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University of Kuopio
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STUDIUM PYRIMIDINOVÝCH NUKLEOSIDŮ MODIFIKOVANÝCH NA CUKERNÉ ČÁSTI JAKO POTENCIÁLNÍCH INHIBITORŮ TYMIDIN (TP) A URIDIN (UP) FOSFORYLÁZY

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Hradec Králové 2008

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INVESTIGATION OF SUGAR MODIFIED PYRIMIDINE NUCLEOSIDES AS POTENTIAL INHIBITORS OF THYMIDINE (TP) AND URIDINE (UP) PHOSPHORYLASE

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This Master Thesis was made at the Department of Pharmaceutical Chemistry in University of Kuopio. I would like to thank all the people, who help and supported me during the whole time of my work. Especially I would like to emphasize my great thanks to Professor Seppo Lapinjoki, head of the department and to Professor Igor A. Mikhailopulo, who was my supervisor. Then I would like to thank Mrs. Tanja Elizarova for her help and advices during the whole laboratory work and for creation of very friendly atmosphere at working place.

I proclaim this master thesis to be my original work, which I have worked out by myself. All of the literature sources used during the elaboration are named in the part of References and they are properly evidenced in the thesis.

In Hradec Králové…….. …….

student´s signature

Abstract

INVESTIGATION OF SUGAR MODIFIED PYRIMIDINE NUCLEOSIDES AS POTENTIAL INHIBITORS OF THYMIDINE (TP) AND URIDINE (UP) PHOSPHORYLASE

Key words: uridine phosphorylase, thymidine phosphorylase, nucleoside, inhibitor, anticancer chemotherapy, substrate specifity

The work deals with the investigation of novel potential inhibitors of recombinant thymidine and uridine phosphorylases from *E. coli* and *Salmonella typhimurium.* The main goals of this study are the determination of kinetic parameters for natural substrates (thymidine and uridine) for recombinant TP and UP, assessment of the role of phosphate anion in the enzymatic transformations and search for new inhibitors of TP and UP. Study of UP and TP substrate specifity and activity under the various reaction conditions is desired to obtain better view to their functions and to design novel structures of potencial inhibitors.

The determination of the Michaelis constant K_M and reaction rate V_{max} was performed at the spectrophotometer *Varian Cary-300 Bio.* The representation of obtained kinetic data we used the Lineweawer-Burk plot. The next point of my work was the determination of the enzymatic activity and their substrate specifity for some pyrimidine analogues by HPLC analysis of reaction mixtures. Tested compounds 2´-O-methyluridine, 1-(ß-D-fructofuranosyl)uracil, 2´-deoxy-2´-fluorouridine, 2´-amino-2´-deoxyuridine, 3´-amino-3´-deoxyuridine and 3´-amino-3´-deoxythymidine showed various abilities to be phosphorylated.

Abstrakt

STUDIUM PYRIMIDINOVÝCH NUKLEOSIDŮ MODIFIKOVANÝCH NA CUKERNÉ ČÁSTI JAKO POTENCIÁLNÍCH INHIBITORŮ TYMIDIN (TP) A URIDIN (UP) FOSFORYLÁZY

Klíčová slova: uridinfosforyláza, tymidinfosforyláza, nukleosid, inhibitor, protinádorová chemoterapie, substrátová specifita

Práce se zabývá studiem nových potenciálních inhibitorů rekombinantní tymidinfosforylázy a uridinfosforylázy z bakterií *E. coli* a *Salmonella typhimurium.* Hlavním předmětem studia je určení kinetických parametrů přirozených substrátů (tymidinu a uridinu) pro rekombinantní TP a UP, určení role fosfátového aniontu v enzymatické reakci a a hledání nových inhibitorů TP a UP. Studium substrátové specifity a aktivity TP a UP za různých reakčních podmínek je důležité pro získání lepšího pohledu na jejich funkce a pro vytvoření nových struktur potenciálních inhibitorů.

Stanovení Michaelisovy konstanty K_M a limitní rychlosti V_{max} bylo provedeno s využitím spektrofotometru *Varian Cary-300 Bio.* Pro grafické znázornění kinetických dat jsme využili Lineweaver-Burkův diagram. Dalším bodem mé práce bylo stanovení aktivity enzymů a jejich substrátové specifity pro některé pyrimidinové analogy pomocí HPLC analýzy reakčních směsí. Testované sloučeniny, 2´-O-metyluridin, 1-(ß-D-fruktofuranosyl)uracil, 2´-deoxy-2´-fluorouridin, 2´-amino-2´-deoxyuridin, 3´-amino-3´-deoxyuridin a 3´-amino-3´-deoxytymidin vykazovaly různě velkou schopnost fosforylace.

Abbreviations

2FUrd	2´-deoxy-2´-fluorouridine
dAdo	2´-deoxyadenosine
dCyd	2´-deoxycytidine
dGuo	2´-deoxyguanosine
D-dRib	2-deoxy-D-ribose
D-dRib-1-P	2-deoxy-D-ribose-1-phosphate
L-dRib	2-deoxy-L-ribose
2Cl-Ade	2-chloroadenine
5´-DFUR	5´-deoxy-5-fluorouridine
KIN59	5´-O-tritylinosine
BAU	5-benzylacyclouridine
BU	5-benzyluracil
TPI	5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]uracil hydrochloride
BBBA	5-m-benzyloxybenzyl barbituric acid acyclonucleoside
BBAU	5-m-benzyloxybenzylacyclouridine
BBU	5-m-benzyloxybenzyluracil
6A5BU	6-amino-5-bromouracil
7DX	7-deazaxanthine
TP65	9-(8-phosphonooctyl)-7-deazaxanthine
ara-C	9-(β-D-arabinofuranosyl)cytosine
ara-G	9-(β-D-arabinofuranosyl)guanine
ara-U	9-(β-D-arabinofuranosyl)uracil
Arg	arginine
CD	cytidine deaminase
FGF-2	fibroblast growth factor-2
Glu	glutamic acid
Gln	glutamine
GA	glutaraldehyde
Gly	glycine
Guo	guanosine
His	histidine
Ile	isoleucine
Lys	lysine
MMP	matrix metalloproteinase
Met	methionine
S_N1	nucleophilic substitution 1
NRTIs	nucleoside analogue reverse transcriptase inhibitors
NP	nucleoside phosphorylase
Phe	phenylalanine
PD-ECGF	platelet-derived endothelial cell growth factor
PNPBs	PNP from *Bacillus stearothermopylus*
Pro	proline

PNP	purine nucleoside phosphorylase
RNA	ribonucleic acid
Ser	serine
Thr	threonine
TP	thymidine phosphorylase
Tyr	tyrosine
UP	uridine phosphorylase
Val	valine
VEGF	vascular endothelial growth factor

Introduction

Analogues of natural nucleosides are very important compounds in the treatment of many viral diseases and also play a significant role in the anticancer and immunomodulating therapy. Thymidine and uridine phosphorylase are crucial enzymes involved in pyrimidine-salvage pathways. They catalyze the reversible phosphorolysis of thymidine to thymine and 2-deoxy-D-ribose and uridine to uracil and ribose.

Thymidine phosphorylase (TP) is highly overexpressed in many tumors, it has angiogenic activity, supports endothelial cell migration and protects cells from the apoptosis induced by hypoxia. The role in tumor development makes the TP as really important target in the treatment of cancer. Together with the finding of angiogenic activity of TP, the research focused on searching of new inhibitors.

Uridine phosphorylase (UP) activity has been also shown to be apparent in various human solid tumors and connected with reducing the effectiveness of several chemotherapeutic pyrimidine nucleoside analogues, such as capecitabine or tegafur, prodrugs of 5-fluorouracil.

The qualities of TP and UP outline interesting possibility of therapeutic intervention in tumor growth by specific inhibition of these two enzymes. We focused on determining of kinetic parameters and substrate specifity of UP and TP. Study of UP and TP substrate specifity and activity under the various reaction conditions is desired to obtain better view to their functions and to design novel structures of potencial inhibitors. With respect to the high similarity in nucleoside binding sites presented in human and bacterial enzymes, we used for our work recombinant TP from *Escherichia coli* and UP from *Escherichia coli* and from *Salmonella typhimurium*.

1 Rewiew of literature

1.1 Thymidine phosphorylase

Thymidine phosphorylase (TP) is an important enzyme involved in the pyrimidine nucleoside salvage pathway. Its main function is the reversible phosphorolysis of pyrimidine 2´deoxynucleosides to 2-deoxyribose-1-phosphate and their respective pyrimidine basis. TP makes the transfer of the deoxyribose from one pyrimidine base to the next out.

thymidine + phosphate

TP

2-deoxyribose-1-phosphate + thymine

It was presumed that, under phisiological conditions, TP always takes effect towards catabolism in contrast to the UP, which is able to act towards both catabolism and anabolism. In the catalytic mechanism, pyrimidine nucleosides are bounded in the high-energy conformation and the flow of electrons from the O5´ of the pentose ring weakens the glycosodic bond to free the pyrimidine.

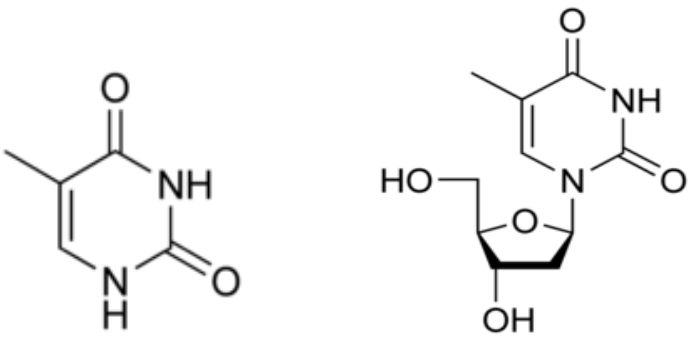


Fig. 1 Structures of thymine and thymidine

1.1.1 Occurrence in nature

Thymidine phosphorylase is of widespread occurrence in microorganisms. [4] This enzyme has been purified from a variety of mammalian tissues, as well as several bacterial sources. [5] TP belongs to the Pyrimidine Nucleoside Phosphorylase family. In lower organisms there is only one enzyme PNP (Pyrimidine nucleoside phosphorylase) which uses both thymidine and uridine as substrates. In mammalian cells there are two enzymes – UP for uridine and TP for thymidine. It was shown that TP is more exacting than UP in discerning of ligands. [2]

Besides the natural 2´-deoxynucleosides thymidine and 2´-deoxyuridine, TP recognises several nucleoside analogues that are being used clinically as antiviral and/or anti-tumour agents, such as 5-(E)-(2-bromovinyl)-2´-deoxyuridine (BVDU), 5-trifluoromethyl-2´-deoxyuridine (TFT, F_3dThd), 5-iodo-2´-deoxyuridine (IDU), or 5-fluoro-2´-deoxyuridine (FdUrd) and many others. [2]

1.1.2 Mechanism of action of TP

The catalytic mechanism of action of thymidine phosphorylase was elucidated by Mendieta *et al.* [6] Their hypothesis that His85 plays an important role in the catalytic mechanism was confirmed. They showed that His85 can be involved in the transfer of the proton from the phosphate to the O2 thymine and also that the enol form of the free base is the most likely intermediate product. The next feature is the ability to stabilize the transition state in process of the catalysis. Furthermore it was observed that His85 is totally conserved in the PNP family.

For determination of mechanism of action they used targeted molecular dynamics simulations and quantum mechanical calculations by comparing the enzymatic reactions of two substrates, thymidine and 2-thiothymidine.

It was found out that the mechanism of phosphorolysis proceeds as two-step reaction like S_N1.

The first step: the O2 of molecule of thymidine interacts with a proton of histidine residue, concretely with a proton from the protonated imidazole ring. This process makes the pyrimidine base a better leaving group. An intermediate oxycarbocation in the sugar is induced by weakened glycosidic bond. The first step usually runs slower, it is rate limiting.

The second step is a fast reaction between this carbocation and the phosphate dianion acting as a nucleophile that gives rise to 2-deoxribose-1-phosphate and the enol form of thymine. In this mechanism, positively charged His85 donates a proton to the thymidine whereas the phosphate dianion is used as the nucleophile for the stabilization of the transition state of the enzyme in the second step. The enol form as the product of the reaction then spontaneously tautomerize to the more stable keto form. [6]

1.1.3 Structural aspects and binding sites of TP

A molecule of thymidine phosphorylase consists of two identical subunits, its molecular mass ranges from 90 kDa in *Escherichia coli* to 110 kDa in mammals. Members of TP family show a noticeable sequence homology. For example, human TP is in 39% homologous with *E. coli* TP.

Each subunit of TP molecule includes a small α domain that contains thymidine binding site and of a large α/β domain where the phosphate binding site is situated. The phosphate binding site is stabilized by a salt bridge with Lys84 and hydrogen bonds made up by rezidues of this α/β domain lining this pocket. The thymine/thymidine binding site in the smaller α domain creates interactions with O2, O4 and N3 atoms of thymidine with Arg171, Ser186 and Lys190. In the human TP dimer interface, the α helices play important role. This interface is formed by α3 helices from each monomer and α1 helix from one monomer connected to α8 and α9 helices from the other monomer.

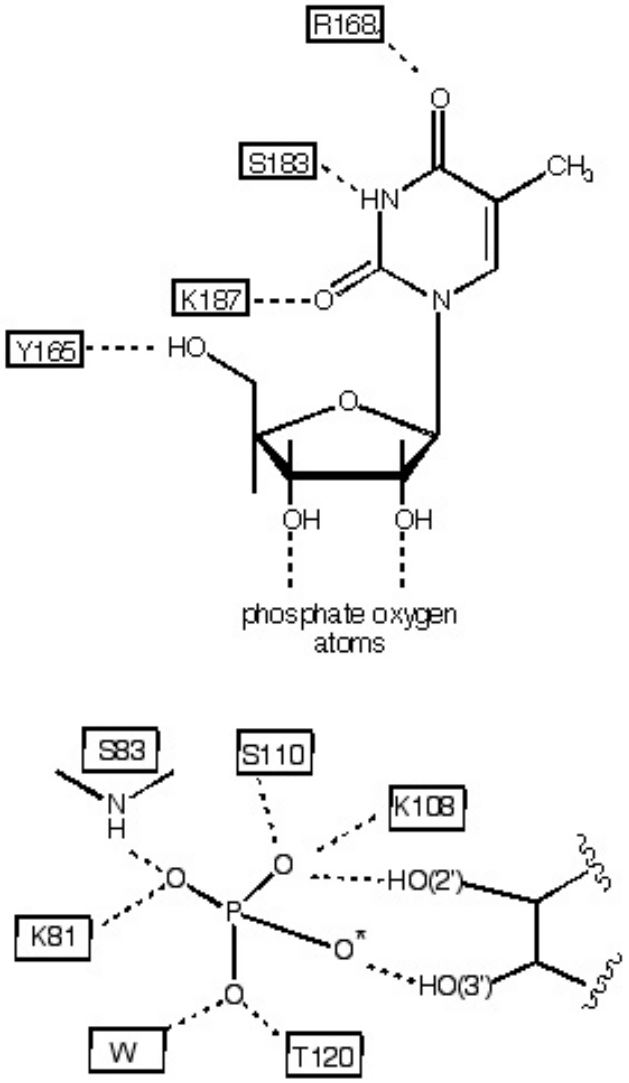


Fig. 2 The residues forming the interactions in the pyrimidine and phosphate binding site of thymidine phosphorylase (pictures available at http://arginine.chem.cornell.edu/Structures/)

There exist some structural differences between human TP, TP from *Escherichia coli* and PNP from *Bacillus stearothermophilus* (PNPBs). The main differences are in the α/β domain where human TP has an additional helix so called α15, an extra turn in α16, and a more prolonged C-terminal end. The small antiparallel β sheet in human TP and in PNPBs is not present in TP from *E. coli*. There exist two forms of active sites: open and closed, which are separated by low-energy barrier. To generate a catalytically active site a domain closure is necessary.

The best accurate structural determination of the active site of the human TP is important for further investigation and prediction of novel structures and

for the searching of the effect of known inhibitory active compounds. The comparison of the human TP, *E. coli* TP and PNPBs has shown that the overall structural similarity between human TP and *E. coli* TP is quite less than between the human TP and PNPBs, while the similarity between the separate domains fits well. [2], [27]

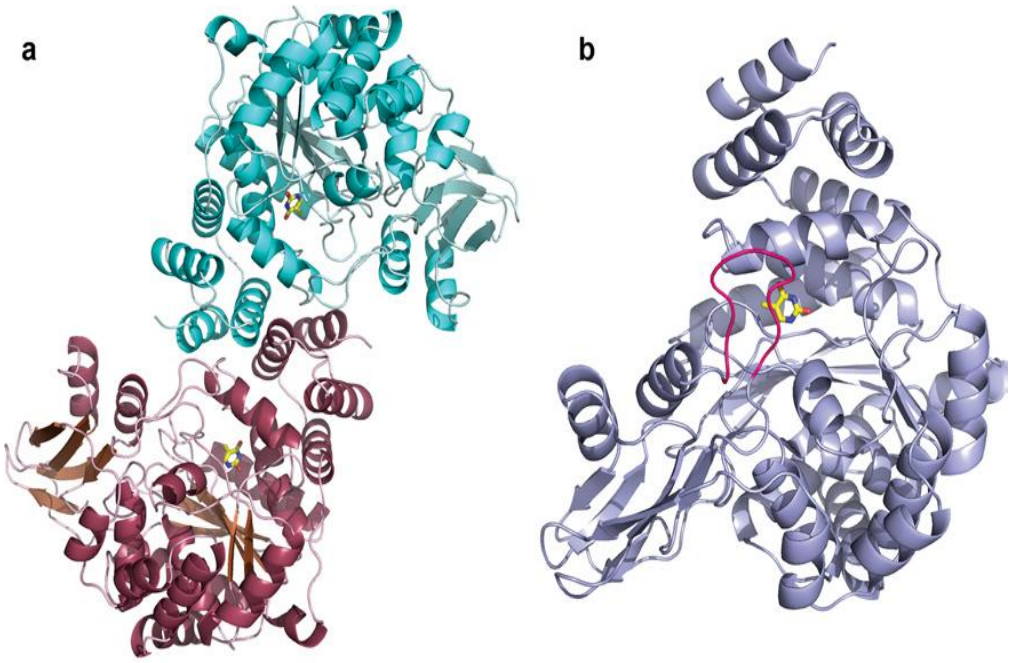


Fig. 3 Crystal structure of human TP. (a)A picture showing the human TP dimer and the dimerization interface; the thymines present in the active sites are shown as sticks. (b)The diagram showing the overall fold of the human TP monomer [8]

1.1.4 TP as PD-ECGF

Previously thymidine phosphorylase was known as *platelet-derived endothelial cell growth factor* (PD-ECGF). In 1987, PD-ECGF was isolated from human platelets and later from placenta tissue. So that TP stimulates endothelial cell migration and angiogenesis.

