

THESIS OF Ph. D. DISSERTATION

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Comparative study of structure and function of the prokaryote *Escherichia coli* and the eukaryote *Drosophila melanogaster* dUTPases

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1. INTRODUCTION AND AIMS

The enzyme dUTP nucleotidohydrolase (dUTPase) catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate, thereby preventing a deleterious incorporation of uracil into DNA (Fig. 1. 1. and 1. 2.). Lack of dUTPase leads to uracil-substituted DNA that perturbs base excision repair, resulting in DNA fragmentation and thymineless apoptosis of the cell. Based on the significant role of the enzyme in DNA metabolism, it was proposed as a novel target for anticancer and antiviral drug design. The enzyme dUTPase is essential for viability of bacteria, so it could also be a good target for the development of antibacterial agents.

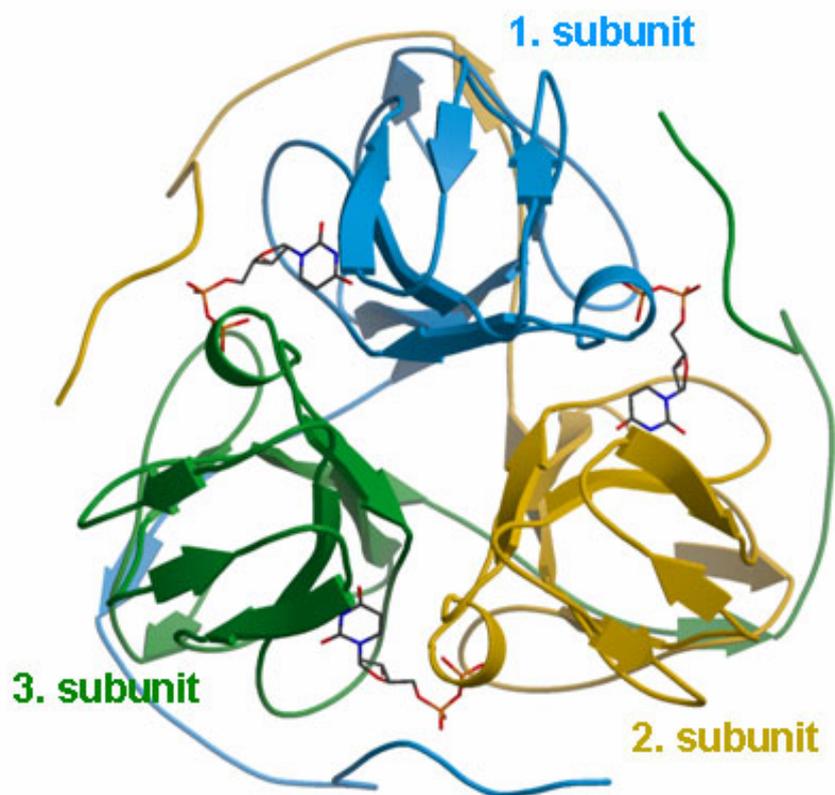


Figure 1. 1. – The crystal structure of homotrimeric FIV (feline immunodeficiency virus) dUTPase:dUDP complex (PDB ID: 1F7R)

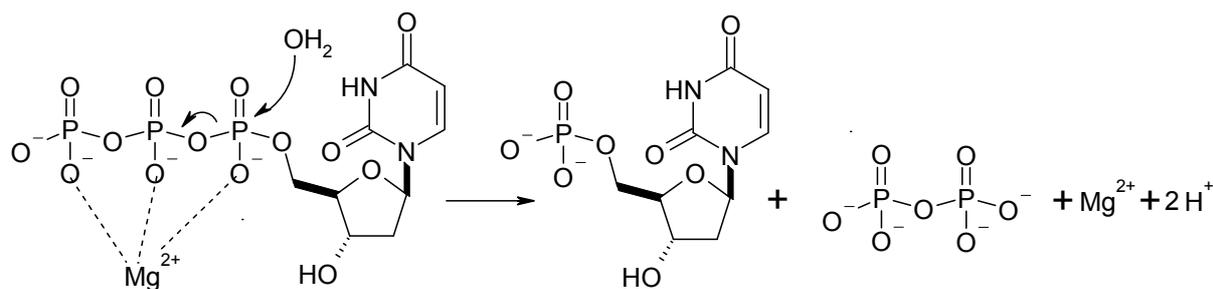


Figure 1. 2. – Reaction scheme of dUTP hydrolysis as catalysed by dUTPases

I intended to characterize the functional evolution of dUTPases by kinetic, ligand binding and crystallographic analysis based on the eukaryote *Drosophila melanogaster* and the prokaryote *Escherichia coli* dUTPases.

