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Application of modified nucleotides in biosynthesis of nucleic acids

SUMMARY OF DOCTORAL DISSERTATION

Natural sciences,
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Modifikuoti nukleotidai nukleorūgščių biosintezėje: taikomieji aspektai

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CONTENTS

LIST OF ABBREVATIONS

HIV-1	human immunodeficiency virus type 1
M.MuLV	Moloney murine leukemia virus
PAA	phosphonoacetic acid
RT	reverse transcriptase

INTRODUCTION

The simplicity and elegance of natural genetic systems inspired scientists since the discovery of DNA double helix structure (Watson and Crick, 1953). Two genetic biopolymers, DNA and RNA, are a universal genetic code based on the rule of complementarity. This simple rule is sufficient to store and propagate the genetic information of all species on Earth. In addition to the four canonical letters of the genetic alphabet, various non-canonical nucleotides have been discovered in the genomic DNA that act as regulatory elements and as a natural defence mechanism (Warren, 1980; Carell et al., 2018). However, that is not enough for biotechnological applications. Many of the major biotechnological processes depend on the ability of DNA polymerases to accept chemically modified nucleotides. This includes DNA and RNA sequencing (Goodwin et al., 2016; Fuller et al., 2016), systematic ligand evolution with exponential enrichment (Sefah et al., 2013) and DNA tagging (Welter et al., 2016). At first glance, it is surprising that DNA polymerases naturally developed to recognize canonical nucleobases are able to use nucleotides with quite significant modifications (Welter et al., 2016; Hottin and Marx, 2016).

First modified nucleotides have been created 50 years ago and used as anticancer or antiviral preparations to suppress various polymerases (Jordheim et al., 2013). Nucleotide modification studies are not limited to polymerase inhibition, but are expanding to the field of synthetic biology, genetic code extension studies (Eremeeva and Herdewijn, 2018), studies of epitranscriptome (Palumbo and Beal, 2018) and the production of modified oligonucleotides (Morihiro et al., 2017). By studying the use of modified nucleotides for DNA biosynthesis, it is possible to better understand the fundamental features of DNA structure and polymerase specificity (Kropp et al., 2018).

Retroviruses have a unique viral reproduction strategy using reverse transcriptase (RT) for DNA biosynthesis from viral RNA. Since in 1981 discovered acquired immunodeficiency syndrome (Gottlieb et al., 1981) and 1983 discovery of a human immunodeficiency virus (HIV) (Barré-Sinoussi et al., 1983), this virus remains a serious problem: 36.7 million infected with HIV, of which 1.8 million die each year (UNAIDS, 2017). Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase is among the most important targets for antiviral treatment. Reverse transcriptase of Moloney mouse leukemia virus (M.MuLV) represents a group of monomeric retrovirus reverse transcriptases. This group includes mouse breast tumour virus, cattle leukemia virus, swine endogenous retrovirus and retrotransposon Tf1 (Hizi and Herschhorn, 2008). These reverse transcriptases exhibit other biochemical properties than the host replication polymerases. The ability to utilize properties of polymerases would allow the development of inhibitors directed against viral polymerases without affecting of host polymerases. At the same time, the ability of polymerases to use alternatively structured nucleotides would allow synthesizing DNA with additional functionalities.

The use of modified nucleotides for DNA biosynthesis has become the subject of synthetic biology research in order to broaden the genetic code and create an artificial functional biological system with wider application possibilities. In this work, the use of modified pyridone nucleosides and nucleotides in DNA biosynthesis, as well as phosphonoacetic acid nucleotide conjugates designed to inhibit DNA polymerases, are discussed.

The specific aim of this study were:

1. To investigate the aspects of pyridone nucleosides and phosphonacetate-conjugated nucleotide applications in DNA biosynthesis.

Tasks:

1. Evaluation of pyridone nucleosides for inhibition of DNA biosynthesis.
2. Determination of the efficiency and specificity of the incorporation of pyridone nucleotides into DNA.
3. Investigation of the incorporation of phosphonoacetate conjugates into DNA and investigation of the mechanism of inhibition of phosphonoacetic acid conjugates.

Scientific novelty

The influence of modified nucleosides and nucleotides on DNA biosynthesis described in this dissertation has not been reported in literature before. This work describes the ability of new acyclic and cyclic nucleoside derivatives bearing a 2-pyridone ring to inhibit Klenow exo- DNA polymerase and M.MuLV reverse transcriptase. A potential M.MuLV reverse transcriptase inhibitor has been identified, the inhibition of which depends on the nature of catalytic metal ions. In addition, 2-pyridone nucleotides were employed for DNA biosynthesis with reduced accuracy and elongation efficiency. Also, the ability of phosphonoacetic acid nucleotide conjugates to inhibit DNA biosynthesis with Klenow exo- polymerase, HIV-1 and M.MuLV reverse transcriptases was evaluated. One of the conjugates tested is able to inhibit Klenow exo- polymerase and HIV-1 reverse transcriptase.

Practical value

During the work, we found a potential M.MuLV reverse transcriptase inhibitor, active in the presence of a with manganese ions, while inhibition is not observed in buffer with magnesium ions.