Angiogenesis and anticancer chemotherapy

Angiogenesis is a complex process requiring cooperation between a planty of celullar factors involving the growth of new blood vessels. There are more than 20 angiogenic growth factors, e.g. vascular endothelial growth factor

(VEGF), angiopoietins, matrix metalloproteinase (MMP) or fibroblast growth factor-2 (FGF2).

Before that the coincidence between TP and PD-ECGF was documented, it was found that TP levels are higher in plasm of pacients with any type of cancer than in healthy individuals and further it is overexpressed at a high level in tumors. Many solid tumours are dependent on density of blood vessels, that is why they produce increased levels of thymidine phosphorylase than non-neoplastic tissues. High concentrations of TP are found in tumours including carcinoma of stomach, colon, ovary and bladder, carcinoma of pancreas, renal carcinoma, oesophageal, breast and lung cancer. TP is also overexpressed in some chronic inflammatory diseases like human atherosclerosis, psoriasis and rheumatoid arthritis. For example, Moghadam *et al.* [28] have found that TP expression is tightly connected to origin of malignancies in breast tumors and assays *in vitro* showed that the growth of carcinoma cells is not supported by TP, whereas the growth *in vivo* is accelerated. [2] [28]

The activity of TP is connected with hypoxia and easier breakout of angiogenesis in malignant and hyperproliferative states. In addition, TP inhibits tumour cell apoptosis. The formation of an intratumoural network of blood vessels is required to provide the growing tumour with oxygen and nutrients. In addition, tumor neovascularization prevents tumours from undergoing necrosis and apoptosis and facilitates the escape of tumour cells into the circulation and subsequent metastasis to distant organs. [4] [27] [28]

The mechanism by which thymidine phosphorylase causes the development of blood vessels is not absolutely clear. But TP enzymatic activity is essential for stimulation of angiogenesis. 2-deoxy-D-ribose as a product of TP enzymatic action was found to produce radicals of oxygen. They induce the secretion of oxidative stress-response angiogenic factors, e.g. VEGF, MMP and interleukin-8. In addition, 2-deoxy-D-ribose is able to activate specific integrins. This reaction connects endothelial cell migration induced by TP to intracellular

signal transduction pathways. That is why the TP enzyme activity which is necessary for angiogenesis should be inhibit by TP inhibitors. [8]

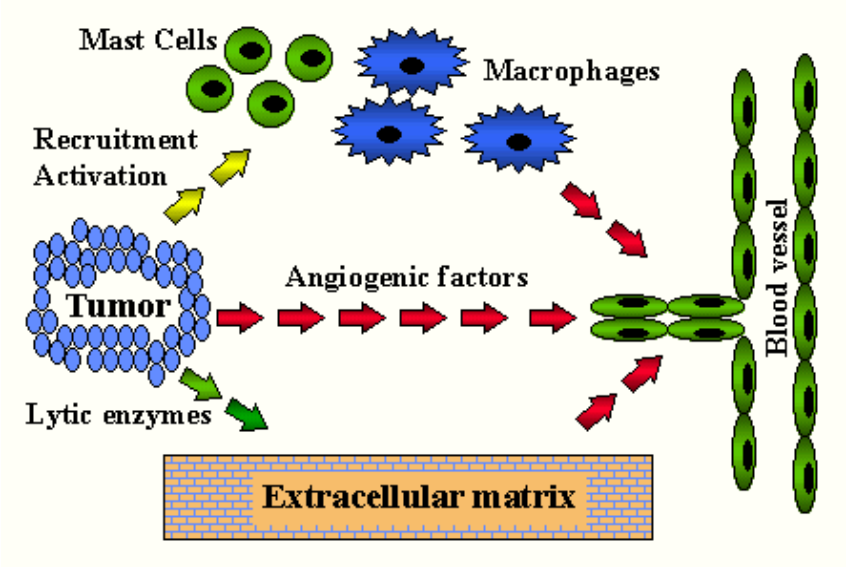


Fig. 4 Not only cancer cells but also inflammatory cells that infiltrate the tumour and extracelullar matrix can be a source of angiogenesis factors. Picture was downloaded from www.med.unibs.it/~airc/cancer.html

Anti-angiogenic therapy, against activated endothelial cells, offers several advantages over therapy directed against tumor cells. Endothelial cells are a genetically stable homogenous target, and spontaneous mutations rarely occur. Anti-angiogenic therapy is directed at activated endothelial cells, its target should be easily accessible by systemic administration. The activated endothelium presents a more specific target because of the expression of specific markers, than the tumor cells, and inhibition of a small number of tumor vessels may affect the growth of many tumor cells.

1.1.5 The identity of TP and gliostatin

Thymidine phosphorylase is also known as ***gliostatin***, initially isolated from human neurofibroma as glial growth inhibitory factor. Gliostatin plays an important role on development and regeneration of the central nervous system. This name, gliostatin, is mainly used in connection with rheumatoid arthritis and cortical neurons. It is integrated probably in the development of the blood-brain barrier [2]

1.2 Uridine phosphorylase

Uridine phosphorylase is a key enzyme involved in the utilization of pyrimidines. It catalyzes the reversible phosphorolysis of ribosides and 2´-deoxyribosides of uracil and their analogues to the relevant nucleobases and ribose-1-phosphate or 2´-deoxyribose-1-phosphate. [9]

Pyrimidine-(2´-deoxy)ribose + phosphate

UP

Pyrimidine + (2´-deoxy)ribose-1-Phosphate

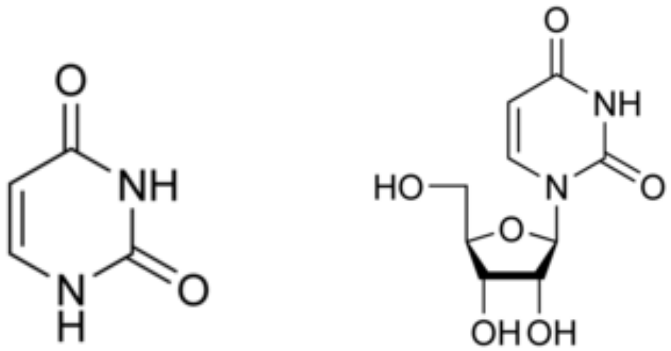


Fig. 5 Structures of uracil and uridine

Uridine phosphorylase is widespread enzyme which has been identified in procaryotes, yeast and higher organisms. The sequence of amino acids is highly conserved among several bacterial, mouse and human enzymes. [10]

Inhibiting UP in human liver raises the blood uridine concentration and in this way is produced a protective effect (so called *uridine rescue*) against the toxicity of the chemotherapeutic agent 5-fluorouracil without decrease of its antitumor activity. In this reason research focused on developing of UP inhibitors is in progress.

1.2.1 Structure of UP and binding sites from *Escherichia coli*

1.2.1.1 Structure of UP

The molecule of UP from *Escherichia coli* is a hexamer 100 Å in diameter and 40 Å thick. Homohexamer is disc-shaped with a long channel opened at each end, which runs through the center of the hexamer. The hexameric quarternary structure of the enzyme is in principle trimer of dimers, where two active sites occur in each dimer and are located at the interface of two monomers. [10]

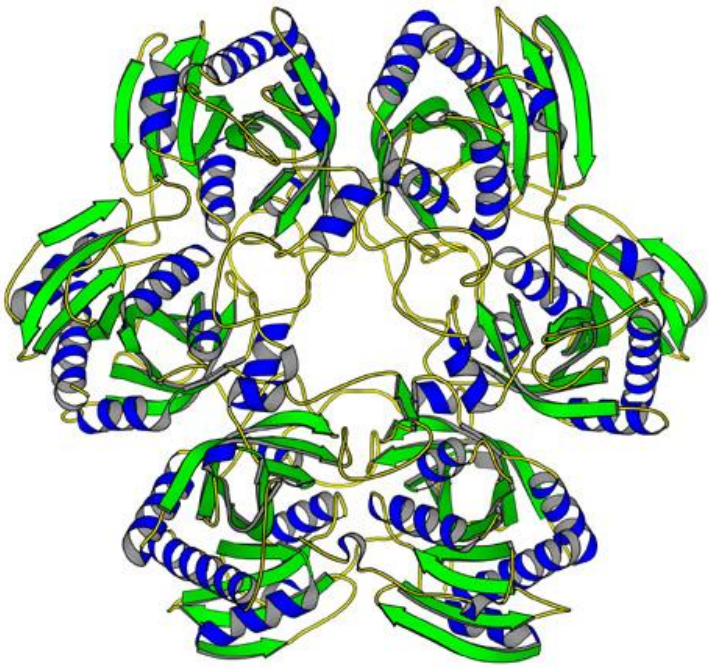


Fig. 6 The active form of the enzyme *E. coli* UP is a hexamer of identical subunits with a molecular weight of 165 kDa. (http://arginine.chem.cornell.edu/Structures/EcUP.html)

A monomer of E. coli UP

A monomer consists of one large β-sheet flanked on one side by four α-helices and on the other side by three α-helices and a shorter β-sheet. [9] The β-sheet centre of UP is bifurcated with α6-helix parting strands β6 and β9. Here α6-helix creates the base of a small β barrel. The central twisted β-sheet is lined with several α-helices with the longest C-terminal helix (α7), packing against two shorter helices, α1 and α4, with each helix paralel with its closest β

strand. At the opposite side of the central twisted β-sheet, α2-helix lies there in a similar way, while α3-helix packs against both α2 and α6-helices. [10]

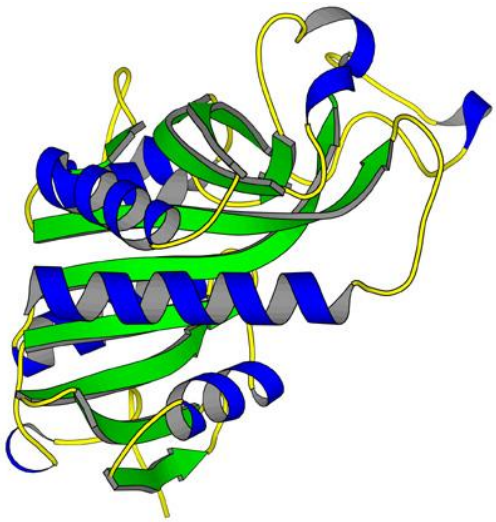


Fig. 7 A monomer unit of E. coli UP includes 253 amino acid residues. (http://arginine.chem.cornell.edu/Structures/EcUP.html)

Structure of dimer and hexamer

The subunit contacts of monomer of the UP dimer are created by quite big amount of residues. Except the Asp160 and His122 the central area of the monomer-monomer interface is constituted of hydrophobic interactions. In the neighbourhood of this central hydrophobic place, there is a group of polar residues that form the remaining monomer-monomer interactions. The loop made up of amino acids 163-185 in UP results in an increased interface area of monomer-monomer contact in contrast to the similar region of PNP. The majority of interactions between the UP dimers that creates the hexamer are formed through hydrophobic residues. The dimer-dimer interface consists of a central hydophobic area with interlocking ridges formed by the 128-134 regions of both dimers. The loop 163-185 is involved in polar dimer-dimer interactions. These residues creates interactions with the monomer of the neighbouring dimer. The side-chain of Arg178 situated at the entrance of the central channel of the hexamer makes up a hydrogen bonding with the Lys181 in the contiguous dimer. Other dimer-dimer interactions are made up by Arg175 and Glu186. [10]

1.2.1.2 E. coli UP active site

The active site contains a highly conserved amino-acid residues. Each active site contains important parts which are given from near neighboring monomer. [9]

The pyrimidine binding site

The uracil binding pocket of an occupied UP active site forms a rectangular slot. Residues Thr95, Gly96, Tyr195, Glu196 and Phe162 form the surface of the active site. The neighbouring region of C5 of uracil is formed by Ile220 and Val221 and the edge consists of Pro229 and Phe7 from the adjacent monomer. These residues form a hydrophobic pocket at the 5 position of uracil. The key residue is Gln166, it is conserved in all UPases. The side-chain of Gln166 makes up two hydrogen bonds to uracil, one of them forms the atom of nitrogen to O2 of uracil and the second bond is formed by the carbonyl oxygen atom to N3 of uracil. The carbonyl makes up the second hydrogen bond to a structural molecule of water, which itself forms a hydrogen bond to O4 of uracil. In the midst of the side-chains Arg223 and Phe162 there is placed the side-chain of Arg168 which forms hydrogen bonds to carbonyls of the main chain. The residues Gln166, Arg168 and Arg223 are conserved in the structures of *E.coli* UP and human UP. [10]

The ribose binding site

The ribose-binding site can accomodate either ribosyl or 2´-deoxyribosyl moiety. [9] This active site lies between the phosphate and uracil binding sites. The ribose is located in the area of active site by a network of hydrogen bonds, mainly by the side-chain His8 and Glu198. The ribose 5´ hydroxyl forms hydrogen bonds with His8 and a molecule of water presented in the active site too. Glu198 creates two hydrogen bonds to the 2´ and 3´ hydroxyl groups of the ribose moiety. The 3´ hydroxyl of ribose forms one hydrogen bond to O3 atom of the phosphate ion. The next important interaction is made up of 2´ hydroxyl of ribose and Met197 and Arg91. Met197 is connected in forming of

the ribose binding pocket and the atom of sulphur of methionine may act in stabilising the C2´-C3´-C4´ area of ribose. The methionine residue is absolutely conserved in the whole NP-I family of enzymes. His8, Arg91, Glu198 and Met197 forming hydrogen bonds are presented in all UPases. [1], [10]

The phosphate binding site

Both subunits of the dimer are involved in binding of phosphate moiety of ribose-1-phosphate. This site is formed by three arginine residues, Arg30, Arg48 and Arg91. Each of them makes two hydrogen bonds with the atom of oxygen of phosphate. Hydrogen bonds are made up also by one side chain hydroxyl and two nitrogen atoms of the main chain (the side-chain hydroxyl of Thr94 and the main-chain nitrogen atoms of Thr94 and Gly26). [10]

Comparison of all known UPases showed, that phosphate-binding site is highly conserved except Arg48, which can be substituted by a lysine [9].

The phosphate binding site of UP and PNP showes hardly identical arrangement of residues because of the evolutionary relationship and conserved function of both enzymes [10], which was further testified by Burling *et al.* [11]

1.2.2 Relationship of *E.coli* UP to human UP

Sequence alignment between UP from *E. coli* and human UP reveals a 21% identity and 35% similarity, however, almost all the active-site residues are absolutely conserved. Only Arg48, which acts in binding of phosphate is replaced by lysine in human UP. [9] Probably these two enzymes have similar quarternary structures. [1]

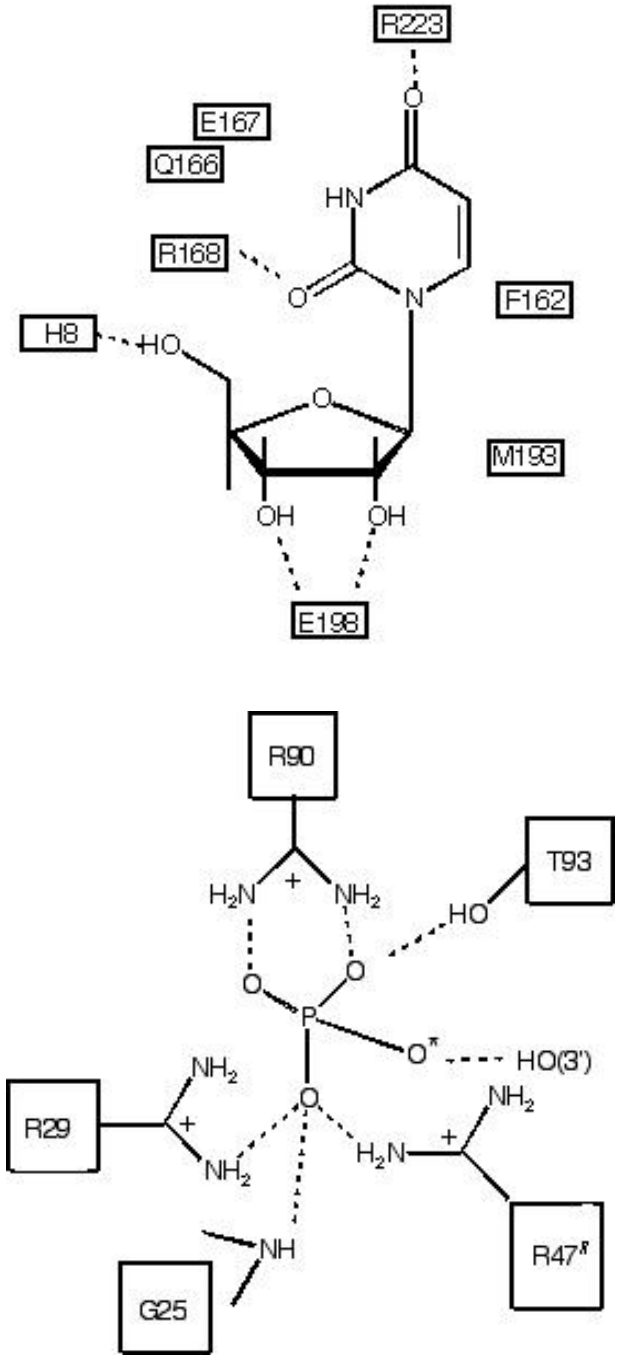


Fig. 8 The residues at the pyrimidine binding site and in the phosphate binding site of uridine phosphorylase (http://arginine.chem.cornell.edu/Structures/StructurePictures/ECUPAS1.jpg, http://arginine.chem.cornell.edu/Structures/StructurePictures/ECUPAS2.jpg).

1.2.3 Similarity between *E.coli* UP and *E. coli* PNP

Uridine phosphorylase from *E. coli* and pyrimidine nucleoside phosphorylase from *E. coli* display significant structural homology. They have hexameric quarternary structure, simillar size and their secondary structural elements show the same position. There is one main structural distinction

between theese two enzymes. The region of residues 165-181 in UP formes prolonged loop, but this region in PNP contains only three amino acids in position 163-165 and forms short loop. This region 163-185 contains Gln166 and Arg168, the key residues in UP specifity. [10], [11]

1.2.4 Mechanism of action of UP

1.2.4.1 Conformational changes in the presence of substrate

The active site undergoes some conformational changes after the binding of a molecule of substrate.