Analysis of the substrate binding and catalytically active pocket of dUTPase may give important additional insights into the functional mechanism of the enzyme and helps us to design effective inhibitors against dUTPases. Two candidates, α,β -imido-dUTP and α,β -methylene-dUTP, were chosen as inhibitors for characterization of the catalytically active pocket of the *Escherichia coli* dUTPase. Both compounds differ from the substrate dUTP in the group located between α - and β -phosphorus. Supposedly, the imido group in place of the oxygen renders the substrate analogue less hydrolyzable. The methylene substitution presumably causes an even less hydrolyzability of the substrate analogue due to the low electronegativity of the carbon atom ($EN_C=2.5 < EN_N=3.0 < EN_O=3.4$).

2. METHODS

Recombinant dUTPases were produced in BL21 *Escherichia coli*. After purification the enzymes were obtained in a relatively good yield (10-20 mg/l) and purity (>95%).

Measurement of dUTPase activity was carried out using a spectrophotometric continuous dUTPase enzyme activity assay convenient for kinetic enzyme characterization. Proton release was followed during the transformation of dUTP into dUMP and PP_i in phenol red indicator assay buffer using a Jasco-V550 spectrophotometer. Initial velocity was determined from the slope of the initial part of the progress curve. This method was used to follow activity during purification and trypsinolysis.

Limited tryptic digestion of *Drosophila melanogaster* dUTPase in the absence or presence of nucleotide ligands was carried out to determine exposed peptide bonds to tryptic cut or flexible protein segments which become conformationally changed or ordered upon ligand binding. Aliquots were taken at different time points for activity measurements and for SDS-PAGE.

Near-UV CD spectra were recorded on a JASCO 720 spectropolarimeter to determine enzyme:ligand dissociation constants. Far-UV CD spectra were recorded on the same instrument to get information about the ratio of secondary structural elements in the enzyme.

3. RESULTS

3. 1. Enzyme-catalyzed production of α,β -imido-dUTP

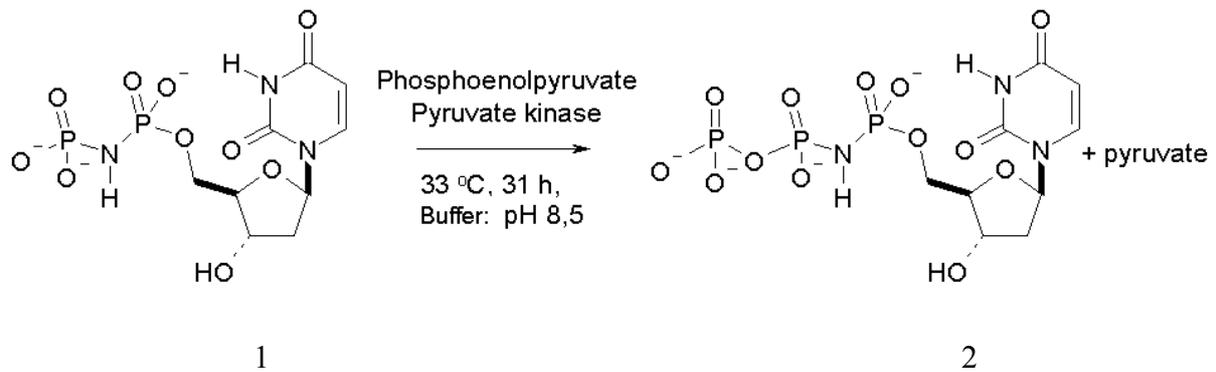


Figure 3. 1. 1. – Enzyme-catalyzed production of α,β -imido-dUTP (2)

I produced α,β -imido-dUTP (2) from α,β -imido-dUDP (1) and phosphoenolpyruvate by the pyruvate kinase-catalyzed reaction shown on Figure 3. 1. 1.

