Analysis of Tautomeric Equilibrium of the Analogue d(dinitro-tC(O))TP During Incorporation by the Klenow Fragment of DNA Polymerase I

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University of Colorado Boulder

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Analysis of Tautomeric Equilibrium of the Analogue d(dinitro-tC\textsuperscript{(O)})TP During Incorporation by the Klenow Fragment of DNA Polymerase I

Arielle Baker

Monday, April 7\textsuperscript{th}, 2014: 4:00pm

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This thesis is submitted in partial fulfillment of the requirements for graduation with honors in the Department of Molecular, Cellular and Developmental Biology at the University of Colorado, Boulder.
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Baker, Arielle Leigh (B.A., Molecular, Cellular and Developmental Biology)
Analysis of Tautomeric Equilibrium of the Analogue d(dinitro-tC(O))TP During Incorporation by the Klenow Fragment of DNA Polymerase I
Honors Thesis directed by Professor Robert Kuchta

The studies outlined in this thesis examine the kinetic parameters of incorporation of the novel nucleotide analogue d(dinitro-tC(O))TP by the Klenow fragment of DNA polymerase I, accomplished through a series of polymerization assays. d(dinitro-tC(O))TP contains an additional pair of nitro groups at the top of the cyclic structure, expanding on the structures of previously studied synthetic nucleotides tC and tC(O) (Stengel et al. 2009). Nitro groups have a large electron withdrawing effect due to the highly electron-withdrawing oxygens, resulting in the compound being recognized more as T than as C when compared to the parent tC and tC(O). KF incorporates d(dinitro-tC(O))TP as efficiently as dTTP opposite a templating adenosine base, and about twice as efficiently as dCTP opposite guanosine.

My studies on d(dinitro-tC(O))TP support the existing hypothesis that selection for the correct nucleotide based on size is only true for analogues closely related to the canonical bases. KF appears to use both the size and hydrogen bonding patterns of incoming nucleotides to distinguish their correctness. The ability of d(dinitro-tC(O))TP to hydrogen bond in a manner similar to its parent compound, cytosine, allows the compound to be incorporated by KF while the bulky cyclic addition is not detrimental enough to sterically inhibit incorporation. The efficient incorporation opposite both A and G is consistent with it existing in roughly equal amounts of the two tautomers, amino (C-like) and imino (T-like). d(dinitro-tC(O))TP is an exciting compound because it retains traditional hydrogen bonding properties, thereby maintaining the identified prerequisite for selection of the correct incoming nucleotide, but expands upon the traditional cytosine structure by expanding into the major groove. Though not therapeutically viable, d(dinitro-tC(O))TP offers interesting insight into the effect of electron-withdrawing groups on tautomeric equilibrium when added to a nucleotide analogue.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CNDAC</td>
<td>1-(2-C-cyanodeoxybeta-D-arabino-pentofuranosyl) cytosine</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytidine triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanosine triphosphate</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNMP</td>
<td>Deoxynucleotide monophosphate</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxythymidine triphosphate</td>
</tr>
<tr>
<td>GW506U78</td>
<td>2-amino-9-beta-D-arabinosyl-6-methoxy-9H-guanine</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>KF</td>
<td>Klenow fragment</td>
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<tr>
<td>NDP</td>
<td>Nucleoside diphosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
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I. Introduction

DNA REPLICATION

Cellular replication, proliferation and survival are contingent upon the process of DNA replication. In many domains of life, there are common features to the replication process, not only in its mechanisms but also in its machinery. This machinery includes DNA helicases, which topologically prepare the DNA helix by unraveling it; single-stranded DNA binding (SSB) proteins, which help to prevent reannealing of the DNA strands after unwinding by helicases as well as prevent destruction of the ssDNA by nucleases; primases, which synthesize a primer on the DNA template to which polymerases can adhere and begin elongation; topoisomerases, which relieve supercoiling stress in the DNA strand generated by helicase unwinding; and DNA polymerases, which synthesize daughter DNAs complementary to each single-stranded parent DNA in a semi-conservative fashion.