A large part of molecule of uridine phosphorylase is changed in conformation after binding of substrate, concretely parts of β5, β6, β7 and β9 sheets, than the N-terminal part of α7 helix and residues 224-230 called a flap. This flap undergoes the longest movement and brings Glu227 closer to the specifity region where Glu227 creates two pairs of hydrogen bonds. One pair of bonds is formed to th eside-chain of Arg168, while the second pair of hydrogen bonds is formed to the main-chain atoms of nitrogen of Tyr169 and Asp170. The flap-movement also encloses the hydrophobic Pro229 in Ile220 and Phe7. There exist several other changes in the active site, the movement of Met234 or the rotation of C-terminal end of α7. [10]

Closed phosphate binding sites display significantly stronger electron density for Arg30, which is stabilised by the pair of hydrogen bonds formed to phosphate oxygens and by enclosure of the mobile region. In the presence of phosphate, Arg91 is able to change its conformation and to make up two hydrogen bonds to phosphate oxygen and to Glu196. A molecule of water forms hydrogen bonds to Glu196 and Arg91. [10]

1.2.4.2 Influence of potassium ions

In the presence of a potassium ion at the monomer-monomer interface there is induced some local change, which causes a stabilisation of dimer of UP and forming its closed conformation. Six atoms of oxygen, three from each monomer, play a role as ligands. The ligands are the side-chain of Glu49, the main-chain carbonyl of Ile69 and the side-chain of Ser73. These ligands are arranged in a octahedral geometry. [10]

The Glu49 side-chain swings into the binding pocket for potassium when the potassium ion is bound and therefore the monomers are able to come closer. If the potassium ion is lacking, the Glu49 jumps away. This motion of Glu49 side-chain has impact on the structure of UP dimer. This side-chain goes between the two monomers and it serves as a wedge to push the monomers apart. It causes the movement of Pro28 and Arg48 apart. [10]

1.2.4.3 Substrate specifity for natural nucleosides

Uridine phosphorylase is specific for utilization of pyrimidines and the inability to metabolize purines is a result of the smaller space of the base binding pocket because of a presence of sequence 163-185. [10] UP from *E. coli* is more specific than the other UPases, it utilizes almost exclusively uridine and its analogues. [9]

Uridine vs. thymidine: The higher affinity of UP for ribosides over 2´-deoxyribosides is probably due to the three hydrogen bonds made up by the OH2´ of the ribosides and residues Arg91, Glu198 and Met197. Therefore the absence of OH2´ group in the molecule of thymidine, together with the small distortions of the uracil binding pocket required for accomodation of the methyl group at the 5 position of the molecule of the base may explain the lower affinity of uridine phosphorylase for thymidine in contrast to uridine.

Uridine vs. cytidine: The molecule of cytidine could not be utilized because the nitrogen atom at the 3 position is at physiological pH deprotonated

and therefore is impossible to create a hydrogen bond to the carbonyl group of Gln166. The 4 position of cytidine contains the amino group, this group shows a δ+ charge on the amino protons, the charge-charge repulsion is generated between this charge and the positive charge of the guanidinium group from Arg168. This repulsion reduces the binding affinity of cytidine. In the structure of uridine there is a negative charge at the 4 position. It means that the conserved residue Arg168 plays an important role in the substrate specifity of uridine phosphorylase by an electrostatic effect. [10]

1.2.4.4 Catalysis mediated by UP

Because of the similarity of structures between uridine phosphorylase and the NP-I family and the high degree of conservation in binding sites, the mechanism of catalysis proceeds in a high probability in the similar way in both enzymes, UP and PNP. At first, the nucleoside reach a shape into high-energy +*anti* conformation. Then this process leads to the positioning of the ribose O4´ between O5´ of the ribose and O1 of the phosphate. This placement creates an *electron push* and evocates an electron flow from O4´. During the catalysis it was observed that nucleophiles of phopshate and pyrimidine stay embedded and the anomeric carbon atom proceeds along the energetically useful trajectory towards the phosphate nucleophile (the anomeric carbon atom is C1´ of the ribose). Protonation of uracil makes the base a better leaving group. A water molecule creates two hydrogen bonds to the O4 of uridine and to the Arg223. In contrast to previously mentioned PNP, in UP, there is unclear where a proton originates if water is not involvedas a proton donor. And the next important mark along the reaction is the geometric arrangement of the side-chains of His8 and Glu80 in the binding of ribose. The side-chain of Glu80 forms hydrogen bonds to His8, Leu9 and Tyr163. His8 is kept in the correct position to create one hydrogen bond to the OH5´ of ribose. It is possible that this arrangement deprotonate the 5´-hydroxyl group of the molecule of ribose to form O5´ more electron-rich and makes the creation of of transition state formation possible. [10]

A comparison of human and *E.coli* UP shows that *sequence identity* exists between these enzymes for all residues that form hydrogen bonds or *π*-stacks to substrate.

Gly26, Arg30, Arg48, Arg91, Thr94 in phosphate binding pocket.

His8, Glu198, Met197 in ribose binding pocket.

Phe162, Gln166, Arg168, Arg223 in uracil binding pocket.

Therefore the complexed *E.coli* UP sructures provide a good model for the human homologue and a reasonable template for structure-based drug design initiatives of inhibitors. [10]

1.3 TP and UP as biocatalysts for the synthesis of nucleosides

The application of enzymes or the whole cells as biocatalysts causes an improvement of synthesis of nucleoside analogues. These methods of preparation of nucleosides are effective, the biocatalysts are more stable under the conditions of reaction and the whole process becomes more cost-effective. [12]

1.3.1 Methods of preparation

Significant progress in the preparation of nucleoside analogues was obtained by combination of chemical and biochemical methods. [12] The chemical synthesis of nucleoside analogues is multistep process and very laborious and complicated. On the other hand, the synthesis using enzymes for the enzymatic transglycosylation of purine basis presents the convenient alternative to chemical methods. Biochemical reactions are joined with using cytidine deaminase, uridine phosphorylase, thymidine phosphorylase and purine nucleoside phosphorylase and the other enzymes. [13]

1.3.2 Biocatalysts

It is possible to use either purified enzymes or whole bacterial cells as biocatalysts for these reactions. Vladimir N. Barai *et al.* [13], [12] have selected three strains of *Escherichia coli* cells expressing significant enzyme activities.

***E. coli* BM-11** – shows broad specifity in relation to nucleoside analogues and was employed for the synthesis of a variety of base- and sugar-modified nucleosides. This strain shows high UP, TP, PNP activities and moreover the high CD activity. It was discovered that the utilization of *E. coli* BM-11 cells cross-linked by glutaraldehyde (GA) shows some experimental advantages over unmodified cells. The cross-linked cells by glutaraldehyde are shown to be more effective over the alginate gel-entrapped cells, are more stable and have better catalytic activity in synthesis of nucleosides than the unmodified cells (cross-linked cells retain operational stability and catalytic

activity up to 65°). Furthermore they could be used repeatedly in reactions performed at 60-65°. [12]

***E. coli* BMT-1D/1A** – is possible to use for the synthesis of thymidine or for example 2-chloro-2´-deoxyadenosine (*cladribine*) (using dGuo, dAdo or dCyd as donors of 2-deoxyribofuranose moiety),

***E. coli* BMT-4D/1A** – the new strain of *E. coli* BMT-4D/1A was found to be very effective for the syntheses of purine nucleoside analogues by usage pyrimidine nucleosides as donators of the carbohydrates for transglycosylations. [13] This strain is more efficient than the others for the synthesis of 2´-deoxy-2´-fluoroguanosine and 2,6-diamino-9-(3-deoxy-β-D-*erythro*-pentofuranosyl)purine. [12]

1.3.3 Synthesis of modified nucleosides

These procedures became effective for the preparation of modified nucleosides with usage of sugar-modified pyrimidine nucleosides as donors of the pentofuranoses.

One example of preparation of nucleoside analogues using bacterial enzymes as biocatalysts is described below:

1.3.3.1 Synthesis of cladribine

The chemical synthesis of *cladribine* is quite laborious. It leads to the origination of two stereoisomers and is necessary to treat the mixture to obtain molecule of *cladribine*.

The first enzymatic approach for the synthesis of *cladribine* (2-chloro-2´-deoxyadenosine) is based on the straight transfer of the 2-deoxyribofuranose from thymidine to 2-chloroadenine (2Cl-Ade). This reaction is catalyzed by *trans*-N-deoxyribosylase. [12]

The second approach seems to be really more advantageous with the action of PNP from *E. coli* BMT-4D/1A cells than the previous approach. *Cladribine* was synthesized by two coupled reversible reaction. The first step of the enzymatic transdeoxyribosylation is the phosphorolysis of 2´-deoxyguanosine (dGuo) in the presence of phosphate ions. This step results in the origination of guanine and the important 2-deoxy-α-D-ribofuranose-1-O-phosphate. The second step rests in the connection between 2Cl-Ade and 2-deoxy-α-D-ribofuranose-1-O-phosphate with the following release of phosphate and forming of the molecule of *cladribine* (Fig. 17(d)). [12]

Reactions used for syntheses of cladribine are examples of the way how to synthesized novel nucleoside analogues through the action of bacterial enzymes. Because of that the study of UP and TP substrate specifity and synthesis of their novel substrates are necessary for further development of purine analogues, hardly synthesized by chemical methods.

1.4 Nucleoside analogues of medicinal importance

1.4.1 Pharmacologically used pyrimidine nucleoside analogues

Several nucleoside analogues are used as antiviral, anticancer and immunomodulating drugs. These compounds are derived from the natural pyrimidine nucleosides as thymidine (more precisely called deoxythymidine), uridine and cytidine. Novel applications of nucleosides are still being found for the clinic usage.

In most cases, nucleosides serves as prodrugs or bioprecursors that have to be activated to their pharmacologically active metabolites by enzymes presented in human tissues. Some of the nucleoside analogues do not require the activation and they are active in themselves. In some cases, these analogues are joined at the substrate-binding site of the enzyme but does not serve as a substrate. Therefore they can be used as an competitive inhibitors in connection with natural substrates. It happens, in a few cases, that the nucleoside does not bind at the substrate-binding site, but is bounded in another part of the molecule of enzyme. This analogues behave as noncompetitive inhibitors and the binding site is called allosteric binding site. [15]

1.4.1.1 Anticancer chemotherapeutics

5-Fluorouracil (5-FU) - An orally-administered chemotherapeutic agent *capecitabine* is the prodrug, which is converted through the *5´-deoxy-5-fluorouridine* to 5-FU (Fig. 9). The enzymes UP and TP were shown to be important enzymes for this activation. 5-fluorouracil, a fluorinated pyrimidine, is a key anti-colorectal cancer drug and it is used in the treatment of breast, head and neck tumors. 5-FU affects the synthesis and repair of DNA and RNA processing in cancer cells. Its antitumor activity is ascribed to thymidylate synthase inhibition. The host toxicity of 5-FU is related to RNA dysfunction

caused by incorporation of this molecule. [16] The typical administration with *leucovorin* (folinic acid) caused the potentiation of the effect of 5-FU.

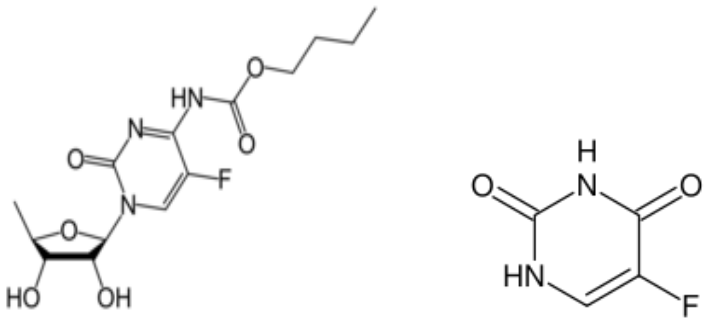


Fig. 9 Structure of capecitabine and 5-fluorouracil

5´-deoxy-5-fluorouridine is an important intermediate in the capecitabine metabolism. Capecitabine is metabolized by PNP, UP and TP. Wan L. *et al.*, [16] reported the importance of tumor necrosis factor-alpha (TNF-α) for the antiproliferative activity of *5´-deoxy-5-fluorouridine* (5´-DFUR). They resulted from the knowledge that the expression of the UP gene was elevated in cancer cells by an incidence of cytokines. Thanks to TNF-α, the sensitivity of cells to 5´-deoxy-5-fluorouridine and its incorporation into RNA and DNA is increased. The antitumor activity of 5-FU targetes the thymidylate synthase inhibition and DNA destruction. That means the more TNF-α exposition is presented the more UP expression is induced and the more 5´-deoxy-5-fluorouridine could be utilized and could increase its cytotoxicity.

Cytarabine (Ara-C) is used for treatment of meningeal leukemia and with the combination with other drugs treats acute myeloid and lymphoblastic leukemia and chronic myelogenous leukemia. Ara-C is converted to *Ara-C triphosphate*, which is an important factor for a blockage of DNA synthesis. [17]

Azacytidine (5-azacytidine) reversibly inhibits DNA methyltransferase, this blocking of DNA methylation causes the activation of tumor suppresor genes. Mainly used in treatment of myelodysplastic syndrome.

Floxuridine (FdUrd, 5-fluoro-2´-deoxyuridine) inhibits thymidylate synthetase leading to the disturbance in DNA synthesis and cytotoxicity. This

agent is an analog of 5-fluorouracil and is of use in treatment of colorectal cancer.

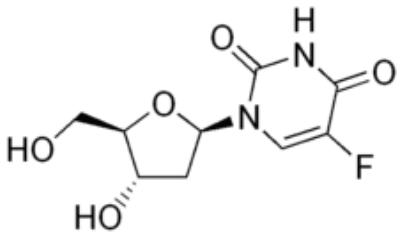


Fig. 10 Structure of floxuridine

Gemcitabine (difluorodeoxycytidine) is able to inhibit ribonucleotide reductase *via* its intermediate metabolites, thereby causing the decreased DNA synthesis. Lung, pancreatic, breast, bladder and oesophageal cancer is sensitive on the treatment.

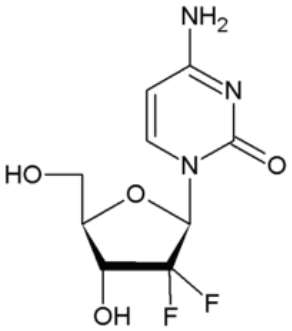


Fig. 11 Gemcitabine

1.4.1.2 Antivirals

Idoxuridine (IDU, 5-iodo-2´-deoxyuridine) serves as antiviral agent against *Herpesviruses*. Idoxuridine breaks the DNA synthesis.

Trifluridine (F_3dThd, 5-tirfluoromethyl-2´-deoxyuridine) like previous nucleoside analogues inhibits competetively the incorporation of natural nucleosides into DNA molecule. It is effective against *Herpes simplex* viruses. [18]

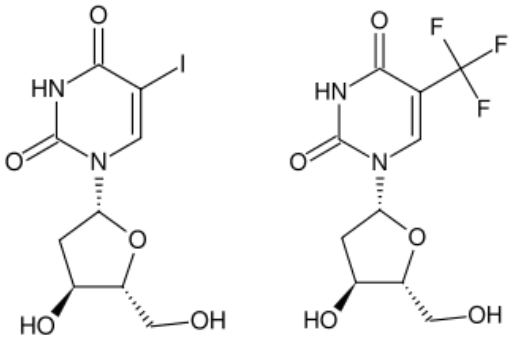


Fig. 12 Idoxuridine and trifluridine.

1.4.1.3 Antiretrovirals

Nucleoside antiretrovirals (NRTIs) are made up of synthetic *dideoxynucleosides*. The major part of NRTIs is made up by pyrimidine nucleoside analogues. The mechanism of action is mediated by the inhibition of reverse transcriptase. The nucleoside have to be activated to the triphosphate metabolite. In the clinical practise, there is used a variety of combination of NRTIs, except stavudine, which is not possible to administered together with zidovudine because of the arisen inhibition of zidovudine. [17], [18]

NRTI	Synonym
Zidovudine	azidothymidine
Zalcitabine	dideoxycytidine
Lamivudine	deoxythiacytidine
Stavudine	dideoxythymidine
Emtricitabine	5-fluoro-dideoxythiacytidine

Table 1 NRTIs, their commonly used names and synonyms.

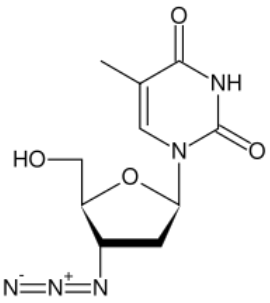


Fig. 13 Antiretrovirals based on the structure of pyrimidine nucleosides. This figure depicts structure of zidovudine, one representative of NRTIs.

1.4.2 Purine nucleoside analogues of medicinal importance

In medicinal practice, there is used a number of purine nucleoside analogues derived from natural purine nucleosides (adenosine and guanosine). They are administered at the same way as pyrimidine nucleoside analogues as antivirals, antiretrovirals and anticancer chemotherapeutics.

1.4.2.1 Antivirals

Acyclovir (acycloguanosine, ACV) – competetively inhibits viral DNA polymerase. .This molecule is efficient against *Herpes simplex*, *varicella-zoster* and other viruses.

Ganciclovir (nordeoxyguanosine, 2´NDG) – the mechanism of action is similar to the inhibition caused by acyclovir and is used for treatment of *cytomegalovirus* infections.

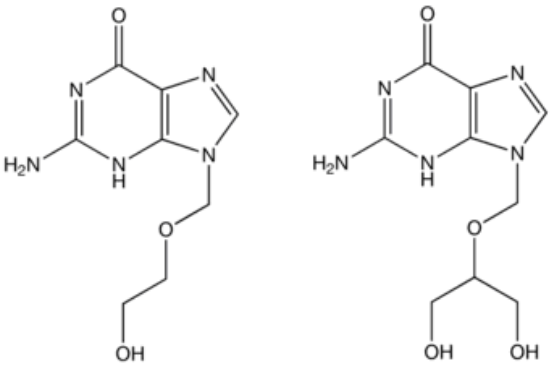


Fig. 14 Antiviral agents acyclovir and ganciclovir.

Vidarabine (adenine arabinoside, ARA-A) – is also inhibitor of DNA polymerase with efficacy against *Herpes viruses*.

Ribavirin (RIBA) – is active against a variety of DNA and RNA viruses, for example *hepatitis C virus*.