This indicates a possible change in the structure of the enzyme, which allows the inhibitor to bind to the enzyme. It is important in study of potential new inhibitors, where the site of the enzyme is formed only by interacting with an inhibitor (Ding et al., 1995; Ren et al., 1995; Tantillo et al., 1994). In addition, the study of the properties of the pyridone nucleotides reveals how nucleobase substitutions influence efficiency of DNA biosynthesis. It is important for the production of aptamers and variously modified oligonucleotides. DNA polymerases selected for the study, Klenow exo- and M.MuLV reverse transcriptase, are enzymes with different properties and applications. Klenow exo- was able to use halogenated pyridone nucleotides more efficiently than M.MuLV reverse transcriptase. This shows the different applicability of enzymes using unnatural nucleotides. In this work, the use of phosphonoacetic acid nucleotide conjugates for inhibiting DNA biosynthesis was evaluated. One of the conjugates tested is able to inhibit Klenow exo- polymerase and HIV-1 reverse transcriptase by altering the tertiary complex conformation. Such conformational changes can slow down DNA polymerases. Therefore, conjugation of these low molecular weight compounds with nucleotides may be the basis for further development of new antiviral drugs.

Defensive Claims:

1. Pyridine nucleosides can inhibit DNA biosynthesis by M.MuLV RT and Klenow exo- DNA polymerase.
2. The DNA polymerases can insert into DNA pyridone nucleotides in a template-specific manner. Pyridone incorparation has little effect on further primer extension using natural nucleotides.
3. M.MuLV RT, HIV-1 RT, Klenow exo- and Taq polymerases incorporate into DNA tested nucleotide conjugates. These compounds can inhibit DNA biosynthesis.

1 MATERIALS AND METHODS

Buffers and solutions. Nucleic acid electrophoresis 15% polyacrylamide gel solution: Rotiphorese® Gel40 (19:1) diluted to 15% with 8 M urea, 89 mM Tris, 89 mM boric acid, 2 mM EDTA; 1x TBE electrophoresis buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA; 45 mM TB electrophoresis buffer: 45 mM Tris, 45 mM Boric Acid; Nucleic acid electrophoresis 8% polyacrylamide gel solution: Rotiphorese® Gel40 (37.5:1) diluted to 8%, 45 mM Tris, 45 mM boric acid; STOP solution: 95% formamide, 0.060% bromophenol blue and xylene cyanol, 25 mM EDTA; 10% acetic acid solution: 100 mL acetic acid was mixed with 900 mL deionized water; 10x reaction buffer A for T4 polynucleotide kinase: 500 mM Tris-HCl (pH 7.6), 100 mM magnesium chloride, 50 mM DTT, 1 mM spermidine; Glutamate buffer: 20 mM sodium glutamate (pH 8.2), 10 mM DTT, 0.5% Triton X-100, 20 mM NaCl, before using add divalent metal salts; Tris-acetate buffer: 33 mM Tris-acetate (pH 7.9), 66 mM potassium acetate, 0.1 mg/mL BSA, before using add divalent metal salts.

DNR primers and templates.
Primers: T7: 5‘TAATACGACTCACTATAGGGAGA3‘**;**
T7-PrA488: 5‘TAAT(A488)ACGACTCACTATAGGGAGA3‘**.**
Templates:
T7-mA:
5‘CCGGAATTAAAATCTCCCTATAGTGAGTCGTATTA3‘;
T7-mT: 5‘CCGGTTAATTTTTCTCCCTATAGTGAGTCGTATTA3‘;
T7-mG:
5‘TTAAGGCCGGGGTCTCCCTATAGTGAGTCGTATTA3‘;
T7-mC: 5‘TTAACCGGCCCCTCTCCCTATAGTGAGTCGTATTA3‘;
T7Tm: 5‘GGTTAACCAAAATCTCCCTATAGTGAGTCGTATTA3‘;
T7Am: 5‘CTCAAAACTTTCTCCCTATAGTGAGTCGTATTA3‘;

DNA substrates: T7/mA: T7 hybridizing with T7-mA, T7/mT: T7 hybridizing with T7-mT, T7/mG: T7 hybridizing with T7-mG, T7/mC: T7 hybridizing with T7-mC, T7/A: T7 hybridizing with T7-mC, T7/A: T7 hybridizing with T7Am, T7/T: T7 hybridizing with T7Tm. The fluorescent tag Atto488 is attached to the fourth nucleotide dT of the T7-PrA488 primer at the 5' terminus.

Introduction of a phosphate group with ^{33}P into the 5' terminus of primers. Reaction mixture is prepared (T7*): 2 µL 10x reaction buffer A for T4 polynucleotide kinase; 1 µL of 10 u/µL T4 polynucleotide kinase; 6 µL of 3.3 µM ^{33}P-γATP; 2 µL 10 µM T7; 9 µL of water. The reaction is carried out at 37 °C for 20 min. Then 1 µL of 0.5 M EDTA was added to the reaction mixture and heated at 80 °C for 10 min. Used immediately or stored at -20 °C.

^{33}P tagged DNA primer hybridization with template and gel filtration. A hybridization mixture is prepared (100 nM DNA substrate): 10 µL T7*; 1.2 µL of 10 µM template; 88.8 µL of water. Heated at 95 °C for 3 min. and then slowly cooled to room temperature. Desalted according to the manufacturer's protocol using a ZebaTM Spin Desalting Columns 7K MWCO.

DNA primer extension reactions. Composition of the reaction mixture: enzyme concentration from 10 to 50 nM, DNA substrate concentration is from 5 to 10 nM, corresponding nucleoside, nucleotide or inhibitor concentration from 0.1 µM to 10 mM. If glutamate buffer is used, 1 mM of $MgCl_2$ is additionally added, if a tris-acetate buffer is used, is additionally added $MnSO_4$ from 0.1 mM for Klenow exo- to 1 mM for M.MuLV RT. Reactions are carried out for 5 ÷ 60 min at 37 °C. If samples are used for native PAGE, they are immediately loaded onto native gel; if used for the denaturing PAGE, samples are supplied with STOP solution and heated at 95 °C for 5 min.