Replication is an intricate process. It requires the perfect orchestration of a number of enzymes, and involves precisely copying genomes containing millions to billions of nucleotides. Despite these factors, replication of the genome is incredibly accurate; rarely do DNA polymerases incorporate the incorrect nucleotide. For A family polymerases, the frequency of error is approximately $10^{-3}$ to $10^{-6}$ errors per nucleotide inserted (Kunkel and Bebenek 1988). Many DNA polymerases also have exonuclease active sites that provide proofreading functions, which will repair many errors by excising incorrectly incorporated nucleotides, thus decreasing the misincorporation rate even further. Finally, repair enzymes that follow the replication machinery carry out a final examination of the replicated DNA, decreasing the frequency of errors to approximately $10^{-9}$ errors per nucleotide inserted (Roberts and Kunkel 1996). It is important to note that while erroneous replication can have deleterious consequences, evolution is founded in erroneous replication.
DNA POLYMERASE

There are several families of polymerases found in a variety of organisms, including A, B, C, X, Y and RT. DNA polymerases are classified according to a variety of structural homologies, both in sequence and in conformation. The studies I conducted utilized the Klenow fragment of DNA Polymerase I (Pol I) from Escherichia coli, an A family polymerase. Pol I was the first identified polymerase (Kornberg 1960). In general, A family polymerases replicate DNA with high fidelity, working with other accessory proteins and subunits to achieve a finished product. Pol I also functions in nucleotide excision repair (Rothwell and Waksman 2005).

DNA polymerases have similar structures across the different families. One distinctive feature of polymerases is their hand-like shape (Figure 1.1).

The hand contains subdomains that resemble a palm, fingers and a thumb (Wang et al. 1997). The palm, similar to a human hand, is situated between the fingers and the thumb subdomains; this contains the active site where dNTPs enter the complex to be catalytically incorporated (Figure 1.1). The palm domains of different polymerase families are largely superimposable (Li and Waksman 2001), highlighting the evolutionary adaptation of the enzyme. The fingers work to recognize incoming nucleotides, and help orient them to bind, while the thumb helps to bind the DNA substrate (Figure 1.1) (Klenow and Henningsen 1970; Wang et al. 1997).

The Klenow fragment of DNA Polymerase I is an important catalytic protein. First discovered in 1970, it was the first solved polymerase crystal structure (Ollis et al.
KF is formed from Pol I by removing its 5’—3’ exonuclease domain. The cleaved enzyme retains 5’—3’ polymerase activity and its 3’—5’ exonuclease domain (Klenow and Overgaard-Hansen 1970; Zhao et al. 2013; Zhao and Guan 2010). The 3’—5’ exonuclease domain can be knocked out, which is the case for the KF utilized in this study.

Because of the importance of ensuring accurate genomic replication, many studies focus on the importance of polymerase fidelity, examining the various mechanisms by which polymerases distinguish between correct and incorrect nucleotides (Prindle et al. 2013; Donigan et al. 2014). Homologies in polymerase structure and function raise the question of why certain types of polymerases are significantly more efficient or accurate than other polymerases (Yao and Müller 2011; Nemec et al. 2014).

NUCLEOTIDE DISCRIMINATION BY DNA POLYMERASES

Nucleotides are compounds naturally created within cells that are utilized in a variety of ways, ranging from traditional RNA and DNA synthesis to metabolism, signaling within the cell, and regulation of enzyme activity (Jordheim et al. 2013). There are four canonical bases found in DNA: guanine, cytosine, adenine and thymine. Specific nucleobases express pairing preferences based on the size and stereochemistry of their nucleotide partners; cytosine and guanine are pairs, and adenine and thymine are pairs (Figure 1.2). Francis Crick and James Watson largely pioneered this research, finding that alternate tautomeric forms of natural nucleotides could account for mutations in DNA (1953). They used a model to determine that cytosine and adenine had the ability to mutagenically pair with each other using amino and imino tautomeric forms, and that thymine and guanine could mutagenically
pair with each other using keto and enol tautomeric forms; because adenine and guanine are purines, and thymine and cytosine are pyrimidines, these tautomeric forms can exist without causing a large structural disturbance (Morgan 1993; Urban et al. 2010; Hirao et al. 2012).

A foundational property of the discovery of DNA structure was the identification of the hydrogen bonding patterns between nucleobases. These distinctive patterns are thought to be a basis upon which polymerases discriminate incoming nucleotides (Moore et al. 2004; Ramirez-Aguilar et al. 2005; Wolfle and Washington 2005; Choi et al. 2009). It appears that A family polymerases use a mélange of positive and negative selectivity in nucleotide discrimination. Pol I, which is a high-fidelity polymerase, does not appear to utilize hydrogen bond pairing to achieve efficient nucleotide incorporation (Chiaramonte et al. 2003; Trostler et al. 2009); these mechanisms will be discussed in detail later.