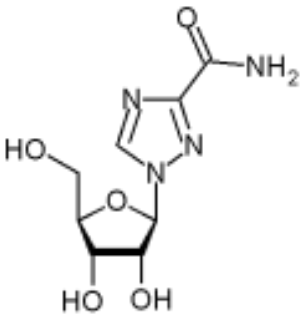


Fig. 15 Ribavirin

Entecavir – makes the reproduction of *hepatitis B virus* slower by interruption of DNA synthesis. [18]

1.4.2.2 Antimetabolites

Mercaptopurine (6-thiohypoxanthine, MP) – this analogue of hypoxanthine shows an antineoplastic and immunosuppresive activity, which is used, as *thioguanine*, for the acute leukemia treatment.

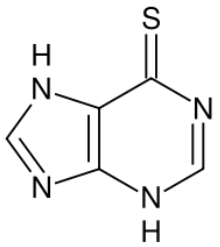


Fig. 16 Mercaptopurine

Thioguanine (TG) - inhibits DNA synthesis.

Fludarabine (2-F-ARA) – is fluorinated analogue of vidarabine affecting the wrong DNA synthesis and inhibiting the tumor cell growth.

Nelarabine – its metabolite Ara-G causes the tumor cell apoptosis.

Clofarabine – is suitable for acute lymphoblastic leukemia treatment.

Cladribine (2-chlorodeoxyadenosine, 2CDA) – caused the wrong synthesis of DNA, depletion of nicotineamide adenine nucleotide and adenosine triphosphate and is administered to patients diseased a hairy cell leukemia. [18]

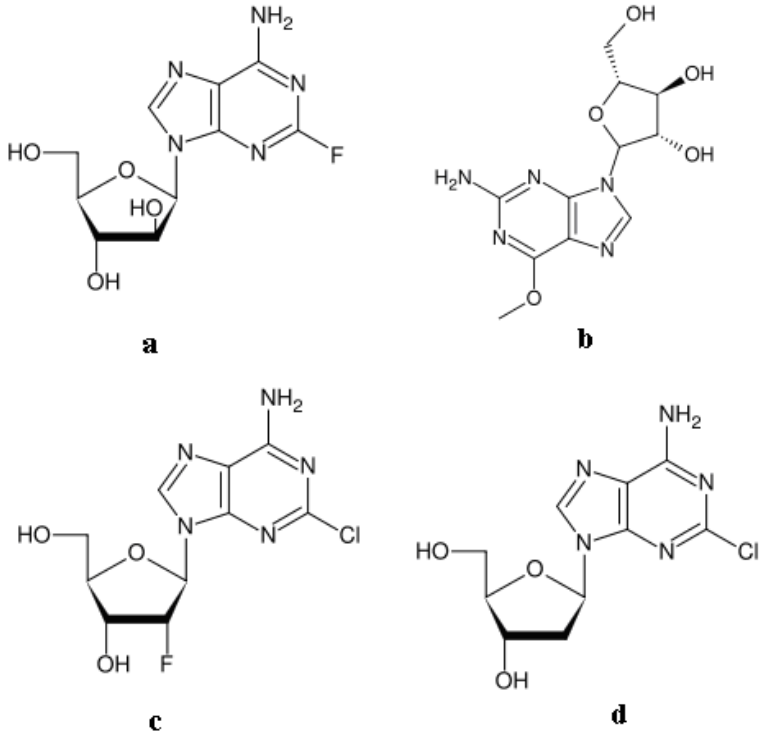


Fig. 17 Fludarabine (a), nelarabine (b), clofarabine (c) and cladribine (d).

1.4.2.3 Antiretrovirals

Didanosine (dideoxyinosine, ddI) – acts as a viral reverse transcriptase inhibitor. [18]

1.5 Development of inhibitors

In many cancers, there is higher level of nucleoside phosphorylases presented than in normal cells. This high level is connected with elevated enzymic activity and with the cytokinin action. Effective inhibitors of these nucleoside phosphorylases are of good promise as potencial anticancer or antiviral drugs. New structures could be acceptable for direct treatment of cancer or for improvement of the effectivity of other anticancer drugs. [14]

1.5.1 Thymidine phosphorylase inhibitors

Inhibitors of thymidine phosphorylase are of good promise for enlargment the effectivity of anticancer chemotherapy by their potential to inhibit neovascularization of tumor tissues.

1.5.1.1 First generation of inhibitors

The first effective TP inhibitors, 6-aminothymine, 6-amino-5-chlorouracil and 6-amino-5-bromouracil, were obtained in the sixties. By virtue of their structure is obvious that free positions 2, 3 and 4 of the molecule of uracil are important for the realization of binding to TP active site and that the presence of Cl, Br or CH_3 substituents in the 5 position causes the increase of inhibitory abilities, which could be explained by the presence of hydrophobic space in TP active site suitable for binding of these substituents. Similar hydrophobic space is probably shown up in the position 6 also, by that reason, a lot of TP inhibitors are 6-amino or 6-methylenamino compounds. [2]

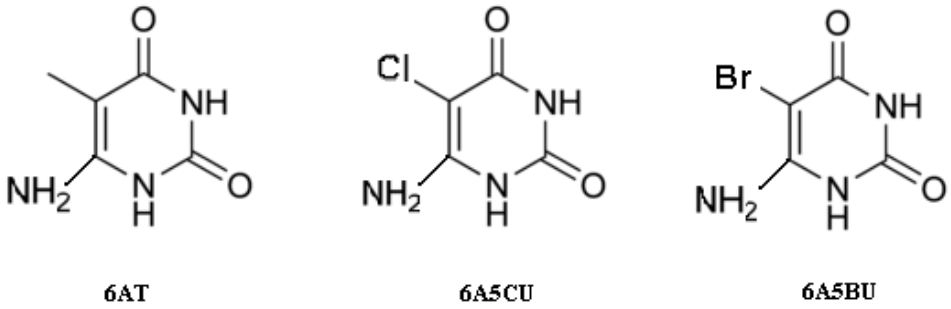


Fig. 18 Prime inhibitors of TP: 6AT (6-aminothymine), 6A5CU (6-amino-5-chlorouracil) and 6A5BU (6-amino-5-bromouracil)

1.5.1.2 6-Aminouracil as basic structure

The next group of TP inhibitors originated by the addition of the second ring on the pyrimidines like 7-deazaxanthine (7DX) and 7-(2-aminoethyl)-deazaxanthine. 7DX was described as the first purine analogue able to inhibit the function of TP. These compounds make up very stable bonds to substrate-binding site of the enzyme. C7 and C8 positions of the pyrrole ring of 7DX have the same function as the methyl group of the thymine molecule at the C5 position. Very potent inhibitory activity against human TP was documented for 6-(2-aminoethylamino)-5-chlorouracil (AEAC) and 5-chloro-6-(1-imidazolylmethyl)uracil (CIMU) (inhibitor of both TP and UP), where is shown the value of basic chain at 6 position. [2], [21]

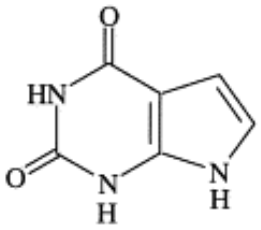


Fig. 19 TP inhibitor 7-deazaxanthine (7DX)

1.5.1.3 Pyridinium-substituted derivatives of uracil and thymine

These bicyclic pyridinium-substituted derivatives of uracil and thymine were prepared as the potencial inhibitors of TP to enlarge the group of

inhibitors based on modified uracil represented by 6-amino-5-bromouracil (6A5BU). Two groups of analogues were synthesized with substitution in C6 and N1 position on the uracil or thymine ring.

Preparation of C6-derivatives gave rise to three groups of uracil/thymine analogues (Fig. 20 **(A)**):

- the direct fusion of pyridine to the uracil ring
- from 6-(chloromethyl)uracil and neat pyridine or 2-amino or 3-aminopyridine were prepared methylene bridged bicyclic compounds or the aminopyridinium compounds
- thymine derivatives were prepared from 5,6-dimethylpyrimidine-2,4-dione giving the methylene bridged pyridinium analogues.

N1 thymine analogues (Fig. 20 **(B)**) forming the fourth group were synthesized of using methylpyridine or ethylpyridine.

More than ten compounds were prepared in this way and were tested for the inhibitory activity to thymidine phosphorylase. 6-methylenepyridinium derivatives were marked as the most effective uracil-based inhibitors of TP. The most active compound was 1-[(5chloro-2,4-dihydroxypyrimidin-6-yl)methyl]pyridinium chloride. Derivatives containing methylene bridge between their two heterocycles were the most potent inhibitors, because this methylene group caused increase of the molecular flexibility and facilitated the binding of molecule into the active site of TP. As was found, free N1 position is also very important for binding of enzyme, because N1-substituted derivatives displayed low inhibitory activity. C5-halogeno substitution of the uracil ring causes the increased acidity of the N1-group and in that way increased polarity of this group and stronger binding with the active site. [22]

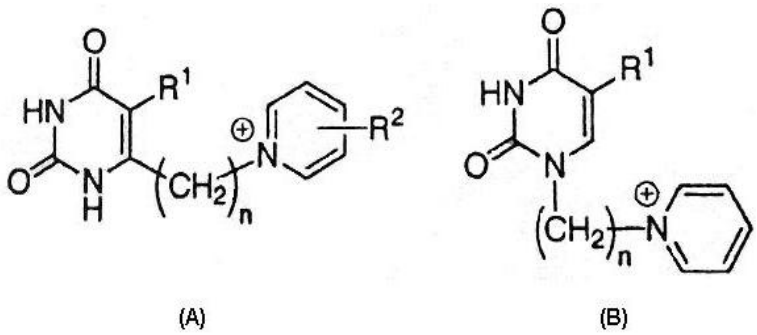


Fig. 20 This picture shows the TP inhibitors, (A) represents general structure of C6-substituted uracil pyridinium derivatives, (B) represents general structure of N1-substituted derivatives. R^1 presents substitution with halogenides or methylene, R^2 subtituents are hydrogen or amino group.

1.5.1.4 Inhibitors substituted with pyrrolidine or guanidine moiety

According to the strucural determination of *E. coli* and *Bacillus stearothermophylus* TP active site closed conformation were designed following molecules. Efficient substitutions at 6 position gave rise to other modifications like 6-methyleneamino and N-methylguanidine uracil derivatives. Indeed, compounds containing 6-methylenepyrrolidine substituents, 5-chloro-(6-methylenepyrrolidine)uracil and 5-bromo-(6-methylenepyrrolidine)uracil, show inhibitory activity, but their relatively high toxicity is disadvantage. Because of that N-methylguanidine and amidino derivatives were synthesized, for example 5-chloro-6-(1-*N*-methylguanidine)uracil hydrochloride or highly interesting 5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]uracil hydrochloride (TPI). Based on the high inhibitory activity of TPI, *N*-(2,4-dioxo-1,2,3,4-tetrahydro-thieno[3,2-*d*]pyrimidin-7-yl)guanidines were established, but their activity against TP was guite lower than TPI exhibited, which was caused by hydrophilic guanidine moiety. According to the *E. coli* TP open-cleft conformational state, other new alternative scaffolds of inhibitors based on 5-substituted *N*-methylhydantoin or 5-substituted 2,4,6-pyrimidinetrione were obtained. The N-methylhydantoin moiety reacts nearby the phosphate-binding site and 2,4,6-pyrimidinetrione moiety racts in the thymine-binding site of the enzyme. [2]

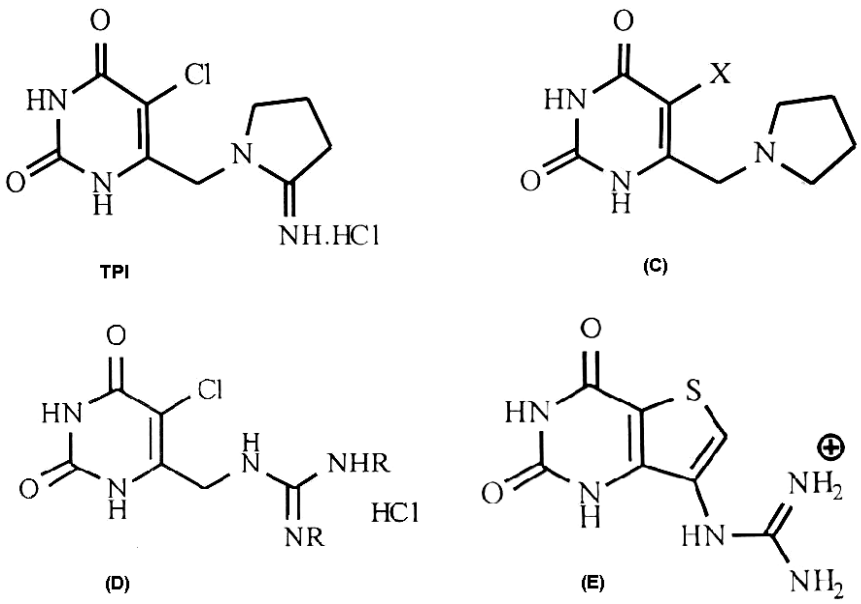
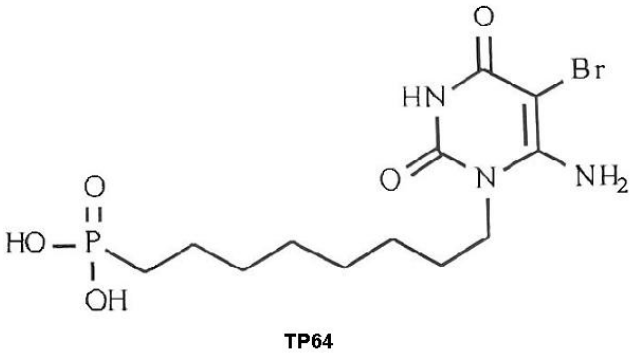


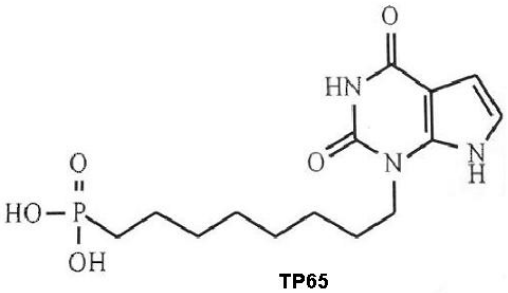
Fig. 21 Inhibitors containing pyrrolidine or guanidine moiety. 5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]uracil hydrochloride (TPI), 5-halo-(6-methylenepyrrolidine)uracil (X=Cl, Br) (C), 5-chloro-6-(1-*N*-methylguanidine)uracil hydrochloride (D) and *N*-(2,4-dioxo-1,2,3,4-tetrahydro-thieno[3,2-*d*]pyrimidin-7-yl)guanidine (E).

1.5.1.5 Multisubstrate inhibitors

These multisubstrate inhibitors interact not only at one binding site but at two binding sites at the same time. According to the open conformation of *E. coli* TP, these substances were developed. The active site in the open (inactive) conformation contains the thymine/thymidine and phosphate binding site, which are approximately 8-10 Å. Based on this distance, compounds involving pyrimidine base, 6-9 atoms long linking chain and the phosphonate group were synthesized to immobilize the enzyme in an inactive conformation. The most active compounds contain the 6A5BU or 7-deazaxanthine moiety. [2]



TP64



TP65

Fig. 22 Examples of multisubstrate inhibitors: 9-(8-phosphonooctyl)-7-deazaxanthine (TP65) and 1-(8-phosphonooctyl)-6-amino-5-bromouracil (TP64).

One of very potent compound is TP65 (9-(8-phosphonooctyl)-7-deazaxanthine) which distinctively inhibited the blood vessel formation in enzymatic assays and is largely better inhibitor than its precursor 7DX. It is good to mention that TP65 was tested for cytotoxic activity against endothelial cell cultures and it was found that this compound is not toxic in the concentration necessary to inhibit blood vessels development. [21]

1.5.1.6 Prodrugs of known inhibitors

Because TP is expressed in various tumors in relatively high levels than in normal tissues, it is supposed to synthesized inhibitors of this enzyme acting only in tumors. It is matter of common knowledge that the environment in tumor tissues is characterized by hypoxia. The following prodrugs have the ability to be reduced to corresponding active compounds.

There are two approaches for the activation:

- *The nitroimidazolylmethyluracil prodrugs* were designed according to the active site structure of *E. coli* TP and due to the supposition that the enzyme is able to distinguish between the nitro and the amino group. Very efficient inhibitors were 5-halo-2-amino-imidazolylmethyluracils in the oposite to their nitroimidazolylmethyluracil prodrugs, which were many times less effective.
- The next approach for the synthesis is based on the ability of the enzyme xanthine oxidase to utilize many compounds because of its broad substrate specifity. High levels of the enzyme are expressed in hypoxic tissues, which means in tumor tissues also. Prodrugs lacking the carbonyl group are oxidised by xanthine oxidase at the 2 and 4 positions of the nucleobase ring to create TP inhibitors like 6A5BU, 7DX or TPI. [2]

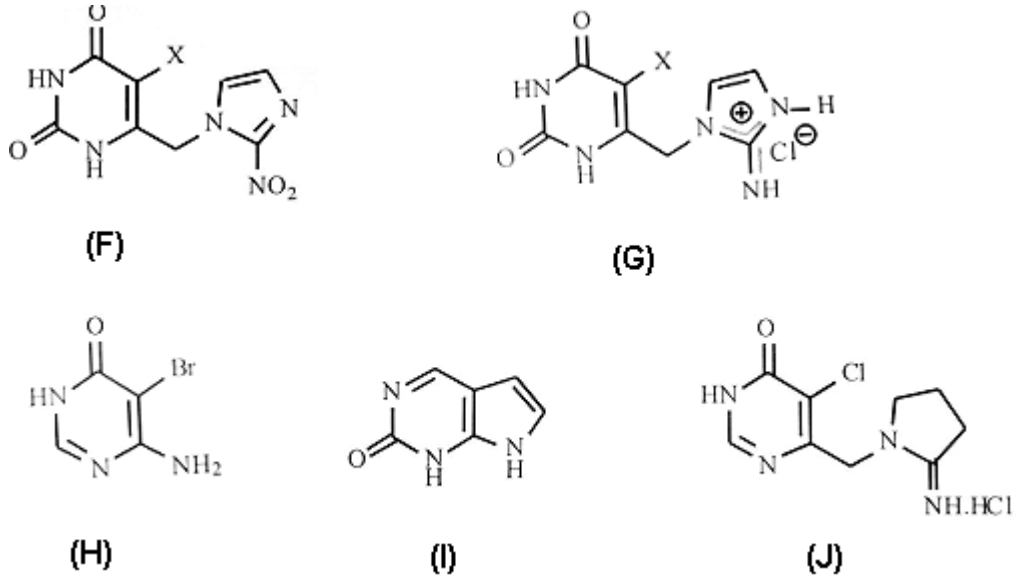


Fig. 23 Prodrugs and their corresponding active molecules: 5-halo-2-nitroimidazolylmethyluracils (F), 5-halo-2-amino-imidazolylmethyluracils (G) and compounds (H), (I) and (J) which serve as substrates for xanthine oxidase.