Electrophoretic fractionation of radioactive DNA fragments in acrylamide gel, visualization and analysis. Electrophoresis of DNA fragments is carried out under denaturing conditions at 8 M urea polyacrylamide gel. To 15 mL of a 15% gel solution, 150 μL of 10% APS solution and 15 μL of TEMED solution is added, mixed, gel solution poured between the two glasses of and the "comb" inserted. When gel is formed, glasses with gel are placed in an electrophoresis apparatus, the unit is filled with 1x TBE electrophoresis buffer solution and combs are removed. Mixture of bromophenol blue and xylene cyanol into gel wells added and electrophoresis is performed at 600 V for 20 min. Subsequently, DNA samples are applied and electrophoresis is performed for 30 minutes at 200 V and then to 600 V until the dye line reaches the bottom of the gel.

After electrophoresis, the gel is soaked in 10% acetic acid for 15 min. Then gel is washed for 15 minutes under running water. The gel is then adhered to Whatman® 3MM paper and the other side of the gel is covered with a polyethylene film. This gel is dried in a vacuum gel dryer. After the gel has dried, it is autoradiographed on a Fujifilm screen and the results are visualized by the Fujifilm FLA-5100 phosphoimager using red laser. The signal of each DNA product is evaluated densitometrically by the *Optiquant* program. *Microsoft Office 2010 Excel* program is employed to estimate the relative incorparation of nucleotides for each DNA product.

Analysis of suspended electrophoretic mobility shift. DNA fragment electrophoresis is performed under native conditions at 8% polyacrylamide gel. To 15 mL of a 15% gel solution, 150 μL of 10% APS solution and 15 μL of TEMED solution is added, mixed, gel solution poured between the two glasses of and the "comb" inserted. When gel is formed, the glasses with gel in between are placed in an electrophoresis apparatus, the block is filled with 45 mM TB

electrophoresis buffer solution and combs are removed. Electrophoresis is performed at 150 V for 20 min. Subsequently, DNA samples are applied at 20 V and electrophoresis is performed at 100 V for 30 min and then at 150 V for 2 hours.

After electrophoresis, the gel is soaked in 10% acetic acid for 15 min. Then gel is washed for 15 minutes under running water. The gel is then adhered to Whatman® 3MM paper and the other side of the gel is covered with a polyethylene film. This gel is dried in a vacuum gel dryer. After the gel has dried, it is autoradiographed on a Fujifilm screen and the results are visualized by the Fujifilm FLA-5100 phosphoimager using red laser.

2 RESULTS AND DISCUSSION

Nucleotide modification studies are expanding into the field of synthetic biology, genetic code extension studies (Eremeeva and Herdewijn, 2018), studies of epitranscriptome (Palumbo and Beal, 2018) and the production of modified oligonucleotides (Morihiro et al., 2017). First modified nucleotides were created half century ago and used as anticancer or antiviral preparates to suppress various polymerases (Jordheim et al., 2013). By studying the use of modified nucleotides for DNA biosynthesis, it is possible to better understand the fundamental features of DNA structure and polymerase specificity (Kropp et al., 2018).

Retroviruses have a unique viral reproduction strategy using reverse transcriptase for DNA biosynthesis from viral RNA. These reverse transcriptases exhibit other biochemical properties than the host replication polymerases. The ability to utilize properties of polymerases would allow the development of inhibitors directed against viral polymerases without affecting of host polymerases. At the same time, the ability of polymerases to use alternatively structured nucleotides would allow synthesizing DNA with

additional functionalities (Schmidt, 2010; Pinheiro and Holliger, 2014).

Human immunodeficiency virus type 1 reverse transcriptase is among the most important targets for antiviral treatment. Reverse transcription inhibiting compounds are divided into several groups: nucleoside RT inhibitors, non-nucleoside RT inhibitors, and the most recent class of RT inhibitors - competitive inhibitors for nucleotides (Menéndez-Arias et al., 2017). Nucleoside and non-nucleoside RT inhibitors form the basis of highly active antiretroviral therapy (Lu et al., 2018). Regularly, new drugs should be included in a set of affordable medicines so that patients do not have unwanted side effects (Lewis et al., 2003; Kohler and Lewis, 2007) or prevent viral resistance to the drug (Meteer et al., 2014). The search for new compounds is ongoing, using alternative structural compounds, such as pyridonic compounds (Medina-Franco et al., 2007; Debnath et al., 2013) or nucleotides with modified bases (Cihlar and Ray, 2010; Vivet-Boudou et al., 2011; Loeb et al., 1999; Harris et al., 2005). Another approach is to combine different inhibitors into a single molecule (Velazquez et al., 1995; Renoud-Grappin et al., 1998; Pontikis et al., 2000) and expect better efficacy in inhibiting reverse transcriptase.

M. MULV RT represents a group of monomeric retroviral reverse transcriptases (Hizi and Herschhorn, 2008) and, although structurally similar to HIV-1 RT (Coté and Roth, 2008), has other biochemical properties. Klenow exo- and Taq DNA polymerases belong to the A family (Braithwaite and Ito, 1993), which would allow to evaluate the specificity of potential viral inhibitors.

This dissertation discusses the use of cyclic and non-cyclic pyridone nucleosides, which are structurally similar to pyridone non-nucleoside RT inhibitors (Medina-Franco et al., 2007; Debnath et al., 2013), as inhibitors of DNA polymerases. The use of an alternate base pair of pyridone nucleotides in DNA biosynthesis has also been

investigated. In this study, we also focused on the phosphonacetic acid nucleotide conjugates designed to inhibit DNA polymerases.