Nucleoside and nucleotide analogues are chemically altered agents that are made to simulate the actions of their physiological counterparts, but with key differences. Perhaps the most famous analogue alteration development came from researchers Gertrude Elion and George Hitchings, who developed, among other drugs, acycloguanosine for the treatment of herpes; their discoveries led to development of AZT, for the treatment of AIDS. Since their remarkable discovery, scientists continue to toy with these useful compounds, producing a variety of novel structures. These alterations can be made on different structural components of the nucleotide. These developments have been built on previous knowledge on how correct nucleotides are selected and incorporated by polymerases. Not only are analogues being tested clinically, but a large number are used clinically as chemotherapeutics for both cancer and viral infection.
The mechanism by which Pol I may in some cases choose its substrate appears to be through evaluation of the shape of the incoming nucleotide (Kool 2002). This mechanism has been explored using analogues of traditional nucleotides, with altered or removed hydrogen bonding capabilities. In addition to shape and hydrogen bond features, investigations have also included consideration of the hydrophobicity and polarity of nucleotides being incorporated. Studies by Urban et al. conclude that primer elongation rate by Pol I decreases significantly when the incoming dNTP is unable to hydrogen bond to its templating base in a manner consistent with canonical Watson-Crick base-pairing (2009; 2010). The acceptors of hydrogen bonds contained within the minor groove appear to be of particular importance to nucleotide incorporation (Seeman et al. 1976; Hendrickson et al. 2004).

Studies thus indicate that the ability of nucleobases to form canonical hydrogen-bonding interactions during incorporation is of paramount importance in determining the efficiency of A family polymerases. Chiaramonte et al., however, showed that Klenow fragment has the ability to incorporate nucleotides whose shapes differ significantly from those of canonical nucleotides (2003). The analogues studied, which are shown in Figure 1.3, have novel and distinct shapes, leading to alternative hydrogen bonding patterns.

These studies help to conclude that polymerases of the A family do not discriminate between nucleobases based solely on shape or hydrogen bond patterns, allowing them to efficiently incorporate a variety of compounds. Knowing what features
A family polymerases do not use to discriminate, termed negative selection, a study by Trostler et al. set out to determine what aspects of the nucleotide is used for discrimination, termed positive selection. Using Pol I of *Bacillus stearothermophilus*, the group studied a number of purine analogue triphosphates (Figure 1.4) in which the N-1, N-3 and N⁶ of the purine ring were systematically replaced with carbons.

This results in a decreased efficiency of correct incorporation opposite T, particularly with substitution of the nitrogen for a carbon in the heterocycle in the 1 or 3 position (2009). This indicates that Pol I does not merely evaluate the shape or the hydrogen bonding ability of a given nucleotide; the composition of the heterocycle plays a key role in ensuring correct incorporation, steering the polymerase away from mutagenic base pairing. Thusly, one can see that it is of paramount importance to consider not only the addition of groups to the canonical compounds, but also the composition of the cyclic portion of the structure itself.

![Purine analogues utilized to probe the roles of specific chemical features of the natural purine nucleobases (Trostler et al. 2009).](image)
THERAPEUTICS

The important role of nucleotides in so many cellular processes has prompted investigation into their clinical applications, and analogues are an excellent avenue for current and future therapeutics. Structural alterations allow researchers to manipulate the nuances of replication and nucleotide metabolism, thereby affecting a plethora of intracellular activities (Jordheim et al. 2013). Notably, analogues can inhibit DNA and RNA synthesis, thus halting cellular division and/or viral replication (De Clerq and Neyts 2004). Nucleotide and nucleoside analogues, then, represent powerful treatments for cancer tumor suppression and anti-viral therapy (Pockros 2013), as well as unconventional approaches to mechanisms involved in polymerase and phosphodiesterase inhibition, epigenetic modulation, immunosuppression, neuroprotection and cardioprotection (Moyle 2000; Squires 2001).

In order for nucleoside analogues to be functional, they must undergo phosphorylation within the cell by nucleoside kinases, (d)NMP kinases and NDP kinase; triphosphorylation is necessary for nucleotides to become active in the body. Some nucleotide analogues already have this phosphorylation completed, and are thus already chemically active, but are unable to enter the cell because of the inability for charged phosphate groups to penetrate the hydrophobic cell membrane (Pockros 2013; Squires 2001). Thus, phosphorylation must be completed once inside the cell, which is a current challenge to clinical application.

THERAPEUTIC MECHANISMS OF ACTION

Nucleotide and nucleoside analogues often behave as anti-metabolites in the process of nucleic acid metabolism. They can utilize the exact metabolic pathways that are used by natural nucleosides and nucleotides (Jordheim et al. 2013). Classical nucleotides are hydrophilic in nature, and are derived from two pathways: the de novo pathway and the salvage pathway. The de novo pathway converts various small molecules into nucleoside triphosphates; this pathway, then, is essential in replication of cells. The salvage pathway, on the other hand, recycles leftover nucleosides from DNA and RNA catabolism (Kufe et al. 1984). Exogenous nucleotides use kinases of the salvage pathway. These analogues are ingested by cells through compound-specific
membrane transport carriers or via passive diffusion across the plasma membrane (Minuesa et al. 2011; Cano-Soldado and Pastor-Anglada 2012). Ongoing research indicates that some antiviral analogues can be taken up by organic anion and cation transporters, in addition to peptide transporters (Roizman et al. 2001), but this data is presently inconclusive.