1.5.1.7 Allosteric inhibitors of thymidine phosphorylase

Allosteric inhibitors are ligands which cause the decrease of the affinity of the enzyme to substrate by binding to the allosteric active site, which is different and topographically distinct from the substrate binding site. These allosteric compounds do not need to keep the similar structure as the natural substrates and can serve for better understanding of enzymatic processes. *N*-phenylhomophtalimide and 5´-*O*-tritylinosine (KIN59) derivatives have been described. [2], [8], [15]

5´-O-tritylated nucleoside derivatives

The inhibitory activity was discovered for the KIN59 which contains nucleobase (hypoxanthine), ribose and a trityl moiety at the 5´-position of the ribose. This interesting compound does not compete neither with natural nucleoside nor with the phosphate at their binding sites, but it acts as noncompetitive inhibitor of TP. KIN59 showed that it is able to completely inhibits the neovascularization without being toxic.

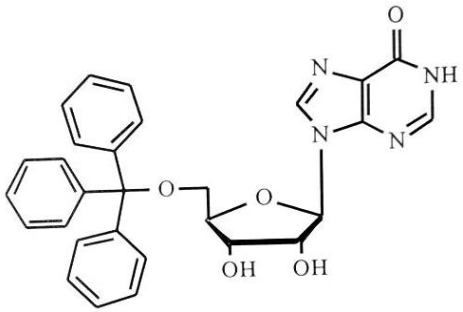


Fig. 24 Basic structure of 5´-*O*-tritylated deriatives KIN59 (5´-*O*-tritylinosine)

The trityl moiety is necessary for causing the antiangiogenic activity. KIN59 became the prototype for synthesis of new compounds based on its structure (as was shown in Fig. 26), which could also show the inhibitory activity *via* the allosteric inhibition. The modifications in the molecule were carried on all parts, the base, sugar and trityl moieties. The insertion of the chlorine atom on the trityl group brought the increase of anti-TP activity (e.g. TP136). The next change at the molecule was methylation of the base, but this

modification did not bring the increase of inhibitory activity (TP140) as compared to KIN59. The methoxy-substitution on the trityl group led to instability of compounds and therefore they were not applicable (TP146, TP147 and D7154) as well as derivatives prepared by replacement of hypoxanthine by the adenine or 6-methyladenine, which were instable also (TA-01 and TP141). The elevation of inhibitory activity against TP was achieved by replacement of hypoxanthine by thymine, while the change by uracil or 5-methylcytosine caused the rise of inhibitory activity on the same level as KIN59. After the removing of the base or changing the base by related hererocyclic substituent, the inhibitory activity disappeared, which means that the presence of heterocyclic base is necessary for the effectivity. Interestingly, the compound TP151 was found to be efficient inhibitor of TP-induced angiogenesis, although it does not inhibit the activity of TP.

Further modifications were performed at KIN59 molecule by Casanova *et al.* [15]. The highly reactive N1 position of nucleobase was substituted by an alkyl, alkenyl or a benzyl group and as the optimal substituent was designated a methylcycloalkyl group with high inhibitory activity. Next, these N1-substituted derivatives underwent the cleavage of the purine ring to subsequent 5-amino-4-(*N*-alkylcarbamoyl)imidazole ribosides (AICAR) (as was shown in Fig. 26). For the elucidation of the importance of sugar moiety to the inhibitory activity, *2´,3´-seconucleosides* were synthesized by oxidative cleavage of the ribose (Fig. 25). With respect to the detection of their inactivity against TP it means that not only the purine base but also the sugar moiety is necessary for the interaction with TP allosteric binding site. [8], [15]

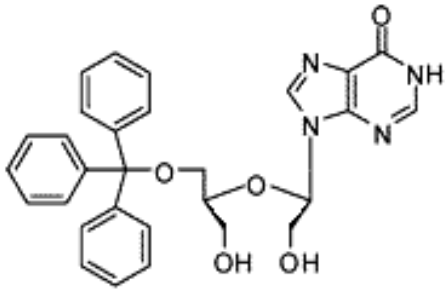


Fig. 25 The KIN59 derivate 5´-*O*-trityl-2´,3´-secoinosine

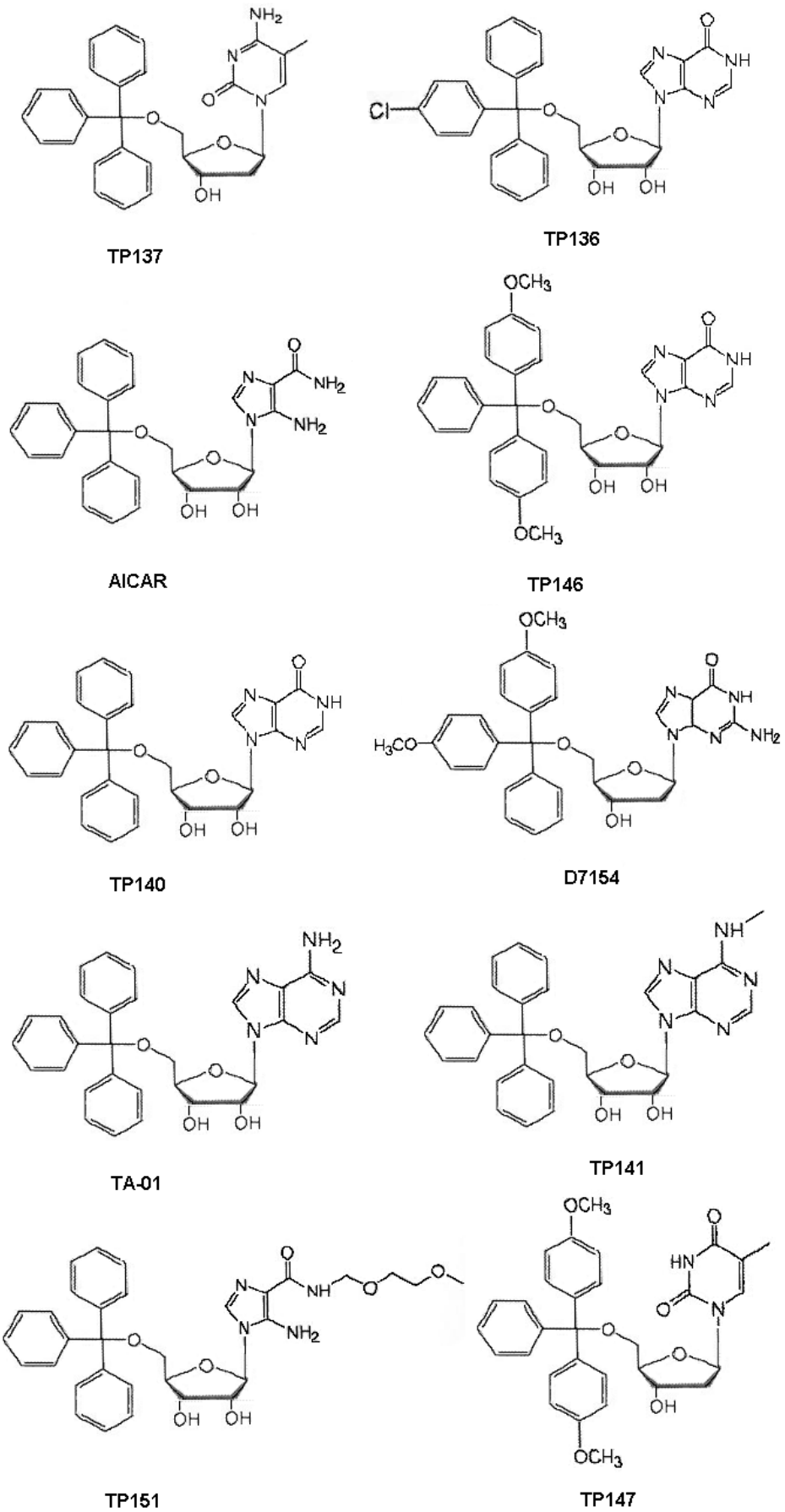


Fig. 26 Several structures of 5′-O-tritylated nucleoside derivatives [8]

So that the trityl group is indispensable for the realization of anti-TP and antiangiogenic activity of these derivatives and the presence of this group is the main requirement for the allosteric binding probably at a lipophylic part of TP. The angiogenesis is stimulated by many other angiogenic factors, not only by TP, therefore the inhibition of blood vessel formation could be caused by interaction of 5´-O-tritylated derivatives with different angiogenic molecules. This possible interaction could be illustrated by the angiogenic activity of TP151, which do not inhibit TP. Therefore, this new group of inhibitors is important for their possible ability to interfere with another angiogenic factors than with TP. The next interesting point is that 5´-O-tritylated derivatives are able to cause destruction of already matured vessels at the area of drug administration. This destruction is not caused by a toxic effect of represented compounds, because the cytotoxicity in various assays was not observed. 5´-O-tritylated nucleoside derivatives show antiangiogenic and vascular-targeted activity, both of these properties may be used for the creation of novel effective class of inhibitors and anticancer drugs. [8], [15]

1.5.1.8 Pentose phosphates and the importance of 2-deoxy-L-ribose as the inhibitor of tumor metastasis

Pentose phosphates represent the basal structures of ribo- and deoxyribonucleotides. Except the oxidative pathway, where the *D*-ribose-5-phosphate (*D*-Rib-5-P) and 2-deoxy-*D*-ribose-5-phosphate (*D*-dRib-5-P) are synthesized from glucose-6-phosphate, the pathway through the action of nucleoside phosphorylases, as TP, UP or PNP is important for their formation. These enzymes are suppliers of the 2-deoxy-*D*-ribose-1-phosphate and *D*-ribose-1-phosphate (*D*-Rib-1-P). The phosphopentomutase transformes *D*-Rib-1-P into the *D*-Rib-5-P leading to glycolysis. The second possibility is the phosphopentomutase reversible conversion of the *D*-dRib-1-P to *D*-dRib-5-P and its resulting cleavage into glyceraldehyde-3-phosphate and acetaldehyde entering the glycolysis or the Krebs cycle. Except their utilization as the energetic sources, their next important pathway is to serve for nucleoside and base interconversion. [31]

By the action of thymidine phosphorylase the reversible conversion of thymidine to thymine proceeds together with release of 2-deoxy-*D*-ribose-1-phosphate (*D*-dRib-1-P). The dephosphorylated product of *D*-dRib-1-P, 2-deoxy-*D*-ribose (*D*-dRib) has interesting qualities, it is able to stimulate chemotaxis, support tubular formation of endothelial cells (even if this ability was formerly ascribed to TP intrinsic action) and give the resistance to tumor cells against apoptosis caused by hypoxia. Through the intervention into the formation or the blockage of *D*-dRib effect could be reached the inhibition of the tumor growth.

Nakajima *et al.* [23] put their mind to the possible inhibition of *D*-dRib by *2-deoxy-L-ribose* (*L*-dRib), the stereoisomer of *D*-dRib. In their performed studies on the mouse models was shown that *L*-dRib is very efficient inhibitor of angiogenesis and also metastasis of tumor cells overexpressing TP. The effectiveness of inhibition of metastasis was higher, compared to tegafur (prodrug of 5-FU). By the action of TP, the release and activation of angiogenic factors VEGF, IL-8 and matrix metalloproteinase-1 proceeds, hence by the help of *L*-dRib their secretion could be decreased. The last important thing is the function of *D*-dRib as the energetic source in hypoxic tissues, it means in tumors also, but *L*-dRib does not serve as the source of energy, so that its administration could cause the lack of energy and restriction of tumor growth.

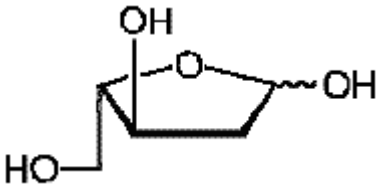


Fig. 27 2-Deoxy-*L*-ribose (*L*-dRib), a stereoisomer of *D*-dRib, can inhibit *D*-dRib antiapoptotic effect and suppress the growth of metastasis.

1.5.2 Uridine phosphorylase inhibitors

Preliminary is necessary to refer that most of the compounds able to inhibit uridine phosphorylase is not able to be inhibitory active against TP. It is assumed that this fact is caused by the presence of larger hydrophobic cavity in UP nucleoside-binding site than the TP contains, namely in adjacencies of C5 of the base, which is obvious from the structures of efficient inhibitors. The most important thing is to preserve the intact C2, N3 and C4 positions of the base ring, positions C1 and C5 could be modified in various ways. Cha S. *et al.* [26] mentioned that the 3´-hydroxyl group of nucleosides analogues has to be preserved for better exteriorization of inhibitory qualities and that the *syn*-conformation round the *N*-glycosidic bond is necessary.

As potent inhibitors of UP were synthesized xylo-, lyxo-, 2,3´-anhydro-2´-deoxy-, 6,5´-cyclo-, and carbocyclic analogues of uridine, but none of these compounds had the ability to inhibit the uridine phosphorylase. Neither the C5 substitution brought the incerase of activity against UP. Which basically means that these molecules do not bind to the enzyme. As the derivatives which proved to be competent for UP inhibition were found acyclouridines and 2,2´-anhydrouridines as described below. [24]

1.5.2.1 5-substituted acyclouridines

As the most important inhibitors of uridine phosphorylase were synthesized specific inhibitors acyclouridine and 5´-substituted analogues of acyclouridine (1-(2-hydroxyethoxymethyl)uracils). After the revelation of the effect and antiherpetic potencial of acycloguanine (known as *acyclovir*, showed in Fig. 14), other structures resembling to acycloguanine were prepared, where the purine was replaced by another base. On the basis of acyclouridine, the methyl-, ethyl-, propyl-, isopropyl- or benzyl- derivatives substituted in C5 position were synthesized (Fig. 28). On the strength of these substitutions it

was shown, that the more the hydrophobic chain is long the lower the inhibition constant (K_i) is from acyclouridine to the propyl derivative according to the theory of hydrophobic region at the active site. But on the other side, the isopropyl- and 5,6-tetramethyl- analogues did not decrease but increase the K_i. These compounds were tested on the *E. coli* and on the rat enzyme (UP). Different values of K_i show the diversities of the structure of active sites of these two enzymes. [25], [26]

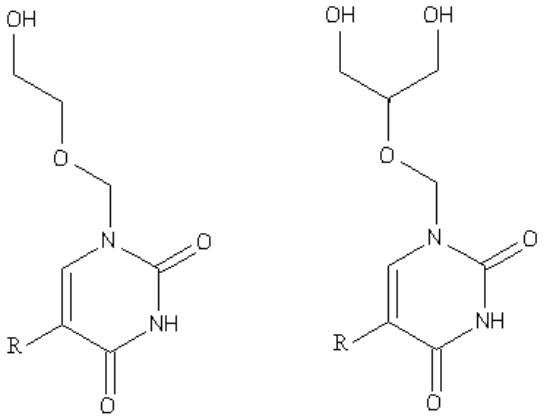


Fig. 28 Structures of acyclouridines: (1-(2´-hydroxyethoxymethyl)uracils and the structure on the right represents hydroxymethyl acyclo compounds 1-(2´-hydroxyethoxy(1´-hydroxymethyl)methyl)uracils)

In respect to the findings that acyclonucleosides are more potent in the inhibition than the non-acyclic compounds, therefore other compunds were synthesized by the combination of acyclomoiety and already known compounds with proved inhibitory activity 5-benzyluracil (BU) and 5-*m*-benzyloxybenzyluracil (BBU). Arised 5-benzylacyclouracil (BAU) is derived from BU, the inhibitory activity against UP was enhanced and the increase of inhibitory potency showed 5-*m*-benzyloxybenzylacyclouracil (BBAU), which was derived from BBU (Fig. 30). Other compounds like HM-BAU = 5-*m*-benzyl-1-(2´-hydroxyethoxy(1´-hydroxymethyl)methyl)uracil and HM-BBAU = 5-*m*-benzyloxybenzyl-1-(2´-hydroxyethoxy(1´-hydroxymethyl)methyl)uracil were prepared by the addition of one hydroxymethylene group to the acyclo moiety at C3´ position. [9], [26]

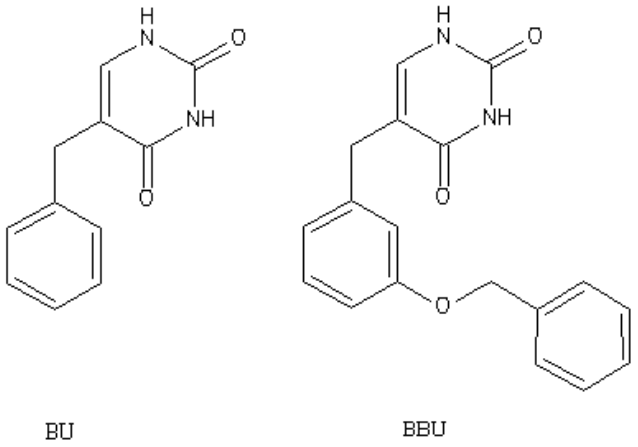


Fig. 29 5-benzyluracil (BU) and 5-m-benzyloxybenzyluracil (BBU)

Basically, many related compounds were synthesized and it is good to mention for example aminomethyl-BBAU, a very potent compound against UP studied on mouse liver. Another acyclonucleosides were similarly potent, for example aminomethyl-BAU and succinyl derivatives. Findings that the 2,2´-anhydrouridine and its derivatives are possible inhibitors of UP gave rise to synthesis of some combinations of these two groups containing BU or BBU and 2,2´-anhydrouridine moiety. These analogues obtained a bit higher inhibitory activity than the previous acyclonucleosides but simultaneously they were not so effective as the best 2,2´-anhydro compound, 2,2´-anhydro-5-ethyluridine.