2.1 Investigation of inhibition of DNA biosynthesis by pyridone nucleosides

Acyclic and cyclic nucleoside analogues (Figure 2.1) were evaluated in the DNA biosynthesis assays. Two DNA polymerases were employed: Klenow exo- and M.MuLV RT. In these assays, DNA substrate was DNA system T7/A, which allows primer extension using dTTP up to four consecutive events. The presence of compounds up to 10,000-fold excess over dTTP in the reaction mix did not inhibit the incorporation of dTTP by all DNA polymerases tested, with exception of compound **7** (not shown). This nucleoside is capable to inhibit DNA biosynthesis of M.MuLV RT, but only using tris-acetate buffer with 1 mM $MnSO_4$. However, phosphonophormic acid inhibits M.MuLV RT about 100 times more efficiently than compound **7** (not shown).

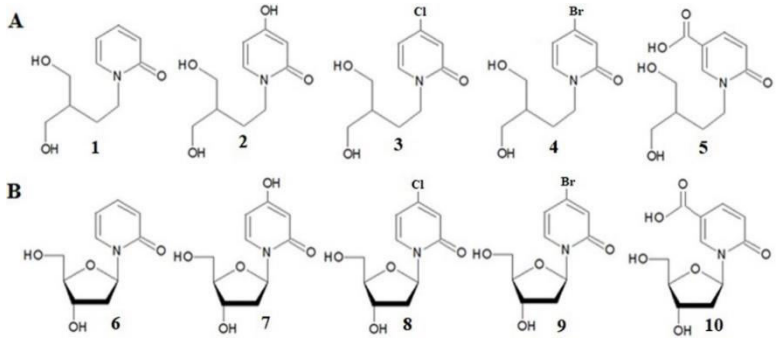

Figure 2.1. Pyridone nucleosides with non-cyclic carbohydrate chain structure (A), and pyridone nucleosides structure (B).

2.2 Use of pyridone nucleotides for DNA biosynthesis

The efficiency of acquisition of five 2-pyridone-based nucleotides (Fig. 2.2) by different DNA polymerases was evaluated. These compounds were used instead of natural deoxynucleoside

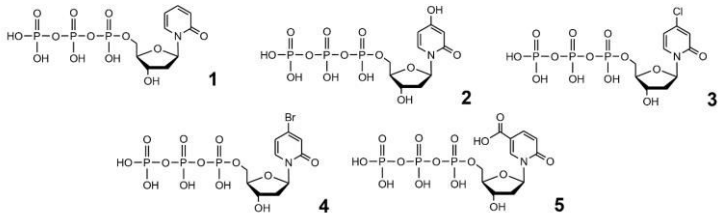

Figure 2.2. Pyridone nucleotide structures. Compounds:
1 – 3-hydroxy-5-(2-oxo-1-pyridyl) tetrahydrofuran-2-yl) methyl triphosphate,
2 – 3-hydroxy-5-(4-hydroxy-2-oxo-1-pyridyl) tetrahydrofuran-2-yl) methyl triphosphate,
3 – 3-hydroxy-5-(4-chloro-2-oxo-1-pyridyl) tetrahydrofuran-2-yl) methyl triphosphate,
4 – 3-hydroxy-5-(4-bromo-2-oxo-1-pyridyl) tetrahydrofuran-2-yl) methyl triphosphate,
5 – 3-hydroxy-5-(5-carboxy-2-oxo-1-pyridyl) tetrahydrofuran-2-yl) methyl triphosphate.

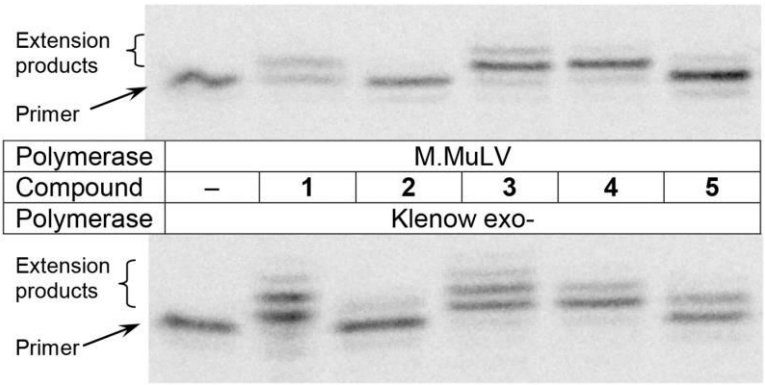

Figure 2.3. Autoradiograms of primer extension reactions. Reactions performed in the presence of 100 μM compounds 1 to 5 (middle row of table) by polymerases disclosed in the rows next to the picture.

triphosphates in a DNA biosynthesis assay. Since nucleobase structures in all instances were clearly different from the natural pyrimidine based moieties, DNA template strand was selected in a