3. 2. *In vitro* analysis of the role of the *Drosophila melanogaster* specific C-terminal dUTPase segment

One of the aims of the present study was to decide whether the *Drosophila melanogaster* specific C-terminal 28-residue extension might have some regulatory effect on enzyme kinetic behavior. The results obtained with the purified recombinant protein constructs (full length and C-terminally truncated proteins, see Fig. 3. 2. 1.) reveal that no such effect could be observed *in vitro*. It is, of course, possible that this region has some potential other role, not accessible in the present experiments conducted in purified systems. Such a role might be to offer a recognition site for some cellular interacting partners.

PSTDFADIPAAK¹²K¹³MK¹⁵IDTCVLR²²FAK²⁵L TENALEPVR³⁵GSAK³⁹AGVDLR⁴⁶SAYDVVV PAR⁵⁶GK⁵⁸AIVK⁶²TD
 Motif I
 LQVQVPEGSYGR⁷⁶VAPR⁸⁰SGLAVK⁸⁶NF IDVGAGVVDEYR¹⁰¹GNLGVVLFNHSDVD FEVK¹¹⁹HGDR¹²³IAQFICER¹³¹
 Motif II Motif III Motif IV
 IFYFQLVMVDK¹⁴²LEDTER¹⁴⁸GEAGFGSTGVK¹⁵⁹DLPAAK¹⁶⁵AQNGNGEK¹⁷³AAEPEGAAPAVAT
 Motif V

Figure 3. 2. 1. - Amino acid sequence of *Drosophila melanogaster* dUTPase. The residues shown as bold letters are conserved in all dUTPases of the trimeric family. The locations of conserved motifs are indicated by roman numbers. The *upper index numbers* account for tryptic sites. The *Drosophila melanogaster* dUTPase-specific C-terminal 28 residues are boxed. The residues confirmed by MS/MS sequencing are underlined.

3. 3. The divalent metal ion as a structural factor in the *Drosophila melanogaster* dUTPase

CD and DSC experiments indicated that Mg(II) binding to *Drosophila melanogaster* dUTPase induces protein conformational changes even in the absence of nucleotide ligands. These results argue for a metal ion site present in the fly enzyme that is accessible in the nucleotide-free protein. I suggest that apart from its universal role of a catalytically important co-factor for dUTPases from all sources, Mg(II) may also have an additional structural role in some of the dUTPases.

3. 4. Conformational shifts in the C-terminus are induced differently in *Drosophila melanogaster* and *Escherichia coli* dUTPases

Detailed studies from several laboratories unanimously indicated that the ordering of the C-terminus of *Escherichia coli* dUTPase requires interaction with the complete triphosphate chain of the substrate. In the present work, experimental evidence from limited proteolysis, CD spectroscopy, and DSC argue in agreement that dUMP and dUDP are also

capable of inducing a significant conformational change upon binding to *Drosophila melanogaster* dUTPase. Limited proteolysis experiments localized this conformational change to the C-terminal conserved motif V (Arg¹⁴⁸ tryptic site, Fig. 3. 4. 1.), removal of which leads to inactivation of the enzyme. Three-dimensional crystal structures of the human and the feline immunodeficiency virus dUTPase in complex with dUTP analogues suggest that the ordered conformation of the C-terminus is realized by its closing over the active site and contacting the bound nucleotide phosphate.



Figure 3. 4. 1. – The two tryptic sites characteristic for the *Drosophila melanogaster* dUTPase are shown in the crystal structure of human dUTPase: α,β -imido-dUTP:Mg(II) complex (PDB ID: 2HQU). The enzyme is represented by ribbon model. Arg¹⁴⁸ and Arg¹³¹ are represented by stick model. The α,β -imido-dUTP-s in the three active sites are shown with stick model. Mg(II)-s are shown with spheres. The Arg¹³¹ is situated about 17 Å away from the nucleotide-binding pocket. (Based on the crystal structure of human dUTPase the distance is 16,3 Å between the Arg¹³¹ α C and the Arg¹⁴⁸ NH1.) Note: In this crystal structure the C-terminal arm of the human dUTPase became ordered on two active sites of three upon ligand binding.