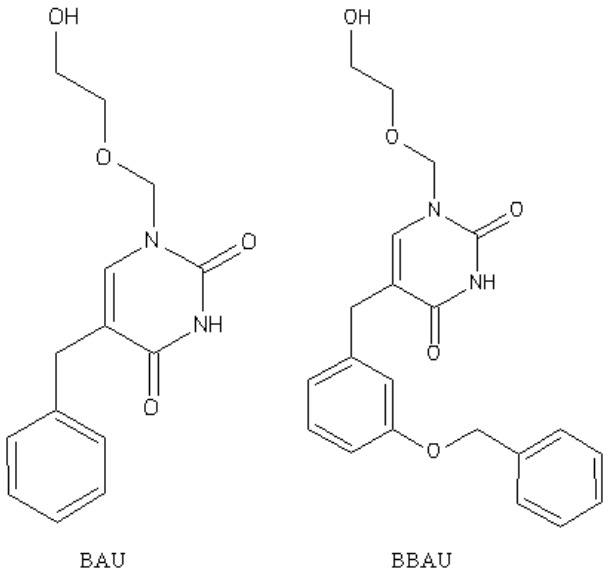


Fig. 30 5-benzylacyclouridine (BAU) and 5-m-benzyloxybenzylacyclouridine (BBAU)

Group of these acyclo nucleosides was further enlarged by 5-phenylthioacyclouridine (PTAU), 5-phenylselenenylacyclouridine (PSAU) and

5-m-benzyloxybenzyl barbituric acid acyclonucleoside (BBBA). In principle, BBBA is BBAU with added =O group at C6 position of uracil ring. The assays, where the inhibitory activity against *E. coli* UP and human UP was designated have shown that BBAU is stronger inhibitor against both enzymes than BAU. PTAU and PSAU have shown stronger activity against human UP than BAU but these two compounds were not better inhibitors than BBBA. [9]

Weiming Bu *et al.* [9] used BAU, BBAU and other compounds for determining of interactions of these inhibitors at the active site of UP and in this way for better understanding of inhibitory abilities in dependence on the structure of inhibitors. The quite large C5 substituent is bonded at the active site by an extra additional hydrogen bonds to the enzyme, in contrast to C5 unsubstituted natural substrates, C5 substituted acyclonucleotides make further interactions to Phe162, Phe7 and Tyr163 in hydrophobic pocket. BBAU and BBBA containing 5-*m*-benzyloxybenzyl moiety make next hydrophobic interactions to Met234 as well.

The binding of inhibitors differs in two general points in contrast to substrate binding as was inscribed in [10], [9] and above in 1.2.1.2 and 1.2.4.

1. The first phenyl ring at the C5 position is close to Phe7 residue belonging to the pyrimidine-binding site. The phenyl ring causes the displacement of Phe7 and its rotation from original position, where Phe7 still undertakes a function in the covering of the hydrophobic pocket but now forms additional herringbone stacking interactions with the first phenyl ring.
2. The loop region from residues 225-230 becomes the second difference in the inhibitor binding. The loop forms closed conformation over the active site in the substrate complex but in the inhibitor complex, the loop does not make so fixed enclosure and the active site remains partially open. Glu227 is involved in establishement of the closed conformation by forming hydrogen bonds to other residues, Tyr169 and Asp170, in contrast to

inhibitor-enzyme complexes, where Glu227 makes no hydrogen bonds with any other residue.

The inhibitors containing benzyloxybenzyl moiety show additional differences, Ile228 and Pro229 shift away from the active site to seat the second phenyl ring, the side chain Met234 rotates and generates hydrophobic interaction with the second phenyl ring.

The acycloribose moiety is so flexible that it could adopt the same *syn*-conformation about the glycosidic bond as sugar ring. This moiety bonds in very similar manner as the ribose or 2´-deoxyribose as was shown in [10], excepting the fact that acycloribosyl moiety creates only two hydrogen bonds to His8 and to water molecule presented in the active site. Which means that the acycloribose is held by weaker power in the active site. Interactions C3´ and C4´ appear to be more variable, presented is just O5´ interaction. And one more exception is BBBA, where only one interaction of O5´ is formed to His8. C1´, O2´ and O5´ fill the same positions both at the inhibitors and at the ribosyl moiety of nucleosides. [9]

Effect on the fluorinated chemotherapeutics

The development of UP inhibitors could be useful for the prevention of degradation of known chemotherapeutics derived from pyrimidine. Concerning the effect of already known inhibitors of UP on the action of fluorinated derivatives in anticancer chemotherapy, it is well-known that they potentiate the effect through the decrease of degradation in organism. One of very interesting molecules is acyclothymidine, the very strong inhibitor of 5´-DFUR phosphorolysis.

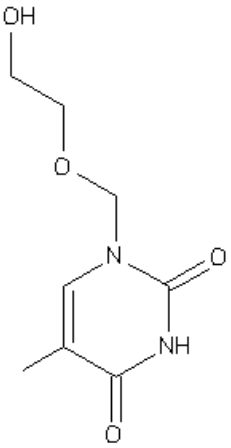


Fig. 31 Acyclothymidine

In the cytotoxicity assays performed for 5-fluoro-2´-deoxyuridine (FdUrd) together with BAU or BBAU the incerease of cytotoxicity caused by these two compounds was not observed but the restriction of uridine intake into the cells proceeds. BBAU also increases the effect of FdUrd on the limitation of tumor growth and following reduction in tumor weight. Further, BAU and BBAU are able to inhibit the transporting of thymidine and adenosine. Only one-third of administered dose is sufficient for reaching the same effect of FdUrd during the BBAU administration than when FdUrd is administered alone. [25] [26]

1.5.2.2 Anhydrouridines

2,2´-Anhydrouridines (Fig. 32) were found to be competitive inhibitors of the rat and *E. coli* UP and none of these 2,2´-anhydro compounds was found to be inhibitor of *E. coli* TP. These derivatives are generaly better inhibitors than their corresponding acyclo- compounds. According to their measured K_i, it is obvious that 2,2´-anhydrouridines are even 33-fold more effective against the rat enzyme than the *E. coli* UP. 2,2´-anhydrouridines are in fixed the high-*anti* conformation, and that is why the distribution of electronic density and the properties are different as compared to uridine. [25]

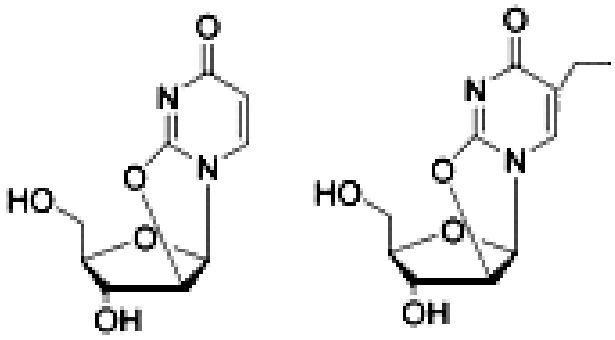


Fig. 32 2,2´-Anhydrouridine and 2,2´-anhydro-5-ethyluridine

C5-substitution of 2,2´-anhydrouridines

These compounds are selective inhibitors of of UP but non-efficient inhibitors of TP. C5-substituted 2,2´-anhydrouridines are substrates of neither UP nor TP. The addition of the ethyl- group at the C5 position led to the higher increase of the inhibitory activity. The resulting 2,2´-anhydro-5-ethyluridine (Fig. 32) obtained much greater inhibitory activity than both the primary anhydro compound and other anhydrouridine derivatives. Next, 5-benzyloxybenzyl substituted compounds were more active than those with 5-benzyl C5-substituted uracil ring. This enhancement of binding of ligands in contrast to 2,2´-anhydrouridine is in agreement with theory of hydrophobic pocket in UP nucleoside binding site, although in the case of anhydrouridines, with the longer C5-chain the inhibitory activity does not increase from ethyl- to benzyloxybenzyl- moiety but the sequence of effectivity is 2,2´-anhydrouridine < 2,2´-anhydro-5-benzyluridine < 2,2´-anhydro-5-(benzyloxybenzyl)uridine < 2,2´-anhydro-5-ethyluridine. As well as the addition of propyl or isopropyl moiety to C5 position led to the decrease of binding with regard to 2,2´-anhydro-5-ethyluridine. The fixed high-*anti* conformation may bear ship to C5 substituents and their orientation at the active site. The large substituents may trouble with access to final conformation, in contrast to previously forenamed acyclonucleosides, which are able to rotate round their *N*-glycosidic bond. [25], [24], [33]

Within the scope of tested compounds, when the inhibitory activity was found for the mouse enzyme, the activity against human UP was demonstrated

also. There exists one exception, 2,5´-anhydrouridine, which shows activity just against the mouse enzyme.

The pentose ring and the influence of hydroxyl groups on the inhibitory activity

Further investigation was focused on the effect of hydroxy groups at the pentose ring. Compounds containing the 5´-hydroxy group show increase of inhibition but some derivatives without this group show the inhibitory activity also, e.g. 5´-deoxy-5-fluorouridine or 2,5´-anhydrouridine. Generally, from this findings emerged the fact that the lack of 5´-hydroxyl group causes the weaker binding to the enzyme. In contrast to the previous group, the 3´-hydroxyl group is essential for the binding of ß-nucleosides to the enzyme. For example 2,3´-anhydro-2´-deoxyuridines lack the binding and even if these compounds are fixed in the *syn*-range, this disability to bind appeared in the ß-lyxo- and ß-xylouracil (shown in Fig. 33), because these compounds do not possess the 3´-hydroxyl group in the correct *ribo*-orientation. The importance of the 2´-hydroxyl group is not very clear, because the 2,2´-anhydrouridines, which do not possess the 2´-hydroxyl group are highly efficient inhibitors of UP and contrary to the arabinosyl uracil, which has the 2´-hydroxyl in the *ara*-configuration and figures as the poor UP inhibitor. [24]

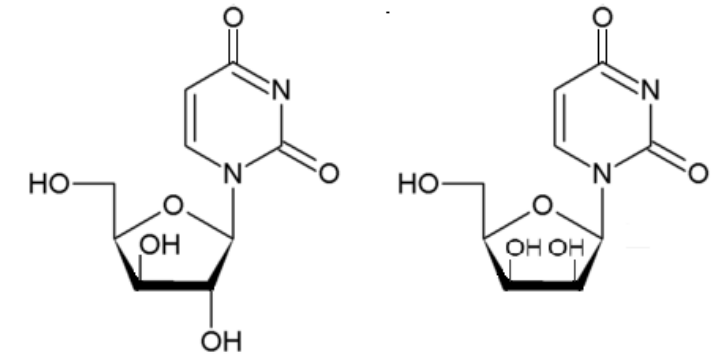


Fig. 33 The chemical structures of uridine analogues modified in the pentose moiety, ß-xylouracil and ß-lyxouracil.

Effect of the conformation around the N-glycosidic bond

According to the *N*-glycosidic bond and its conformation, four groups of uridine derivatives could be described as presented in. [24]

- Derivatives fixed in the *syn*-range (2,2´-anhydro-, 2,3´-anhydro-2´-deoxy- and 2,5´-anhydro-)
- Derivatives fixed in the *anti*-range (6,5´-cyclo-)
- Compounds not fixed (ribosyl-, 2´-deoxyribosyl-, 5´-deoxyribosyl-, arabinosyl-, xylosyl-, lyxosyl- and acyclo-)
- Derivatives without *N*-glycosidic bond (cacbocyclic-)

The best derivatives with demonstrated inhibitory activity were 2,2´-anhydrouridines followed by acyclonucleosides and ribosides. The inability of binding of the carbocyclic compounds (Fig. 34) is due to the lack of N-glycosidic bond as in true nucleosides. The 6,5´-cyclonucleosides lost their ability to bind also due to their *anti*-conformation of *N*-glycosidic bond.

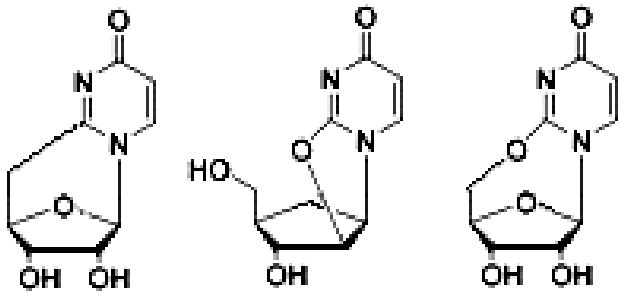


Fig. 34 6,5´-Cyclouridine, carbocyclic-2,2´-anhydrouridine and 2,5´-anhydrouridine

As well as the 2,2´-anhydrouridines, the 2,5´-anhydrouridines are fixed in the *syn*-conformation around the *N*-glycosidic bond and therefore they are inhibitory active against UP also.

Uracil nucleosides occupy mainly the *anti*-conformation and therefore they are more rigid in the formation of bounds to the enzyme active site than their corresponding acyclonucleosides.

All of these facts point to the importance of the *N*-glycosidic bond in the *syn*-conformation, which is necessary for nucleosides to bind to the enzyme.

Differences between the α- and ß-anomers

On the whole, α-anomers form the weaker bound than their ß-anomers. Basically the 5´-hydroxyl group in the α-anomers fills the very similar position in the binding site of the enzyme as the 3´-hydroxyl group in the ß-anomers, but the 5´-hydroxyl is not able to accomodate the exact or strict position, and that is why its binding becomes weaker than in ß-anomers due to the steric blockage, which makes the 3´-hydroxyl at the *xylo*-configuration in the α-xylo and α-lyxo-compounds and because of this 3´-hydroxyl group the 5´-hydroxyl is not able to bind. This fact was observed for α- and ß-anomers of the 2´-deoxyuridines and xylo- and lyxo- compounds. [24]

1.5.3 Novel nucleosides

1.5.3.1 Nucleosides based on 2´-deoxy-2´-fluororibosides

The atom of fluorine substituted for hydroxyl group in the pentosyl moiety of nucleosides causes the manifestation of important biological activities. Analogues with *2´-deoxy-2´-fluoroarabinosyl* moiety show activity against *herpes simplex, simian varicella, varicella-zoster, human cytomegalo, Epstein-Barr, hepatitis B* viruses. Some of them have also antileukemic activity.

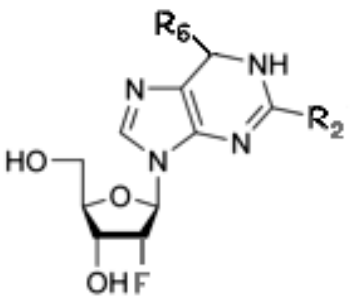


Fig. 35 Purine 2´-deoxy-2´-fluoronucleoside

2´-deoxy-2´-fluororibosyl moiety containing analogues show also activity against some viruses like herpes simplex, pseudorabies, equine abortion, influenza or varicella-zoster. 2´-deoxy-2´-fluorouridine (2FUrd, depicted in Fig. 38) is a substance used for the enzymic transfer of pentosyl moiety for the creation of other purine analogues. Transfer of the pentosyl moiety from 2´-deoxy-2´-fluorouridine to purines involves two coupled reactions using UP or TP and PNP as biocatalysts. These new structures (substituted in 2 and 6 position with amino-, hydrogen-, fluoro-, methyl- or methoxy- group) were tested for the antiinfluenza activity. Purine 2´-deoxy-2´-fluroribosides containing 2-amino substituent on the purine ring appear as more potent against the antiinfluenza virus than 2-unsubstituted congeners. 2´-fluoro analogue of 2´-deoxyguanosine and its counterparts embody the most potent activity. Though 2-amino-6-substituted analogues have not shown significant activity, they are not useless, because they can be converted by the metabolic pathways into other efficient analogues. [19]

1.5.3.2 3´-amino-2´,3´-dideoxyribonucleosides

The next group of interesting nucleosides is made up by 3´-amino-2´,3´-dideoxyribonucleosides (Table 2) synthesized by Krenitsky *et al.* [20] by which the attractive biological effects were discovered. As the basic structures for the syntheses corresponding 2´-deoxyribonucleosides were used.

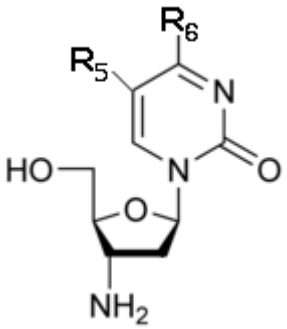


Fig. 36 The basic structure of 3´-amino-2´,3´-dideoxyribonucleosides.

$3NH_2$Thd and $3NH_2$Urd were synthesized by a quite laborious chemical method through the tritylation of the 5´-hydroxy group of ribose moiety followed

by the chlorination in 3´-position, then through the deblocking of 5´-hydroxy position. Arisen *threo*-3´-chloro derivatives converted to the *erythro*-3´-azido derivatives and the last step was the subsequent catalytic hydrogenation. Synthesis of $3NH_2$IUrd was performed by the same way as previous nucleosides, $3NH_2$Thd and $3NH_2$Urd, but under the milder conditions of the reactions because of the presence of 5-iodo substituent compared to $3NH_2$BrUrd, $3NH_2$ClUrd and $3NH_2$FUrd which were synthesized enzymaticaly. The procedure used TP (purified from *E.coli*) as the catalyst. Aminopentosyl moiety was transfered from $3NH_2$Thd to corresponding 5-halogenouracil. 3´-azido-2´,3´-dideoxyuridine became the starting material for the synthesis of $3NH_2$Cyd, it was aminated in the 4-position and subsequently hydrogenated to $3NH_2$Cyd.

Name of nucleoside	Abbreviation	Substituents	
		R_5	**R_6**
3´-amino-2´,3´-dideoxythymidine	**$3NH_2$Thd**	CH_3	OH
3´-amino-2´,3´-dideoxyuridine	**$3NH_2$Urd**	H	OH
3´-amino-2´,3´-dideoxy-5-iodouridine	**$3NH_2$IUrd**	I	OH
3´-amino-2´,3´-dideoxy-5-bromouridine	**$3NH_2$BrUrd**	Br	OH
3´-amino-2´,3´-dideoxy-5-chlorouridine	**$3NH_2$ClUrd**	Cl	OH
3´-amino-2´,3´-dideoxy-5-fluorouridine	**$3NH_2$FUrd**	F	OH
3´-amino-2´,3´-dideoxycytidine	**3´-NH_2-dCyd**	H	NH_2

Table 2 Pyrimidine 3´-amino-2´,3´-dideoxyribonucleosides, which were synthesized by *Krenitsky et al.* [20] from corresponding 2´-deoxyribonucleosides.