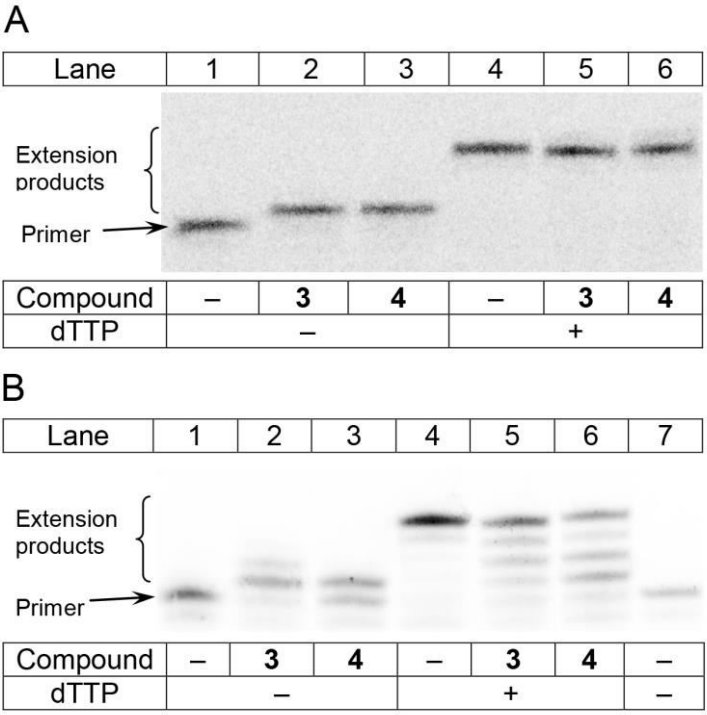

Figure 2.4. Autoradiograms of primer extension reactions by the use of (A) Klenow exo- in the presence of compounds 3 or 4; (B) M.MuLV in the presence of compounds 3 or 4. Under completion of the first reaction, dTTP was added and primer extension was prolonged, as indicated by bottom row of the table.

way to provide platform for incorporation of residue, complementary to any of natural nucleobases; therefore, four separate assay systems were used. Compounds **1**, **3**, **4** and **5** were found to be accepted for incorporation into DNA structure by Klenow exo- DNA polymerase and M.MuLV RT tested (Fig 2.3). Compound **2** was accepted only by Klenow exo-. While compounds **3** and **4** were readily accepted by Klenow exo- and to the lesser extent by reverse transcriptase M.MuLV, significantly lower yet observable incorporation was observed for other compounds. For all compounds incorporated, the following nucleobases on template strand were invariantly preferred: A≳ G> T> C.

Limited primer extension events observed by virtue of compounds **3** and **4** prompted us to evaluate such extended DNA for a chain termination property. We performed DNA primer extension by Klenow exo- and M.MuLV RT and compounds **3** and **4** under conditions designed to obtain uniform primer extension by single nucleotide. Upon completion of the reaction, aliquots of these samples were supplemented with dTTP and reaction set to proceed (Fig. 2.4). The primer extension proceeded completely up to the allowed limit of 4 consecutive residues. The observed complete primer extension, independent of the initial extension step, proves the ability of involved DNA polymerases to employ 4-chloro- and 4-bromo-2-pyridone nucleobase-terminated DNA for further extension.

2.3 Investigation of inhibition of DNA biosynthesis by nucleotide conjugates

In this study, we focused on phosphonoacetic acid-nucleotide conjugates designed to achieve a high affinity for the target polymerases (Figure 2.5). All DNA polymerases differed in their ability to accept the selected conjugates as a substrate (Figure 2.6). In the case of all polymerases tested, only the incorporation of PA5TT was comparable to that of dTTP. In contrast, PA5T was accepted exclusively by HIV-1 reverse transcriptase, and only a negligible amount of this compound was incorporated by M.MuLV and Klenow

exo- after a prolonged incubation. Whereas Taq polymerase showed no ability to incorporate PA5T into DNA. Performance of PAT was found to be ambiguous. HIV-1 reverse transcriptase as well as the Klenow exo- polymerase accepted the nucleotide, although at a slightly lower rate. In the case of both M.MuLV reverse transcriptase and Taq DNA polymerase, however, only a negligible amount of PAT was incorporated.

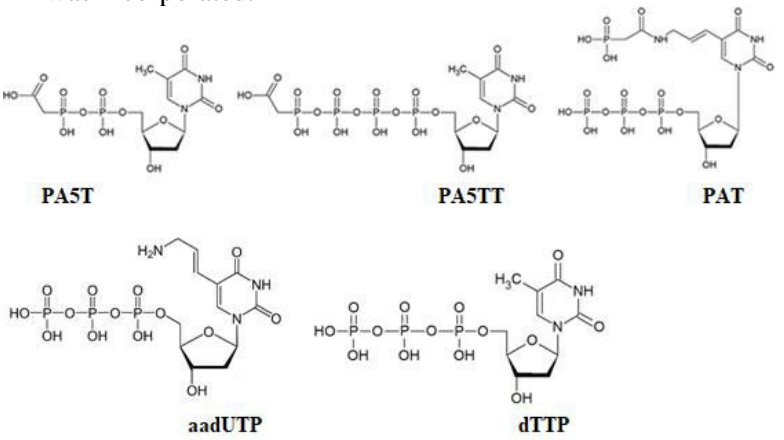

Figure 2.5. Structures of modified nucleotides with phosphonoacetic acid (PAA) and dTTP.

Due to the poor DNA incorporation, PA5T and PAT were suspected to inhibit DNA polymerases, and these compounds were selected for competitive primer extension with dTTP. PA5T did not exhibit inhibition with either Klenow exo- DNA polymerase, M.MuLV RT or HIV-1 RT (Ravoitytė, 2014), even when the concentration was 1000 times higher than dTTP, while phosphonoacetic acid (PAA) at this concentration is able to inhibit DNA biosynthesis. Klenow exo- polymerase, M. MuLV RT and HIV-1 RT (Ravoitytė, 2014) were able to incorporate PAT. DNA products differed between the DNA polymerases investigated, especially towards M. MuLV RT. However, selected PAT concentrations were too high to monitor the inhibition of DNA biosynthesis.