3. 5. Binding of dUDP and α,β -imido-dUTP in the active site of *Drosophila melanogaster* dUTPase triggers allosterism in the enzyme

In addition to protection at the tryptic site in the conserved motif V, and indicative of the closed enzyme conformer, binding of dUDP or α,β -imido-dUTP to *Drosophila melanogaster* dUTPase also exerted significant protection at an additional cleavage site. The latter site was identified by mass spectrometric analysis of tryptic digests as the Arg¹³¹-Ile¹³² peptide bond that is situated about 17 Å away from the nucleotide-binding pocket (Fig. 3. 4. 1.). This result demonstrates that nucleotide-binding induced conformational changes are coupled between the active site and the inner treefold interaction surface of the homotrimer.

In contrast to the above discussed results, the only tryptic cleavage site accessible to limited digestion experiments in *Escherichia coli* dUTPase is the one in motif V.

3. 6. Identification of the nucleophile water molecule

As we could not crystallize the homotrimeric form of the *Drosophila melanogaster* dUTPase we did not have the possibility to realise a comparative study between the pro- and eukaryotic dUTPase active sites. Therefore analysis of the active site of the *E. coli* enzyme was performed. I produced the inactive AspIIIAsn mutant *Escherichia coli* dUTPase. Analyzing the crystal structures of the mutant and wild type *Escherichia coli* dUTPases in complex with α,β -imido-dUTP, Barabas et al. presented a description of the catalytic pathway via identification of the nucleophile water molecule and characterization of interactions responsible for building up the required active site arrangement and initiating the reaction.

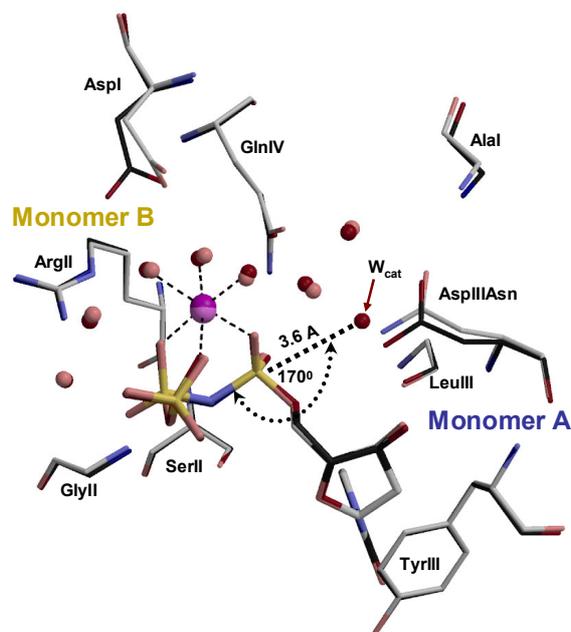


Figure 3. 6. 1. - Superimposed structures of wild type (*dark tones*) and AspIIIAsn mutant (*light tones*) dUTPase: α,β -imido-dUTP:Mg(II) complexes. Note that the only remarkable difference between the superimposed structures is the disappearance of W_{cat} from the mutant complex. Mg(II)-s are represented with big spheres, water molecules are represented with small spheres.

3. 7. Synthesis of α,β -methylene-dUDP

3. 7. 1. Synthesis of 5'-*O*-tosyldeoxyuridine

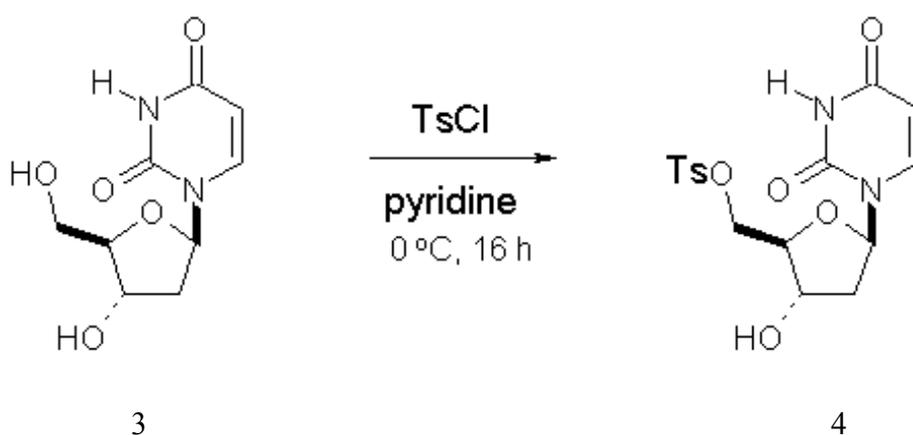


Figure 3. 7. 1. 1. - Synthesis of 5'-*O*-tosyldeoxyuridine (4)

I synthesized the 5'-*O*-tosyldeoxyuridine (4) as shown in Fig. 3. 7. 1. 1.