These nucleosides were tested for the ability of inhibition of the growth of mammalian cells, bacteria and viruses *in vitro*. Only $3NH_2$FUrd was quite effective against *adenovirus*. Tests for the antibacterial activity performed with $3NH_2$FUrd against some Gram-positive bacteria (*Staphylococus aureus, Streptococcus faecalis* and *Streptococcus agalactiae*) showed the inhibition of organisms, whereas the activity against Gram-negative bacteria was not demonstrated. The other 3´-amino-2´,3´-dideoxyribonucleosides showed the inhibitory activity against neither Gram-positive nor Gram-negative organisms. $3NH_2$Cyd showed cytotoxicity to human sternal marrow cells and to mouse connective tissue cells, while $3NH_2$Thd is also toxic but one-third as $3NH_2$dCyd. Important thing in $3NH_2$FUrd is that this compound is nontoxic *in vivo* and have

antiviral and antibacterial abilities in concentracions, which do not inhibit the growth of mammalian cells. [20]

1.5.4 TP inhibitors and their drawbacks

TP inhibitors and their usage have also some disadvantages, because these compounds can inhibit TP directly thereby they can cause the accumulation of thymidine in plasm, which cause toxic effect (previously have been found that the low TP activity is connected with pathological changes in the brain and muscles). Moreover, the TP activity is very important for the activation of known antitumor drugs (e.g. 5´-deoxy-5-fluorouridine – 5´-DFUR), so the administration of inhibitor together with that drug could cause some problems and it is necessary to have respect to this fact. [23]

2 Experimental part

2.1 The aims of study

This work largely deals with studies on substrate specifity of the *E. coli* recombinant thymidine phosphorylase (TP) and recombinant uridine phosphorylases from *E. coli* (UP) and two from *Salmonella tymiphurium* (UP1 and UP2).

The main goals of this study are:

1. Determination of kinetic parameters for natural substrates (thymidine and uridine) for recombinant TP and UP;
2. Assessment of the role of phosphate anion in the enzymatic transformations;
3. Search for new inhibitors of TP and UP.

2.2 Materials and methods

2.2.1 Chemicals

- Uridine phosphorylases (UP, UP1, UP2) - prepared in the Department of Biotechnology (Shemyakin-Ovchinnikov Institute of Bioorganic chemistry, Russian Academy of Sciences, Moscow, Russia; Head of the Department Prof. A. I. Miroshnikov; [Esipov R. S.; Gurevich A. I.; Chuvikovsky D. V.; Chupova L. A.; Muravyova T. I.; Miroshnikov A. I.; Protein Expr. Purif. 2002, 24, 56-60.])
- Thymidine phosphorylase (TP) - prepared in the Department of Biotechnology (Shemyakin-Ovchinnikov Institute of Bioorganic chemistry, Russian Academy of Sciences, Moscow, Russia)
- Potassium dihydrogen phosphate (KH_2PO_4) – Merck, Germany
- Acetonitrile (MeCN) – Merck, Germany
- Natrium thiophosphate (Na_3PO_3S) – Merck, Germany
- $2NH_2$Urd – 2´-amino-2´-deoxyuridine
- $3NH_2$Urd – 3´-amino-3´-deoxyuridine
- 2FUrd – 2´-deoxy-2´-fluorouridine
- $3NH_2$Thd – 3´-amino-3´-deoxythymidine
- 2-O-MeUrd - 2´-O-methyluridine
- AraFrU – 1-(ß-D-fructofuranosyl)uracil
- Uridine (Urd)
- Thymidine (Thd)

2.2.2 Equipment

- Laboratory digital pH-meter Orion, model 420A, US
- HPLC column – Waters C18; 5 µm; 4,6 x 150 mm

- *Varian Cary-300 Bio* spectrophotometer (software Cary WinUV version 3.0 (182) – used for determining an enzyme kinetics run and kinetic parameters K_M and V_{max}

2.2.3 Kinetic measurements

The best way to obtain enzyme kinetic data is to use Michaelis-Menten equation and Lineweaver-Burk plots. The estimation of K_M of phosphorolysis of uridine by UPases and of thymidine by TP was obtained by spectrophotometric estimation of the reaction rate at 265 nm for TP and 260 nm for UPases where the maximum absorbtions were observed.

Reaction conditions: Into the cuvette, the substrate, K-phosphate buffer and stock solution of enzyme were added. The enzyme was added for the initiation of reaction and than immediately measured.

At first we prepared stock solutions of buffer, substrate and enzymes:

- K-phosphate buffer: 5mM solution (0.68 g was dissolved in 1.0 l), pH = 7.0
- The amounts of substrate added into reaction mixture, uridine or thymidine, varied from 20 to 60 µl of 100 µM stock solution (0.025 mg in 1 ml of KPB).
- Stock solutions of enzymes: 0.95 mg of protein was dissolved in 1.0 ml of KPB (5 mM, pH = 7.0) that corresponds to 28.5 U. 20 µl of this stock solution is taken into reaction, i.e., 0.57 U of enzyme is taken into reaction.

Reaction mixture: Substrate (20, 30, 40, 45, 50, 55 or 60 µl) + 20 µl of enzyme + 5 mM KPB in exact amount for refilling to 1.0 ml.

The mixture was mixed and immediately measured. To obtain K_M and V_{max} values, we used the Lineweaver-Burk plots.

2.2.4 Substrate specifity

The phosphorolysis of our tested compounds by TP and UP was measured by high-performance liquid chromatography (HPLC) analysis. This HPLC analysis have been performed in isocratic elution on the reverse phase column.

HPLC system: buffer A: 5 mM KPB (pH = 5.5) and buffer B: 0% with one exception for AraFrU where the isocratic elution ran in buffer A: 5 mM KPB (pH = 5.5) and buffer B: 3%

For single injections on the column were prepared reaction mixtures containing tested substrate, enzyme and K-phosphate buffer (KPB) or thiophosphate buffer (Na_3PO_3S) as a solvent.

Stock solution of the substrate was prepared by dissolving of 1.0 mg of substrate in 1.0 ml 5 mM KPB. Concentration of enzymes varied for UP from *E. coli* (3.9 mg in 25 ml 5 mM KPB or 7 mg in 1.5 ml 5 mM KPB) stock solutions. UP1(2) was used only in concentration 7.2 mg in 1.5 ml 5 mM KPB in stock solution. Stock solution of TP contains 3.9 mg in 25 ml 5 mM KPB.

Into the Eppendorf tubes we put 20 µl of substrate stock solution, 20 µl of enzyme stock solution and refilled with the 5 mM KPB to 1.0 ml of total volume. We prepared KPB solutions with various pH for our reactions. Reaction mixtures were performed in pH 6.5, 8.0 or 9.0. The 5 mM thiophosphate buffer (all measurements with this buffer were carried out in pH = 8.0) was prepared for investigation of the role of phosphate in reactions catalyzed by UP and TP and was added into reaction mixtures in the same way as KPB.

Freshly prepared reaction mixtures were injected at HPLC column in several time intervals, immediately after the addition of enzyme, after 0.5 hour, 1.0 hour, 1.5 hour and further injections were dependent on already observed activity.

HPLC analysis was performed under the conditions of isocratic elution on the UV-based detection was performed at 254 nm. A flow rate was set up to 1.0 ml/min.

3 Results

3.1 Kinetic measurements

Kinetic parameters (Michaelis constant K_m, maximum rate V_{max} and catalytic constant k_{cat}) were assignated for phosphorolysis of uridine by uridine phosphorylases and thymidine by thymidine phosphorylase and compared with published data. Kinetic parameters are shown in **Table 3**. All these data have been obtained in 5 mM K-phosphate buffer (pH=7.0). We have previously shown that the 5 mM buffer has some experimental advantages over the 50 mM buffer for an enzymatic synthesis of nucleosides.

Substrate/Enzyme	K_M, μM	V_{max}, μM min^{-1}	k_{cat}, sec^{-1}
Thymidine/TP [2]	95	25	50
Uridine/UP [3]	80	36	77
Uridine/UP1	73	48	69
Uridine/UP2	194	74.5	112

Table 3 Kinetic paramerets of enzymatic phosphorolysis of thymidine and uridine by the recombinant thymidine phosphorylase (TP, *E. coli*) and uridine phosphorylases from *E. coli* (UP) and from *Salmonella typhimurium* (UP1 and UP2) [1]

[1] All the measurements have been performed in 5 mM K-phosphate buffer (pH= 7.0) at 23°C.

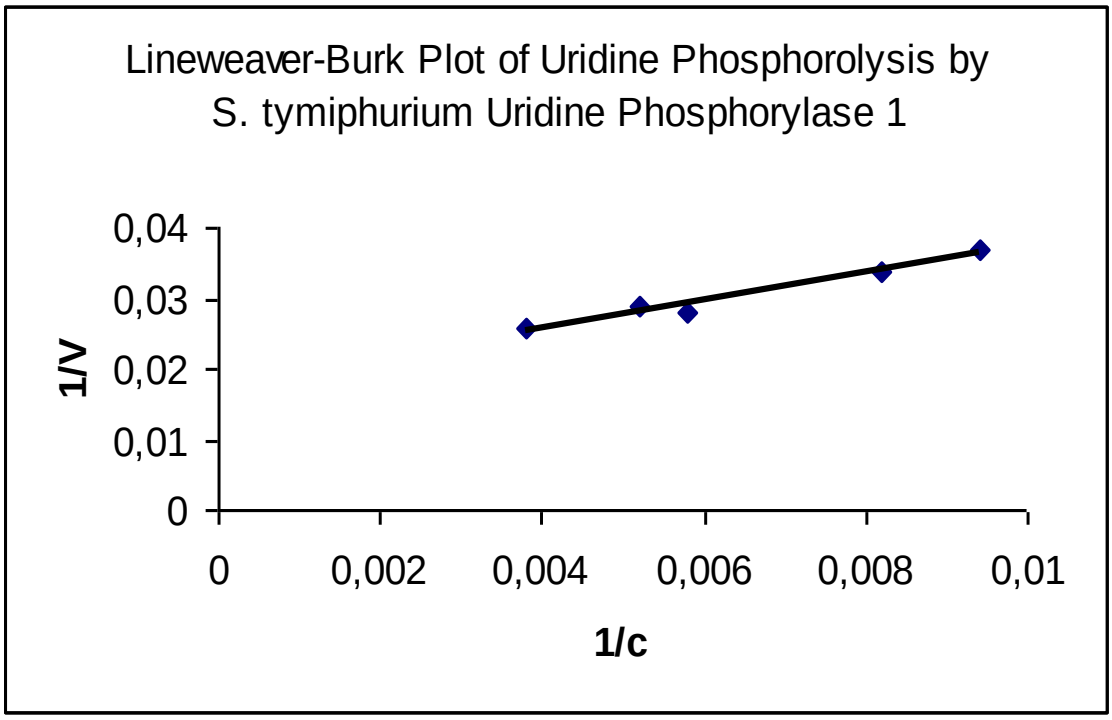
[2] Lit. Data observed for TP by Panova N.G. *et al.* [14]: K_M = 300 μM; V_{max} = 119 μM min^{-1}; k_{cat} = 198 sec^{-1}

Under conditions of the present study the phosphorolysis of thymidine by TP is reached after one hour a 1:1 equilibrium of thymidine and thymine as it followed from the HPLC data of the reaction mixture. Establishment of the equilibrium is accompanied by the drop of absorption at 266 nm from 2.24 to

2.038 ($\Delta\varepsilon$ = 850 $M^{-1}cm^{-1}$). The molar extinction coefficient for thymidine was found to be ε = 9,480 M^{-1} cm^{-1}. All the measurements have been performed at the λ = 265 nm.

[3] Lit. Data observed for UP by Panova N.G. *et al.* [14]: K_M = 80 μM; V_{max} = 180 μM min^{-1}; k_{cat} = 98 sec^{-1}.

In all the experiments with UPases, practically complete phosphorolysis was observed after 1 h and the $\Delta\varepsilon$ value was determined to be 1,840 $M^{-1}cm^{-1}$. During the phosphorolysis, displacement of the maximum absorption from 261 to 259 nm was observed; all the measurements have been performed at the λ = 260 nm.

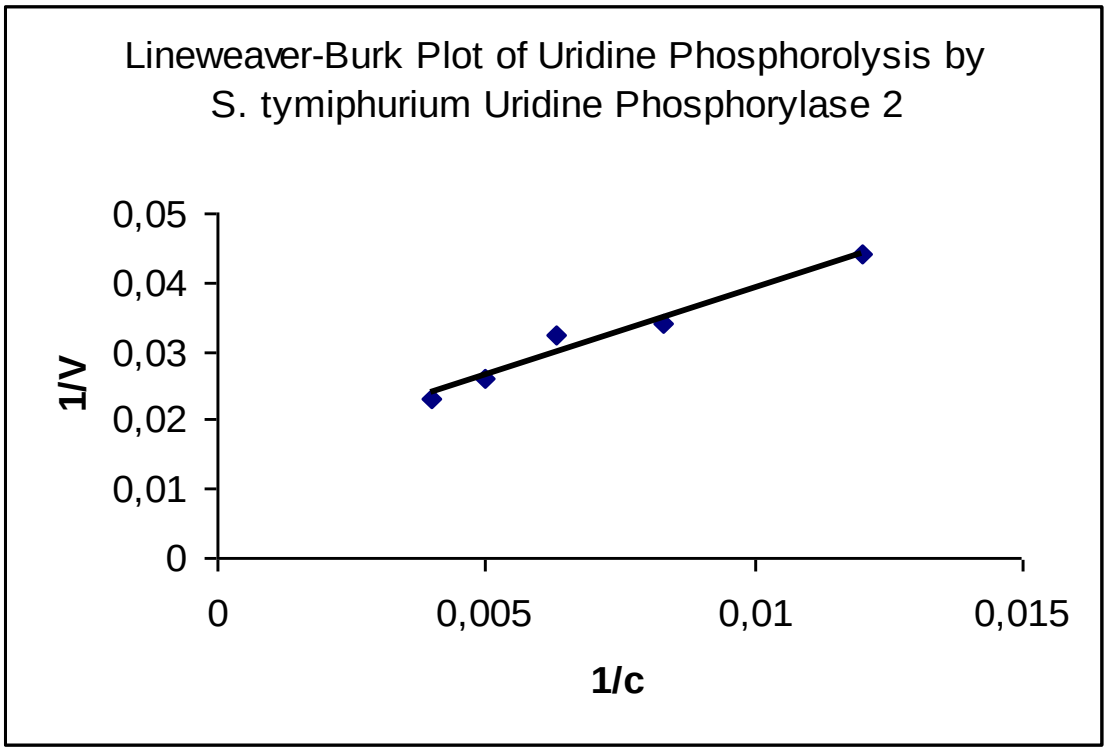


Graph 1 shows the behaviour of uridine phosphorolysis, 1/c [μM^{-1}], 1/V [min. μM^{-1}].

K_M = 73 µM

V_{max} = 48 µM min^{-1}

k_{cat} = 69 sec^{-1}

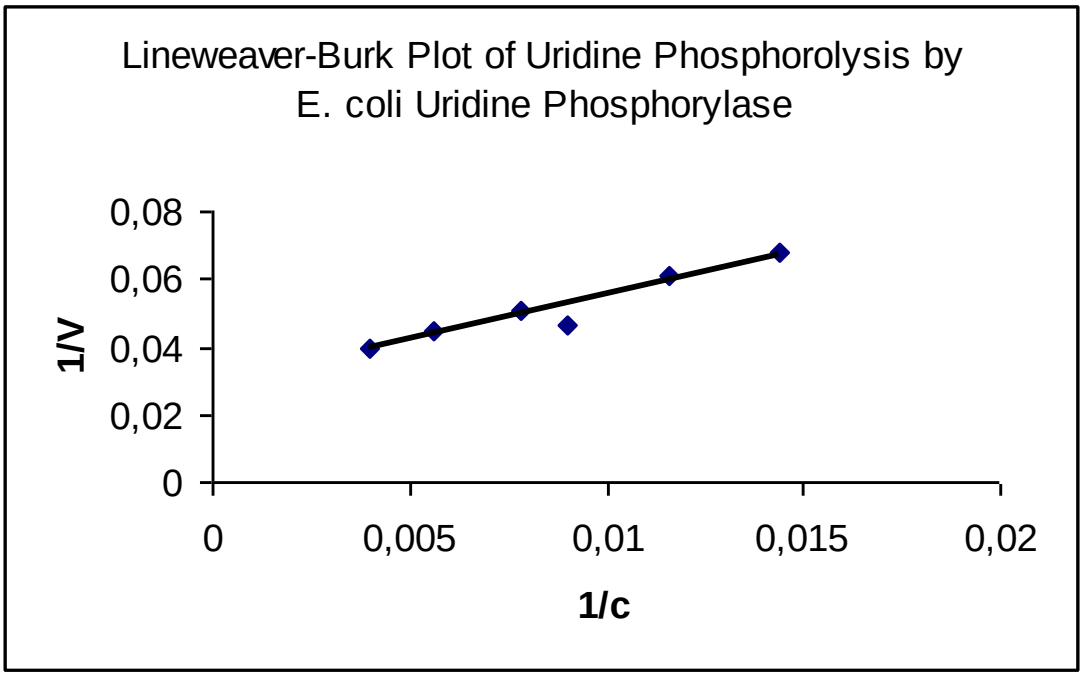


Graph 2 shows the behaviour of uridine phosphorolysis by UP2, 1/c [μM^{-1}], 1/V [min. μM^{-1}].

K_M = 194 µM

V_{max} = 74.5 µM min^{-1}

k_{cat} = 112 sec^{-1}

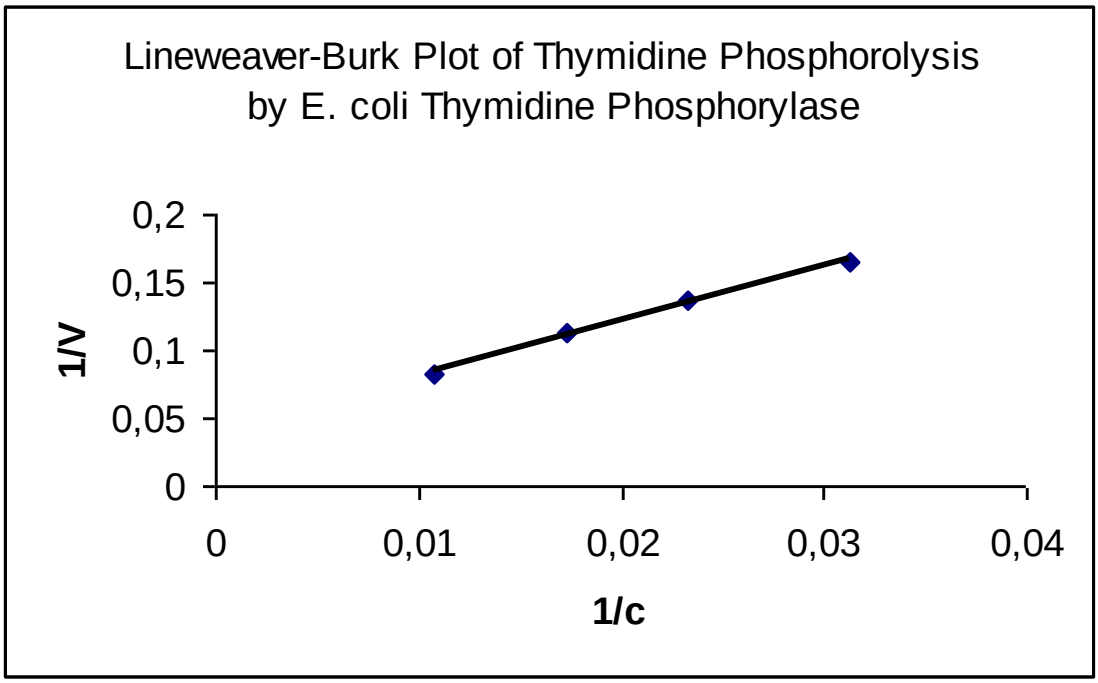


Graph 3 shows the behaviour of uridine phosphorolysis by UP, 1/c [μM^{-1}], 1/V [min. μM^{-1}].