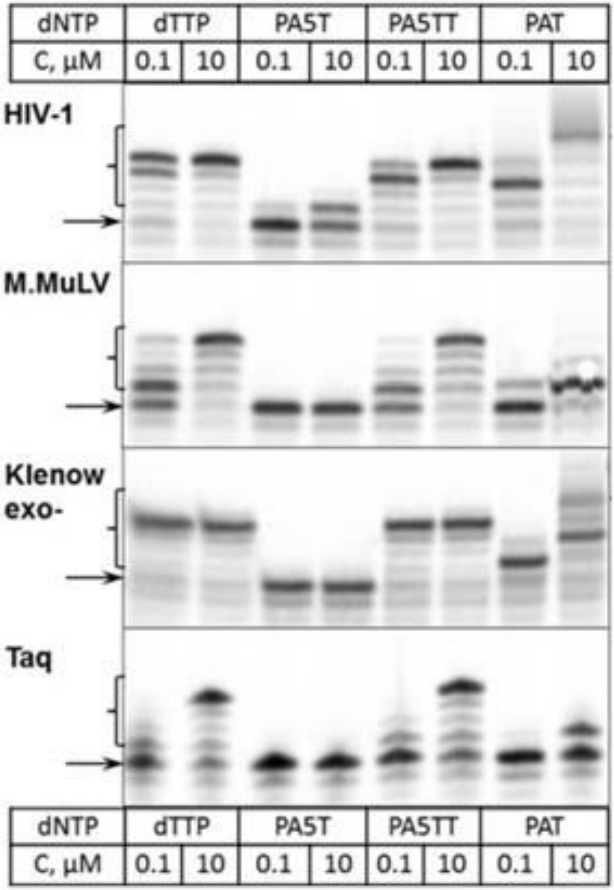

Figure 2.6. Extension of the DNA substrate in the presence of various nucleotides. The concentrations are given in the table, above and below the corresponding lanes.

To decipher the reason(s) behind the reduced processivity of polymerases upon incorporation of PAT, the primer extension assays were performed with either PAT alone, a mix of 5-aminoallyl-dUTP (precursor of PAT) and PAA, or 5-aminoallyldUTP alone. The performance of HIV-1 and Klenow exo- polymerases was evaluated by densitometric analysis of the formed products. Both the amount

and a relative distribution of the reaction products within a given sample were calculated. In the case of PAT and 5-aminoallyl-dUTP, the pronounced difference in the pattern of product formation during primer extension reactions was observed. As seen in Figure 2.7, upon the incorporation of 5-aminoallyl-dUTP, the formation of higher molecular mass products was observed, starting from the lowest concentrations of the nucleotide and irrespectively of the presence of PAA in the reaction mixture (Fig. 2.7). Such pattern is typical for the polymerases that incorporate nucleotides in a processive manner. Compared to 5-aminoallyl-dUTP, a lower yield, particularly at the lowest concentrations of the compound, was observed during the incorporation of PAT. Compared with 5-aminoallyl-dUTP, the primer extension reaction in the presence of PAT was highlighted by the prevalence of less-extended products, independent on the PAT concentrations employed. The inhibition of the primer extension in the

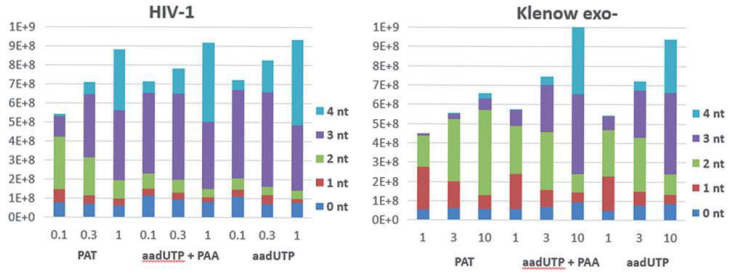

Figure 2.7 Primer extension in the presence of PAT and 5-aminoallyl-2‘-deoxyUTP (aadUTP) with or without the equimolar amounts of PAA. Polymerases used in the primer extension reactions are indicated above the graph. PAT, aadUTP and PAA concentrations in mM are given below the graph. For calculation of reaction outcome, a number of incorporated nucleotides is taken into account and is represented by the total height of the bar. The internal fractions of the bar represent a distribution of the respective products and are indicated in graph legend.

presence of free PAA was not observed; moreover, a minor stimulation of the reaction took place in the case of Klenow exo-.