3. 7. 2. Synthesis of α,β -methylene-dUDP

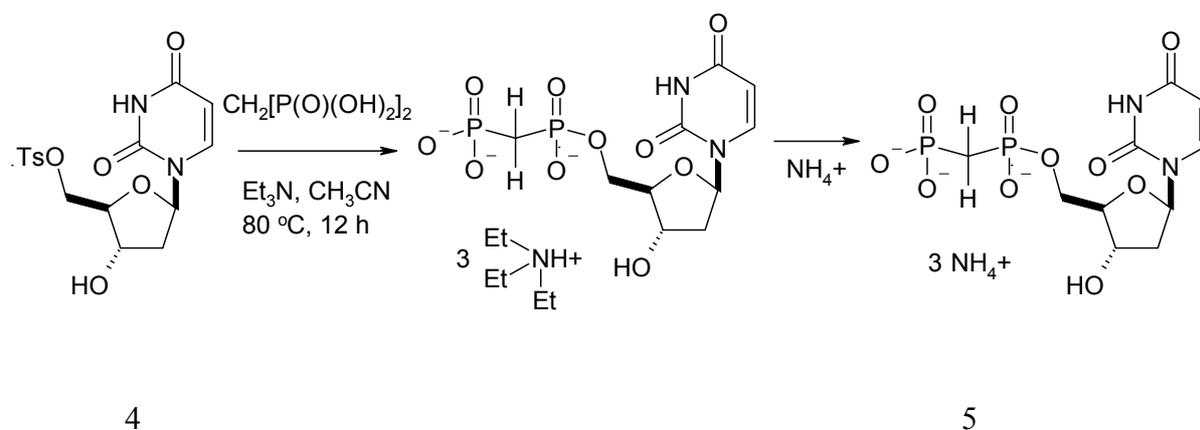


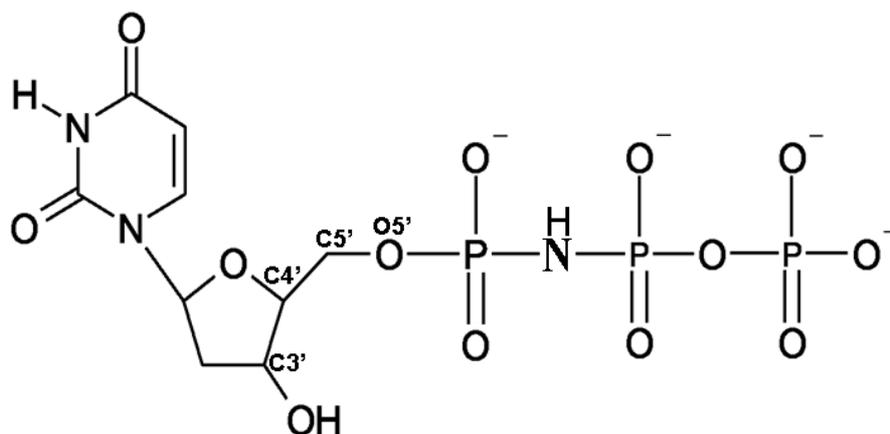
Figure 3. 7. 2. 1. - Synthesis of α,β -methylene-dUDP (5)

I synthesized the α,β -methylene-dUDP (5) as shown in Fig. 3. 7. 2. 1.

3. 8. Methylene substitution at the α - β bridging position within the phosphate chain of dUDP profoundly perturbs ligand accommodation into the dUTPase active site

We present structural and functional data suggesting that the methylene substitution in α,β -methylene-dUDP induces significant distortion of the phosphate chain binding conformation within the active site of dUTPase. There is a difference between the α -phosphate site of α,β -methylene-dUDP (*trans* conformation concerning the C3'-C4'-C5'-O5' dihedral angle) and the α -phosphate site of dUTP, α,β -imido-dUTP and dUDP (*gauche* conformation) bonded in the active site of *E. coli* dUTPase (Fig. 3. 8. 1.). This distortion stabilizes the α -P atom at a site that is incompetent with the incoming nucleophilic attack initiating hydrolysis of the substrate analogue. The binding mode also interferes with accommodation of the divalent metal ion cofactor and decreases binding affinity approximately 20-fold.