K_M = 80 µM

V_{max} = 36 µM min^{-1}

k_{cat} = 77 sec^{-1}



Graph 4 shows the behaviour of thymidine phosphorolysis, 1/c [µM^{-1}], 1/V [min. µM^{-1}].

K_M = 95 µM

V_{max} = 25 µM min^{-1}

k_{cat} = 50 sec^{-1}

3.2 Experiments with UP

In the following part of our work, we illustrate the substrate specifity of investigated enzymes. We have compared the similarity of UP from *Escherichia coli* (UP) and *Salmonella typhimurium* (UP1 and UP2).

3.2.1.1 Studies on 2´-O-Methyluridine and 1-(ß-D-fructofuranosyl)uracil

2´-O-Methyluridine (2´-O-MeUrd) and **1-(ß-D-fructofuranosyl)uracil** (AraFrU) devoid of both substrate and inhibitory activities for our tested uridine phosphorylases from *E. coli* and *S. typhimurium*. The activity was tested at pH = 6.5, 8.0 and 9.0 with 5 mM KPB. The thiophosphate buffer was not used for these two compounds.

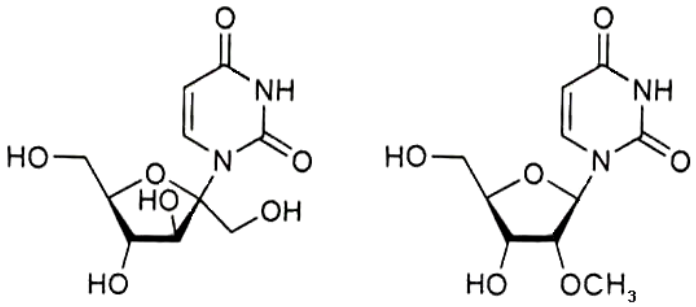


Fig. 37 No substrate ativityfor uridine phosphorylases for E. coli and S. typhimurium was found in our measurements for AraFrU and 2-O-MeUrd.

But these two compounds were not found to have some effect on the activity of enzymes as is shown below in Table 4 and in Table 5.

2´-O-Methyluridine (2´-O-MeUrd)				
enzyme	**pH**	**Retention time (R_t, min)**	**reaction time**	**product %**
UP	6,5	7,72	30 min	−
UP	8,0	7,84	20 (50) hours	−
UP2	8,0	9,96	22 hours	−
UP	9,0	10,42 (10,19)	17 (40) hours	−

Table 4

1-(ß-D-fructofuranosyl)uracil (AraFrU)				
enzyme	**pH**	**Retention time (R_t, min)**	**reaction time**	**product %**
UP	6,5	2,25	30 min	-

Table 5

UP - Recombinant Uridine Phosphorylase from *E. coli*
(stock solution = 7 mg in 1,5ml KPB)
UP2 - Recombinant Uridine Phosphorylase from *S. typhimurium*
(stock solution = 7,2 mg in 1,5ml KPB)

For the reaction was used volume 20 µl of the stock solution of enzyme, i.e., 0.57 U of enzyme is taken into reaction mixture.

HPLC system:

- all reactions for 2´-O-MeUrd have been preformed in isocratic elution B 0%, buffer A: 5 mM KPB (pH 5.5)
- the system for AraFrU was as follows: buffer A: 5 mM KPB (pH 5,5); buffer B: 5 mM KPB in 60% MeCN (pH 5,5); isocratic B 3%

3.2.1.2 Studies on 2´-deoxy-2´-fluorouridine

Reaction mixtures with **2´-deoxy-2´-fluorouridine** were prepared for both types of buffer (5 mM KPB and Na_3PO_3S) and also here the third type of buffer was used (50 mM Na_3PO_3S). The pH values were 6.5, 8.0 and 9.0. Apparently, 2FUrd was phosphorylated only in Na_3PO_3S in pH = 8.0 but in very low yield, the other values of pH were not found to be suitable for reaction

process. After the analysis of reaction mixtures containing 20 µl of enzyme solution, where the yield of product was very poor, we prepared and measured the process of phosphorolysis with higher amount of enzyme stock solution (80 µl). Reaction mixtures prepared in this manner did not cause the increase of yielded product but the reaction proceeded faster. Table 6 represents our obtained results.

2´-Deoxy-2´-fluorouridine (2FUrd)					
enzyme/volume	**buffer**	**pH**	**Retention time (R_t, min)**	**reaction time**	**product %**
UP (20µl)	KPB 5 mM	6,5	7,86	50 hours	–
UP (20µl)		8,0	7,93	50 hours	–
UP (20µl)		9,0	7,87	17 hours	–
UP (20µl)		9,0	7,82	40 hours	–
UP2 (20µl)		8,0	7,49	21 hours	0,60%
UP (20µl)	Na_3PO_3S 5 mM	8,0	7,86	24 hours	0,57%
UP2 (20µl)		8,0	7,86	24 hours	0,44%
UP (80µl)		8,0	7,90	1,5 hour	0,51%
UP2 (80µl)		8,0	7,95	1,5 hour	0,39%
UP (80µl)	Na_3PO_3S 50 mM	8,0	7,89	2,5 hours	0,57%
UP2 (80µl)		8,0	7,90	2,5 hours	0,44%

Table 6

UP - Recombinant Uridine Phosphorylase from *E. coli*
(stock solution = 7 mg in 1,5 ml KPB)

UP2 - Recombinant Uridine Phosphorylase from *S. typhimurium*
(stock solution = 7,2 mg in 1,5 ml KPB)

HPLC system: all the reactions have been performed in isocratic elution B 0%, buffer A: 5 mM KPB (pH 5.5)

Injected volumes of reaction mixtures were 20 µl and 80 µl respectively.

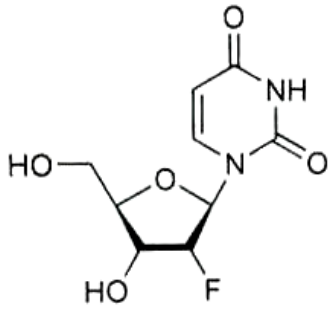
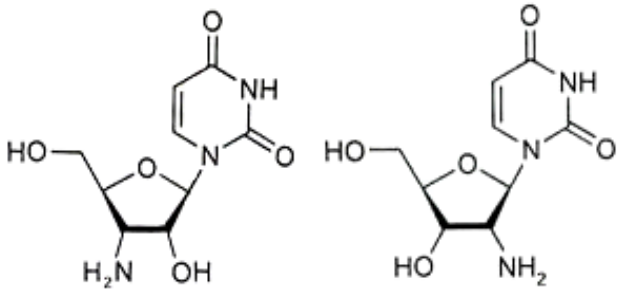


Fig. 38 2´-deoxy-2´-fluorouridine (2FUrd)

3.2.1.3 Results for aminouridines

Our work was focused on two uridine derivatives, **2´-amino-2´-deoxyuridine** ($2NH_2Urd$) and **3´-amino-3´-deoxyuridine** ($3NH_2Urd$).



2NH2Urd **3NH2Urd**

As is obvious from our obtained data, $2NH_2Urd$ is a quite good substrate for phosphorolysis made by both UP from *E. coli* and UP2 from *S. thyphimurium* in pH = 8.0. Results obtained for $2NH_2Urd$ are shown below in Table 7.

2´-Amino-2´-deoxyuridine ($2NH_2Urd$)					
enzyme	**buffer**	**pH**	**Retention time (R_t, min)**	**reaction time**	**product %**
UP	KPB 5 mM	6,5	3,36	30min	–
UP2		8,0	3,50	22 hours	60,40%
UP		8,0	3,44	1 hour 20 hours	20,40% 86,00%
UP	Na_3PO_3S 5 mM	8,0	3,36	1,5 hour 20 hours	66,20% 81,60%
UP2		8,0	3,39	1,5 hour 20 hours	65,00% 68,20%

Table 7

Injected volumes of reaction mixtures in column were 20 µl.

UP - Recombinant Uridine Phosphorylase from *E. coli*

(7 mg in 1,5 ml KPB)

UP2 - Recombinant Uridine Phosphorylase from *S. typhimurium*

(7,2 mg in 1,5 ml KPB)

HPLC system: all the reactions have been performed in isocratic elution B 0%, buffer A: 5 mM KPB (pH = 5.5)

3´-Amino-3´-deoxyuridine ($3NH_2Urd$)					
enzyme	**buffer**	**pH**	**Retention time (R_t, min)**	**reaction time**	**product %**
UP (20µl)	KPB 5 mM	8,0	3,16	20 hours	9,80%
UP2 (20µl)		8,0	3,08	3,5 hour 20 hours	4,50% 23,90%
UP2 (80µl)		8,0	3,04	22 hours	23,00%
UP (80µl)		8,0	3,08	22 hours	36,60%
UP (20µl)	Na_3PO_3S 5 mM	8,0	3,10	3 hours	-
UP2 (20µl)		8,0	3,05	3 hours	-

Table 8

UP - Recombinant Uridine Phosphorylase from *E. coli*

(stock solution = 7 mg in 1,5 ml KPB)

UP2 - Recombinant Uridine Phosphorylase from *S. typhimurium*

(stock solution = 7,2 mg in 1,5 ml KPB)

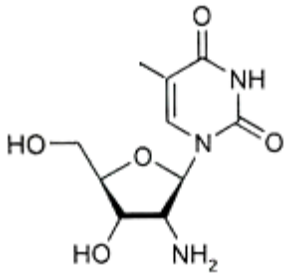
HPLC system: all the reactions have been performed in isocratic elution B 0%, buffer A: 5 mM KPB (pH = 5.5)

In the case of $3NH_2$Urd, indeed, the reaction proceeded but slowly and with quite low final yield (Table 8).

3.3 Experiments with TP

3.3.1.1 Phosphorolysis of 3´-amino-3´-deoxythymidine

Substrate specifity of thymidine phosphorylase from *E. coli* was studied in the case of **3´-amino-3´-deoxythymidine** ($3NH_2$Thd) at pH = 6.5 or 8.0 in prepared reaction mixtures. Table 9 presents our results achieved by HPLC analysis.



3NH2Thd

3´-Amino-3´-deoxythymidine ($3NH_2$Thd)					
enzyme	**buffer**	**pH**	**Retention time (R_t, min)**	**reaction time**	**product %**
TP	KPB 5 mM	6,5	4,02	20 hours	25,50%
TP		8,0	4,02	45 min	13,60%

Table 9

TP – thymidine phosphorylase from *E. coli*

HPLC system: all the reactions have been performed in isocratic elution B 0%, buffer A: 5 mM KPB (pH = 5.5)

3.3.1.2 The role of pH and phosphate

In to gain further insight into the substrate activity of aminodeoxy nucleosides, we have investigated two aspects of this reaction, 1 – the role of the pH of the medium, and 2 – the role of the phosphate.

1. pH – we have performed our measurements at three different values of pH, 6.5, 8.0 and 9.0.
2. Buffer – two types of buffers were involved in our measurements, 5 mM K-phosphate buffer (for reactions at all kinds of pH values) and 5 mM thiophosphate buffer (only for measurements at pH = 8.0)

All obtained data are depicted above in Table 5-10.

4 Discussion

Determination of kinetic constants

We have measured kinetic parameters in 5 mM KPB, pH = 7.0. In the most publications the 50 mM buffer was employed in kinetic studies by other authors leading to somewhat different results as is shown above. 50 mM K-phosphate buffer, pH = 7.5 for UP and pH = 6.5 for TP, was used for determination of kinetic parameters by Panova *et al.* [14]. Zinchenko *et al.* [34] used 50 mM KPB at pH = 7.0.

2-O-MeUrd and AraFrU

No formation of nucleobases was observed either in variations of pH or after quite long incubation time (even 50 hours). That data clearly show that the bulky substituent at the C2´ and C1´ carbon atoms, respectively, preclude the formation of productive enzyme-analog complex and proceeding of phosphorolysis.

2FUrd

2´-Deoxy-2´-fluorouridine displayed very low substrate activity for TP and UPases, which is in accord with reported data on the synthesis of purine 2´-deoxy-2´-fluoro-ß-D-ribonucleosides using 2´-deoxy-2´-fluorouridine as a donor of the pentofuranose residue in the enzymatic transglycosylation reaction. (Tuttle J. V. *et al.* [19], Zaitseva G. V. *et al.* [36])

The very low substrate activity of 2´-deoxy-2´-fluorouridine for both enzymes may be explained by higher electronegativity of C2´ fluorine atom and by the shortening and strengthening of glycosidic bond upon replacement of the 2´-hydroxyl group of uridine by a fluorine atom. This glycosidic bond is hard to cleave for enzymes.

2NH2Urd and 3NH2Urd

As is clearly visible from our findings, reactions proceeded only in pH = 8.0 and is much faster in the presence of Na_3PO_3S than in KPB under the same conditions, but finally the phosphorolysis stopped at the same yield – the reaction got into an equilibrium faster.

It was previously shown in experiments with UP and TP within the whole *E. coli* cells (50 mM K-phosphate buffer, pH=7.0) that substrate activities of 2´-deoxyuridine, 3´-deoxyuridine, 2´-amino-2´-deoxyuridine and 3´-amino-3´-deoxyuridine are 35.5%, 6.4%, 11.6% and 0.6% compared to uridine, activity of which was calculated as 100%. From these data it remained unclear the reason for an activity drop by going from uridine to 2´-deoxyuridine and further 2´-amino-2´-deoxyuridine, and, moreover, very low activity of 3´-amino-3´-deoxyuridine vs 3´-deoxyuridine. [34]

The intact cells of *Erwinia herbicola* AJ 2803 have been employed by the Japanese workers as a biocatalyst for the synthesis of 2´-amino-2´-deoxyinosine using chemically prepared 2´-amino-2´-deoxyuridine as a donor of the pentofuranose moiety and hypoxanthine (3.23:1.0 ratio, mol) as an acceptor in 34% isolated yield based on the acceptor. [38] Recently, the results of studies have been reviewed by Utagawa [39].

$3NH_2$Thd

Our measurements showed that TP is able to phosphorylased 3NH2Thd but in not very good portion of product. After measurements with TP we tried to test this compound in reactions with UP from *E. coli* and UP1 and UP2 from *S. typhimurium.* $3NH_2$Urd devoid of activity for both types of enzyme.

It was also shown that 3´-amino-3´-deoxythymidine is a very poor substrate of TP from *E. coli.* Krenitsky *et al.* [20] have used this $3NH_2$Thd as donor of carbohydrate moiety in the reaction of enzymic transglycosylation.

Later, 3´-amino-3´-deoxythymidine was used by Zaitseva G. V. *et al.* [37] as a donor of the pentofuranose moiety in the transglycosylation of adenine and guanine and it was found that it is poor substrate also.

The role of pH and thiophosphate buffer

It was found that pH plays very important role, viz., at pH = 6.5 the enzymatic phosphorolysis proceeds extremely slowly, whereas at pH = 8.0 the reaction is going with reasonable rate (which is apparent for $3NH_2$Urd and $2NH_2$Urd).

Inorganic phosphate is an essential co-factor to complete the reaction. In pH = 6.5 we supposed that the amino group in $3NH_2$Urd and $2NH_2$Urd exists in $–NH_3^+$ form and makes a strong ionic bond with $-PO_4^{3-}$, but our results are different. In $3NH_2$Urd in Na_3PO_3S there was no reaction observed. Because we can suppose that $–PO_3S^{3-}$ makes stronger bond (ionic) than $–PO_4^{3-}$ and therefore there is no reaction. In pH = 8.0 the amino group exist as $–NH_2$. It makes hydrogen bond, which is weaker than the ionic bond for binding $-PO_4^{3-}$ and $–O^-$ can have influence to C1´ of ribose moiety (to glycosidic bond). Probably because of that the reaction of $3NH_2$Urd proceeded in KPB but in thiophosphate buffer not.

The amino group in C2´ of ribose is stereospecific for reaction. The reaction of $2NH_2$Urd in Na_3PO_3S buffer proceeds much more faster than in KPB but after 20 hours the reaction was on the same level as reaction in KPB (round 80%). The acceleration of reaction rate in Na_3PO_3S buffer is the result of forming stronger hydrogen bond between $–O^-$ of the buffer and $–NH_2$ of the ribose (hydrogen bond is a presumption for the effectiveness of phosphorolysis). Lower pH values cause the formation of ionic bonds and the reaction can not proceed.

To understand the role of phosphate, we investigated the reaction in 5 mM thiophosphate buffer taking into account that thiophosphoric acid is more acidic as phosphoric, on the one hand, and sulphur atom is more nucleophilic

than oxygen. [35], [30] However, we have not observed essential differencies in the reaction rate in both buffers.

We have not found some efficient inhibitor, all of our tested compounds were either substrate or had no influence on the enzyme function. We added the natural substrate (Urd or Thd) into reaction mixture containing modified nucleoside and enzyme and we monitored if the enzyme is able to do the phosphorolysis of natural substrate in the presence of modified nucleoside. By all of tested compounds the enzyme was not able to react.

5 Conclusions

The most important feature of ivestigated enzymes consists in high sensibility to structural changes of substituents at secondary carbon atoms of the pentofuranose ring.

The important observation is that aminodeoxy nucleosides studied by us showed that they are not substrates in the ammonium form of TP and UPases, whereas display satisfactory substrate activity in the amino form. Whereout we can designate the importance of pH in our performed reactions for the activity of enzymes.

The most important conclusion of this study consists in gaining further insight into the mechanism of functioning TP and UP that is very important in design of preparative synthesis of nucleosides using these enzymes as biocatalysts.

The detailed understanding of TP and UPs functions could be instrumental in syntheses and development of new inhibitors of these enzymes and in following research or development of new potencial anticancer chemotherapeutics.

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