylate synthase family. This new enzyme class is an attractive target for development of antibiotics.

2.2.2 Synthesis of 3´- triazole thymydine nucleoside analogs and their us as substrates for human and Ureaplasma parvum thymidine kinase for structure-activity investigations (Paper II)

Both thymidine kinase from *Up* and humans have been crystallized and although the overall structures are similar, some small differences exists. The TK1 family accepts 3´-substituted dT analogs, such as AZT with good activity. AZT can be converted to a closed cyclic form called triazole. Although antimicrobial properties were observed with triazole compounds, 3´-triazole dT analogs lack antiviral activity indicating low activation by intracellular TK. Thirteen click chemistry-synthesized 3´-triazole dT analogs were provided from our collaborators; the Agrofoglio research group. These analogs were used for exploring the substrate and inhibition properties of hTK1 and *Up*TK. The enzymatic properties were then correlated to Arguslab-docked structure complexes for activity-structure relationship analysis.

In this study we show that both kinases were able to phosphorylate many 3´-substitutions to some extent and many achieved 2-10 fold higher efficiency with *Up*TK than with hTK1. Except AZT, compound **13** was the best substrate for both enzymes. The IC_{50}-values for these analogs revealed that hTK1 was more inhibited by these compounds than *Up*TK. However, none of these analogs were very good inhibitors, but may serve as lead compounds for future drug development. Compound **13** was docked in the active site of both enzymes, the results suggested that the 3´-substitutions of **1-13** may be positioned in two different conformations; conformation A, seen in *Up*TK; the 3´-substitution was positioned between the lasso loop and P-β hairpin, and part of the 3´-substitution was most likely exposed to solvent. In conformation B observed in hTK1; the 3´-substitution stretched from the lasso loop through the P-β hairpin. In this case, there is a Tyr 61 after two additional serines 63-64 providing potential hydrogen donor interactions with the fluorine atom of **13**. This may explain the increased compound selectivity for hTK1compared to *Up*TK. The study here illustrated small functional difference in the TK1 family due to residue

diversity in the loop and P-β hairpin. This new information may contribute to development of future agent against *Ureaplasma* and other bacteria.

2.2.3 Computational docking analysis: AZMT docked with *Up*TK and hTK1active sites

Small differences in the substrate specificity of hTK1 and *Up*TK were observed in an earlier study (Carnrot *et al.*, 2003), particularly with substitutions at the 5-position, 3-position of the base and 3´-position of the sugar. The majority of the tested nucleoside analogs showed lower IC_{50}-values with hTK1 than *Up*TK. However, the 3´-Azido-methyl deoxythymidine (AZMT) (figure 7B), which is an analog of the known anti HIV drugs AZT (Mitsuya *et al.*, 1985), showed 6-fold lower IC_{50}-values for *Up*TK than hTK1 (11 μM compared to 62 μM) (Kosinska *et al.*, 2005).

The Arguslab program was used to dock AZMT into the available structures of *Up*TK (2UZ3), chain B, and hTK1 (1W4R), chain A. These structures were used because there are no TK complexes available with the natural substrates dT and ATP bound simultaneously in the active site. Another problem is the absence of visible lasso loops and β-p hairpins in several structures, but both are visible in these cases. It is believed that the monomeric TK remain mostly unchanged during the catalytic process (Kosinska *et al.*, 2007; Segura-Pena *et al.*, 2007a; Segura-Pena *et al.*, 2007b; Birringer *et al.*, 2005; Kosinska *et al.*, 2005; Welin *et al.*, 2004). The dTTP-TK complexes therefore represent the best structures for docking. The Mg^{2+} ion in hTK1 (1W4R) chain A was adapted from the hTK1 (1XBT) structure, because of the absence of a Mg^{2+} ion. The latter hTK1 structure show a disordered lasso loop, β-phosphate hairpin and was not used for docking. Water molecules were removed from TK structure, because the TK1 family of enzymes only interact through main-chain hydrogen bonds with the substrate. Furthermore, the active site is buried from the direct access of water (Birringer *et al.*, 2005; Welin *et al.*, 2004). The binding site was defined by the coordinates of dTTP and docking settings were set to Argusdock with "high" and "flexible ligand docking" or set to the alternative GA docking engine with default settings.

The template structures in figure 9A and B illustrates how the feedback inhibitor dTTP is position within the active site of *Up*TK and hTK1 and acts as a bisubstrate analog. The Mg^{2+} ion, a participant in the reaction, is in front of the triphosphate groups of dTTP. The triphosphate groups also

interact with the ion and enzyme amino acids, but this is not relevant for the present study and is not illustrated in the figures. There are only two hydrogen bonds between the pentose sugar and enzyme residues, involving the 3´-OH to Gly182 (*Up*TK) or Gly176 (hTK1), and 5´-OH to the Glu 97 (*Up*TK) or Glu 98 (hTK1). Glu97/98 are involved in the transfer of γ-phosphate group from donor (ATP) to the acceptor (dT), (figure 5).