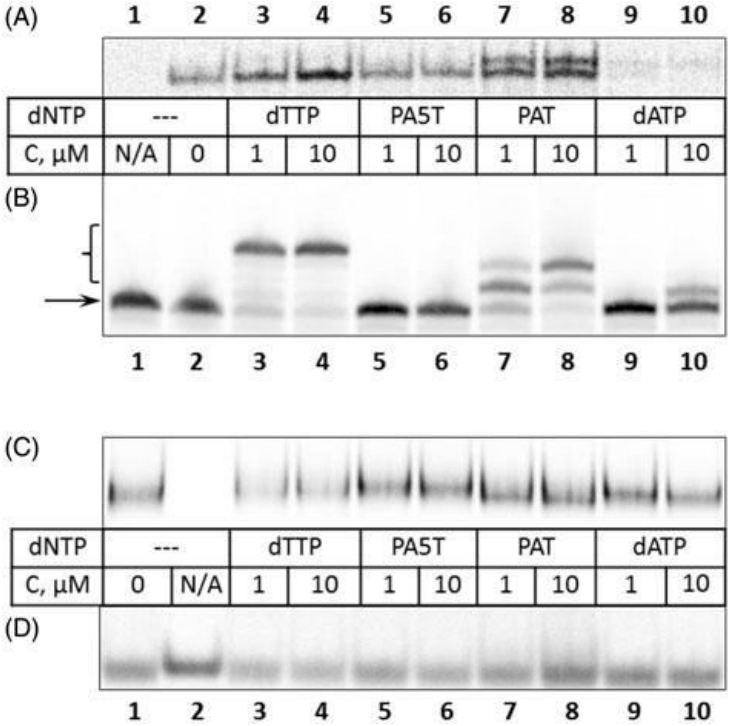

Figure 2.8. Equilibrium (A, C and D, gel shift mode) and product composition analysis (B) in the presence of various nucleotides. The nucleotides and their concentrations are listed in the table between panels, N/A – no protein added. A. Binding of Klenow exo– to the DNA substrate under catalytic conditions as analysed by native electrophoresis. Only bound fraction of DNA is shown. (B) Analysis of the products of primer extension by Klenow exo–. The samples from panel A were resolved on a denaturing gel. Arrow indicates the position of the unreacted primer strand, bracket – the products of primer extension. C. Binding of HIV-1 reverse transcriptase to the DNA. The bound fraction of DNA is shown at 2-fold extended

vertical axis. (D) Unbound fraction of DNA substrate in HIV-1 polymerase binding assays, presented in C.

The binding of polymerase to a DNA substrate in a catalytically competent complex mode was evaluated in order to investigate the overall conformation of the ternary complex (Fig. 2.8). In the absence of nucleotide triphosphates, the binding of Klenow exo- to the DNA substrate was found to be somewhat weaker under the conditions employed (Fig 2.8 A, lane 2). In contrast, the presence of nucleotide triphosphates either enhanced binding or stimulated the formation of complexes of different mobility (Fig 2.8 A, lanes 3, 4, 7 and 8). Notably, the complexes of reduced mobility were observed upon incubation with PAT and dATP (Fig 2.8 A, lanes 7, 8, 9 and 10). The latter compound was incorporated into the primer rather inefficiently, as evidenced by denaturing gel electrophoresis (Fig. 2.8 B, lanes 9 and 10). In comparison, an incorporation of PAT was more efficient (Fig. 2.8 B, lanes 7 and 8). Our results indicate that, in the presence of DNA and PAT, the catalytically competent ternary complex includes Klenow exo- polymerase with altered conformation. The analysis of ternary complexes formed by HIV-1 reverse transcriptase also indicated a conformational irregularity (Fig. 2.8 C, lanes 3–10). In addition, the smears migrating just above free DNA substrate indicate that the ternary complexes assembled in the presence of PAT may be meta-stable, disassembling shortly after the loading of samples on a gel (Fig. 2.8 D, lanes 7 and 8).

2.4 Discussion

The ability of pyridone nucleosides to inhibit M.MuLV RT and Klenow exo- catalysed DNA biosynthesis was investigated. Unfortunately, in none of the investigated cases, significant inhibition of dTTP incorporation into DNA was observed with except for compound **7** (4- hydroxy 2-pyridone deoxyriboside), able

to inhibit M.MuLV RT when the reaction mixture contained a buffer with manganese ions. No inhibition was observed in the presence of a buffer with magnesium ions. Magnesium ions are mostly used *in vitro* and it is believed that *in vivo* reverse transcriptases also use them for DNA biosynthesis. *In vitro* M.MuLV and other gamaretroviruses are capable of using manganese ions for DNA biosynthesis (Herschhorn and Hizi, 2010). Manganese ions are associated with reduced accuracy of DNA polymerases due to the ability of manganese ions to bind triphosphates tighter and stabilize tautomeric bases (Vashishtha et al., 2016). The ability of compound **7** to inhibit M.MuLV RT in buffer with manganese ions indicates a possible change in the enzyme structure that allows the inhibitor to bind to the enzyme. However, further research is required to find out the precise mechanism of this pyridone nucleoside inhibition.

The pyridone nucleotides were investigated for reactions carried by DNA polymerases. The selected DNA polymerases - Klenow exo- and M.MuLV RT - are enzymes with different properties and applications. Klenow was able to use halogenated pyridone nucleotides against template nucleotides A and G, while M.MuLV RT capability was much weaker. This shows the different applicability of enzymes using artificial base pairs. Although the incorparation by the Klenow exo- polymerase can increase the error rate and induce mutagenicity, *in vivo* DNA polymerase I or bacterial repair systems could eliminate the incorrectly incorporated nucleotides.

Investigating the properties of pyridone nucleotides helps to reveal the effect of nucleobase substitutions on the efficiency of DNA biosynthesis. Similar compounds have been studied to determine the influence of fluoro substituents on aromatic ring formation on base pairs. These pairs were less stable than fluoropyridones and heteropairs, with the exception of G and A. Base pair stability is greatly influenced by hydrophobic interaction and possible hydrogen bonds between functional groups (Hwang et al.,

2007). Incorporation of halogenated pyridone nucleotides is more effective than unmodified or 4 hydroxy pyridone nucleotides. This indicates that chlorine or bromine groups interact more effectively with the active centre of the DNA polymerase. Typically, halogen atoms in drug-type molecules are implicated in indirect hydrophobic interactions or fill in voids that they tend to occupy and do not participate in stabilizing interactions. Electron-rich groups like oxygen, nitrogen and sulphur atoms, as well as aromatic π-electron systems in proteins, halogen atoms, when structurally possible, can form halogen bonds with surrounding amino acids (Parisini et al., 2011). However, studies with base pairs in which functional groups have been replaced to form halogen bond instead of hydrogen bonds indicate that the halogen bond is weaker than the hydrogen bond or is structurally more difficult to form (Parker et al., 2012; Tawarada et al., 2008). The introduction of halogen groups can increase DNA stability (Ramzaeva et al., 2014) or the effectiveness of inhibitors (Herman et al., 2012).