A



B

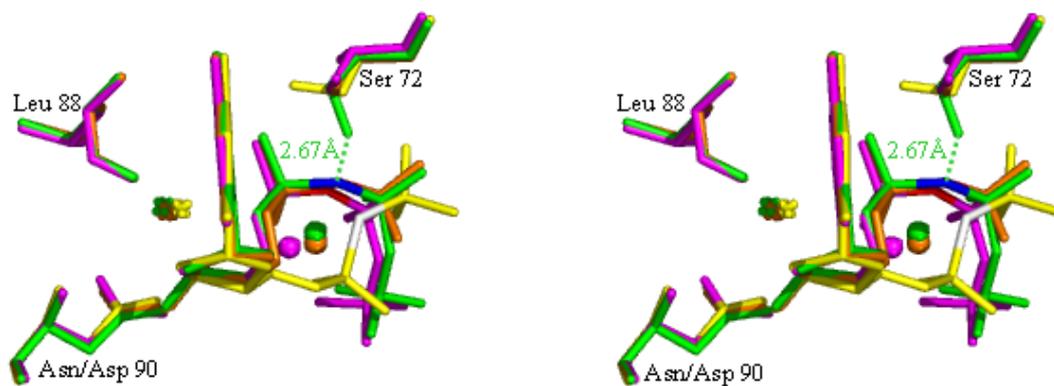


Figure 3. 8. 1. – (A) The C3'-C4'-C5'-O5' atoms of the nucleotide ligands are shown in the α,β -imido-dUTP molecule. (B) Stereo view of superimposed structures of *E. coli* dUTPase:dUDP:Mn(II) (*gauche*) (PDB ID: 2HR6), *E. coli* dUTPase: α,β -methylene-dUDP (*trans*) (PDB ID: 2HRM), inactive mutant *E. coli* dUTPase:dUTP:Mg(II) (*gauche*) (PDB ID: 1RNJ), *E. coli* dUTPase: α,β -imido-dUTP:Mg(II) (*gauche*) (PDB ID: 1RN8). Attacking water molecules are represented as stars, metals as spheres.

4. APPLICATIONS

There are differences between the trimer interface channels of the prokaryotic (*Escherichia coli*, *Mycobacterium tuberculosis*) and the eukaryotic (*Drosophila melanogaster*, *Homo sapiens*) dUTPases. A specific drug molecule binding in the prokaryotic dUTPase channel might disorder the dUTPase active site with little or no chance of inhibiting the eukaryotic enzyme. In human therapy it would be beneficial if the inhibitor drug were designed to be specific for bacterial enzymes (for example *Mycobacterium tuberculosis*) as dUTPase is encoded in the human genome and provides an essential housekeeping function for host.

Methylene analogues of dUTP do not present strong binding inhibitors against dUTPase possibly due to the altered binding mode of the phosphate chain of α,β -methylene-dUDP. Therefore, the effective concentration required for drastic inhibition of dUTPase would be in the millimolar range within the cells. Such high concentration would easily lead to many additional interactions with other proteins that bind nucleotide phosphates resulting in sub-optimal specificity. Results also indicate that methylene analogues may not faithfully reflect the catalytically competent conformation; and their use as substrate-mimicks requires caution. The binding affinity of α,β -imido-dUTP is relatively high, but this substrate analogue was shown to be hydrolyzable. Therefore it is not suggested as an optimal lead-molecule for inhibitor design. However it was shown that the imido analogue bind in the active site of dUTPase in a similar manner as dUTP, hence it seems to be an adequate substrate analogue to analyze the possible dUTPase:dUTP interactions.

5. PUBLICATIONS

5. 1. Publications included in the dissertation

1.

Kovári J, Barabás O, Takács E, Békési A, Dubrovay Zs, Pongrácz V, Zagyva I, Imre T , Szabó P and Vértessy BG

Altered Active Site Flexibility and a Structural Metal-binding Site in Eukaryotic dUTPase: KINETIC CHARACTERIZATION, FOLDING, AND CRYSTALLOGRAPHIC STUDIES OF THE HOMOTRIMERIC DROSOPHILA ENZYME.