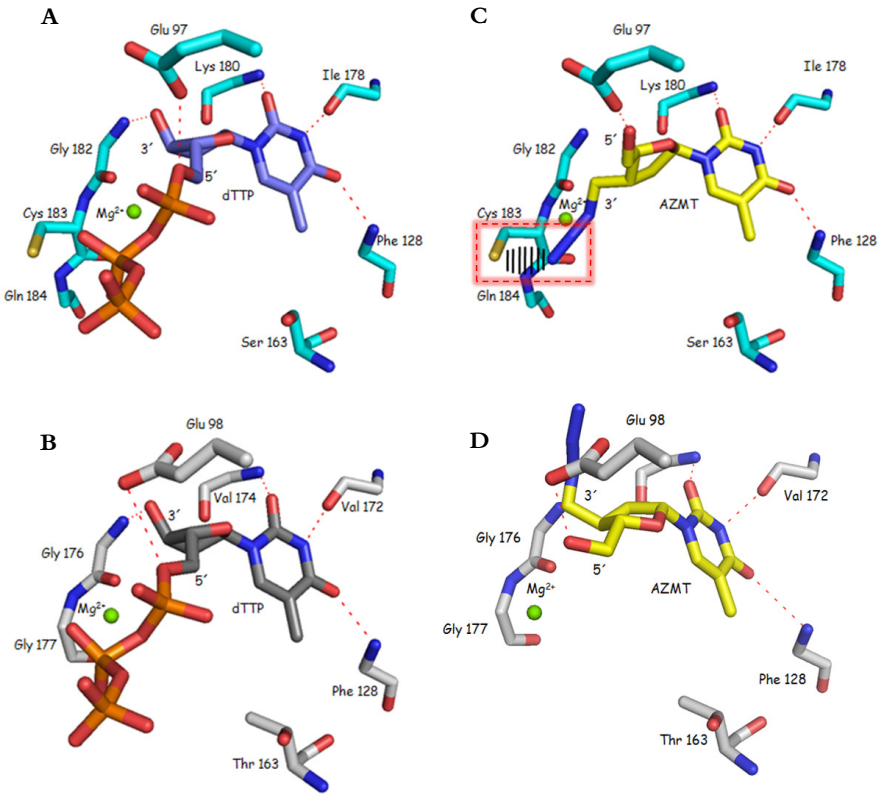


Figure 9. The 3D structure complexes of **A**) dTTP-*Up*TK1 (2UZ3) and **B**) dTTP-hTK1 (1W4R) and the modeled **C**) AZMT-*Up*TK and **D**) AZMT-hTK1 complexes. Hydrogen bonds with main chain residue are indicated with red and the assumed interaction between AZMT and Cys 183 is illustrated with black stripes within the red dashed box.

Kosinska *et al.*, (2005) observed that Glu97 was slightly closer to the 5´-OH in dT-*Up*TK structure compared to dTTP-*Up*TK complex. The difference is small and is not included in this study. The base of dTTP is located in a hydrophobic pocket and forms three similar hydrogen bonds in both enzymes; 2-OH to residues Lys 180, 3-N to Ile 178, 4-OH to Phe 128 for *Up*TK. In hTK1 the residues corresponds instead to Val 174, Val 172 and Phe 128 (Figure 9A and B).

The overall conformation of sugar and base of AZMT remained the same when docked in *Up*TK and hTK1, and it retained most hydrogen bonds as the template dTTP-TK1 complex. However, the 3´- of AZMT is substituted with a methyl-azido moiety and this substitution abolishes the hydrogen bond that normally occurs with 3´-OH of dT. When analysed further, the azido moiety shows two interesting conformations depending on the amino acids in the lasso loops. *Up*TK lasso loop display a unique Cys 183, which apparently interacts with the azido moiety (figure 9C). The azido moiety is on the other hand in an opposite position in hTK1, in the space between the lasso loop and the P-β hairpin (figure 9D).

Earlier studies have shown that the azido-group has inhibitory effects on cystein proteases like caspases, where it apparently interacting with the catalytic cystein (Yang *et al.*, 2008; Le *et al.*, 2006). A Cys-azido bond probably explains the 6-fold lower IC_{50}-value in *Up*TK than hTK1 (Kosinska *et al.*, 2005). When this was repeated, similar results were obtained with regard to relative activities and a similar IC_{50}-value found for hTK1, however, the IC_{50}-value was about 10-fold higher (11 to 100 μM) with *Up*TK (data not shown). At present we do not know the reason for this discrepancy, but it is possible that AZMT has been converted to another compound during storage. Further studies are needed to clarify this discrepancy.

The *Up*TK Cystein is unique in comparison to other TKs (figure 7) and is therefore interesting as a drug-target, especially with SH-group containing nucleoside analogs since it is possible that a disulfide bond could be formed and lock the *Up*TK lasso loop possible inactivating the enzyme.

3 General concluding remarks and future perspectives

New antibiotics are needed to keep in pace with rising bacterial resistance or else our future health and economic situation is at stake. Awareness of antibiotic resistance and acceptance for the necessary preventive measures can only be obtained by better information to the general public.

This thesis investigates the possibilities to use known DNA biosynthesis pathways as medical targets. It concerns two enzymes from *Ureaplasma parvum* (*Up)* in thymidylate synthesis; a putative thymidylate synthase (UU572) and *Up* thymidine kinase (*Up*TK). In addition, a human thymidine kinase 1 (hTK1) was also used in parallel with *Up*TK for exploring differences between them. Expression problems in *E. coli* with *Ureaplasma/Mycoplasma* proteins cause serious obstacles to further biochemical studies. However, codon optimization may facilitate *Mollicute* protein studies. The discovery of thymidylate synthase (TS) activity in *Ureaplasma* and some *Mycoplasma* species, despite the absence of ThyA and ThyX homologs, demonstrates the existence of a new TS family. Once again nature is full of surprises.

Although *Up*TK and hTK1 have similar structures, small differences in the amino acid sequences of their active sites, are important for nucleoside analogs selectivity. Molecular docking analysis, combined with biochemical investigations, demonstrates a valuable approach for explaining the structure-activity relationship of these two enzymes.

The results from the basic research described in this thesis have contributed to a better understanding of two *Mollicute* pyrimidine salvage enzymes and their potential as anti-*Mollicute* medical targets for future drug development.

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