Phosphonoacetic acid observes antiviral activity against *Herpesviridae* members (Overby et al., 1977), when its analogue, phosphonoformic acid, has a broad antiviral activity against various *Herpesviridae* and *Retroviridae* members (Öberg, 1982). To achieve better affinity with selected DNA polymerases, PAA is conjugated to different nucleotide positions. During DNA extension, the low molecular weight inhibitor conjugated to the nucleotide should be in the active centre of polymerase. This inhibitor, close to the catalytic amino acids, and the high local concentration will compensate for the low affinity of the phosphonoacetic acid. However, it has been found that the structure of the prepared 5' phosphate region of conjugates is important for DNA polymerase recognition. PA5TT, which has an intact 5' triphosphate structure, was far more efficiently incorporated than PA5T.

The nucleobase C5 position of pyrimidine is suitable for incorporation of various functional groups into DNA. PAA is

conjugated to the 5-aminoalyl group following many other nucleobase modifications, in terms of size and charge patterns, which are efficiently utilized by DNA polymerases (Hollenstein, 2012; Welter et al., 2016; Hottin and Marx, 2016). The results showed that with PAT or during incorporation of PAT into DNA, the tertiary complex of Klenow exo- DNA polymerase and HIV-1 RT has an alternative conformation. HIV RT during DNA polymerization may have different conformations - open (Ding et al., 1998) and closed (Hsiou et al., 1996), which determine the activity of the enzyme. HIV-1 RT with non-nucleoside RT inhibitors acquires an alternative conformation (Esnouf et al., 1995), which is not catalytic. PFA and probably PAA attaches to the HIV-1 RT and blocks at the state of pretranslocation (Marchand et al., 2006). Incorparation of PAT into DNA can potentially block HIV-1 RT at the state of pretranslocation. Klenow exo- alternate tertiary complex with PAT resembles tertiary complexes, when non-complementary nucleotides are incorporated. This suggests that incorporation of PAT into DNA reduces polymerization and increases exonuclease activity. Klenow fragment with 3' - 5' exonuclease activity or DNA polymerase I should have even lower ability to use PAT. Further research is required to find out the exact changes induced by PAA. Therefore, conjugation of these low molecular weight compounds with nucleotides may be the basis for further development of new antiviral drugs.

CONCLUSIONS

1. Pyridone nucleosides do not inhibit DNA biosynthesis, with exeption for the compound **7** (4- hydroxy 2-pyridone deoxyriboside). Compound **7** is able to inhibit M.MuLV RT in the presence of manganese ions.
2. Klenow exo- ir M.MuLV RT can insert one or more pyridone nucleotides into DNA. Compounds **3** and **4** (4-chloro and 4-bromo pyridone nucleotide) are the most effectively used, the preferred nucleobases in the template are: A≳ G> T> C. Incorparation of pyridone nucleotide has little effect on further primer extension by natural nucleotides.
3. M.MuLV RT, HIV-1 RT, Klenow exo- and Taq DNA polymerases showed a similar tendency for nucleotide uptake: dTTP≈ PA5TT> PAT> PA5T.
4. PA5T does not inhibit DNA extension using dTTP of all tested DNA polymerases, and PAT can be incorparated during DNA biosynthesis along with dTTP.
5. Klenow exo- polymerase and HIV-1 RT form a ternary complexes of alternative conformation with PAT.

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1. **Mikalkėnas, A.**, Ravoitytė, B., Tauraitė, D., Servienė, E., Meškys, R., and Serva, S. (2018). Conjugation of phosphonoacetic acid to nucleobase promotes a mechanism-based inhibition. Journal of Enzyme Inhibition and Medicinal Chemistry, 33(1), 384–389. doi:10.1080/14756366.2017.1417275
2. **Mikalkėnas, A.**, Ravoitytė, B., Tauraitė, D., and Serva, S. (2017). Pyridone-based nucleotide analogues accepted for DNA biosynthesis. Biologija, 63(1). doi:10.6001/biologija.v63i1.3473
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The material presented in the dissertation was presented at international conferences

1. **A. Mikalkėnas**, B. Ravoitytė, D. Tauraitė, J. Jakubovska, R. Meškys, S. Serva. Pyridone-based Nucleotide Analogues as Substrates of DNA Polymerases. XIV International Conference of the Lithuanian Biochemical Society. 28-30 June, 2016. Druskininkai, Lithuania. Poster presentation.
2. **A. Mikalkėnas**, B. Ravoitytė, D. Tauraitė, E. Servienė, R. Meškys, S. Serva. DNA polymerase inhibition with derivatives of pyrophosphoric acid analogues *in vitro*. XV International Conference of the Lithuanian Biochemical Society. 26-29 June, 2018. Dubingiai, Lithuania. Poster presentation.

3. **A. Mikalkėnas**, B. Ravoitytė, D. Tauraitė, E. Servienė, R. Meškys, S. Serva. Conjugation of small molecule inhibitor to nucleobase promotes a mechanism-based inhibition. 43rd FEBS Congress. 7-12 July, 2018. Prague, Czech Republic. Poster presentation.

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