J Biol Chem **279**, 17932-44. (2004)

2.

Kovári J, Imre T , Szabó P and Vértessy BG

Mechanistic studies of dUTPases

Nucleosides Nucleotides Nucleic Acids **23**, 1475-9. (2004)

3.

Kovári J, Barabás O, Varga B, Békési A, Tölgyesi F, Fidy J, Nagy J, Vértessy BG

Methylene substitution at the α - β bridging position within the phosphate chain of dUDP profoundly perturbs ligand accommodation into the dUTPase active site

Proteins, to be published (2007)

4.

Kovári J

THE POTENTIAL ROLE OF dUTPase INHIBITION IN CHEMOTHERAPY

PERIODICA POLYTECHNICA SER. CHEM. ENG. VOL. 49, NO. 1, PP. 60-61 (2005)

5.

Barabás O, Pongrácz V, **Kovári J**, Wilmanns M and Vértessy BG

Structural Insights into the Catalytic Mechanism of Phosphate Ester Hydrolysis by dUTPase.

J Biol Chem **279**, 42907-15. (2004)

6.

Barabás O, Dubrovay Zs, Harmat V, **Kovári J**, Takács E, Zagyva I, Náray-Szabó G, Vértessy BG

Structural studies of *Drosophila Melanogaster* dUTPase

Acta Cryst. A58, C96 (2002)

5. 2. Oral and poster presentations included in the dissertation

1.

Kovari J., Barabas, O., Merenyi, G., Zagyva, I., Vértessy, B. G.

dUTPase mRNA silencing triggers apoptosis in cancer cells

31st FEBS Congress, Molecules in Health & Disease, Istanbul, Turkey, 2006. 06. 24-29.,

poster

2.

Kovari J., Barabas, O., Merenyi, G., Zagyva, I., Vértessy, B. G.

dUTPase mRNA silencing triggers apoptosis in cancer cells

Magyar Biokémia Egyesület Vándorgyűlése, Pécs, 2006. 08. 30 – 09. 02., poster

3.

Kovári J., Barabás O, Nagy J, Vértessy BG

A dUTPáz gátlásának potenciális szerepe a rákterápiában

2nd CONFERENCE OF PHD STUDENTS AT FACULTY OF CHEMICAL ENGINEERING, Budapest University of Technology and Economics, November 24, 2004, oral presentation

4.

Kovári J., Takacs E, Imre T , Szabo P and Vértessy BG

Mechanistic studies of dUTPases

Joint 11th International and 9th European Symposium on Purines and Pyrimidines in Man, 2003. 06. 9-13., Netherlands, Egmond aan Zee, Hotel Zuiderduin, poster and oral presentations

5.

Kovári J., Békési A, Takács E, Szavicskó I, Pongrácz V, Barabás O, Szabó P, Vértessy BG

Ecetmuslica dUTPáz: Eukarióta modell az enzimműködés evolúciójának tanulmányozására

A Magyar Biokémiai Egyesület Molekuláris Biológiai Szakosztályának 7. munkaértekezlete, Keszthely, 2002. 05. 14-17., poster

6.

Kovári J, Békési A, Barabás O, Takács E, Szavicskó I, Szabó P, Vértessy BG *Drosophila melanogaster* dUTPase: a preventive DNA repair factor

EACR 17, 17th Meeting of the EUROPEAN ASSOCIATION FOR CANCER RESEARCH,
8-11 June 2002, GRANADA, poster

5. 3. Other publications

1.

Békési A, Zagyva I, Hunyadi-Gulyas E , Pongrácz V, **Kovári J**, Nagy ÁO, Erdei A,
Medzihradzky KF and Vértessy BG

Developmental Regulation of dUTPase in *Drosophila melanogaster*.

J Biol Chem **279**, 22362-70. (2004)

2.

Faigl F, Thurner A, Tárkányi G, **Kovári J**, Mordini A, Tóke L

Optical Resolution and Enantioselective Rearrangements of Amino Group Containing
Oxiranyl Ethers

Tetrahedron: Asymmetry, **13**, 59-68 (2002)