Once ingested, the analogues undergo a series of phosphorylation events, allowing di- and tri-phosphorylated analogues to collect in the target cells. Without these phosphorylation steps, the drugs remain inactive (Jordheim et al. 2013). The active analogues are then readily available for incorporation into DNA and RNA strands being synthesized, or for inhibition of enzymes other than polymerases, or oftentimes for both. Many analogues, due to the structural and chemical changes they possess, cause elongation to halt, as the polymerase enzyme cannot extend the strand past such a compound (Pockros 2013). Typically in replication of the viral genome, incorporation of analogues causes the cell to recognize the strand as severely mutated, causing replication termination of the viral DNA or RNA, called error catastrophe (De Clerq and Neyts 2004; Leyssen et al. 2008;). Researchers postulate that analogues results in a chain-termination event that prevents progression of the cell cycle S phase (Ewald et al. 2008), but further research in this area is needed in order to draw more decisive conclusions.

It is important to understand the structural and mechanistic basis for nucleotide and nucleoside analogues in order to develop new compounds. Such considerations have fostered the creation of agents that interact more efficiently with membrane transporters, or that are less liable to degradation (Jordheim et al. 2013). New compounds can be developed with enhanced properties that increase the efficiency and rate of success of nucleotide and nucleoside analogues as therapeutics (Pockros 2013). Research in the nucleotide and nucleoside analogue field has seen the development of many new nucleoside drugs, several of which are presently in early-phase clinical trials, and a significant number that have become regularly prescribed drugs (Jordheim et al. 2013). I will briefly review the history and present state of such therapeutics, to set the stage for the investigation of d(dinitro-tC(0))TP. Nucleoside and nucleotide analogues fall into two primary therapeutic categories: anticancer compounds and antiviral compounds.
II. Anticancer Nucleotide and Nucleoside Analogues

The first anticancer nucleoside analogue that targeted DNA polymerases was cytarabine (Figure 2.1) for acute myeloid leukemia; approval of this compound by the US Food and Drug Administration (FDA) in 1969 initiated a cascade of compound development for anticancer therapeutics (USA National Institutes of Health 2011).

In terms of anticancer nucleoside analogues, as of 2007, nine are currently approved and on the market: cytarabine, fludarabine, cladribine, gemcitabine, clofarabine, nelarabine, capecitabine, floxuridine, and deoxycoformycin (Nekhai et al. 2007). These drugs treat specific types of cancer, and are largely derivatives of deoxycytidine, deoxyadenosine or deoxyguanosine (Van Rompay et al. 2003; Galmarini et al. 2010; Senanayake et al. 2011). Two additional compounds, azacitidine (Kaminskas et al. 2005) and decitabine (Stewart et al. 2009; Jordheim et al. 2013) are known as demethylating agents and act through a different mechanism to prevent proliferation in cancer cells.

Presently, many consider existing anticancer agents to not be impactful enough to merit continued clinical treatment. Cytarabine, the most powerful clinically used compound, provides cures of acute myeloid leukemia in fewer than 30% of all adult patients (Kantarijian et al. 2012). A couple of other cytotoxic nucleoside analogues have been developed (Jordheim et al. 2013), but do not impact the patient long-term, simply prolonging survival, but not ultimately curing the cancer.

A few of the newly developed nucleoside analogues have the same cytotoxic mechanisms as those of older nucleoside analogues. One such compound is 1-(2-C-cyanodeoxybeta-D-arabino-pentofuranosyl) cytosine (Figure 4), also referred to as CNDAC (Jordheim et al. 2013), from which an orally administered drug has been created: sapacitabine (Figure 2.2) (Chaio et al 2013).
CNDAC is a cytarabine analogue that functions by cleaving the DNA strand through nucleophilic attack of the cyano groups. Human cancer xenografts responded well \textit{in vivo} to both CNDAC and sapacitabine. This has resulted in the initiation of sapacitabine’s clinical testing, as well as in combination with other anticancer agents, and appears to have a beneficial effect in patients being treated for chronic lymphocytic leukemia, acute myeloid leukemia, as well as some small tumors, largely by inhibiting the polymerases that copy genetic information. By disallowing these processes to occur, sapacitabine prevents the growth of tumors (Kantarjian et al. 2012).

Another realm of anticancer analogue research is focused on inhibiting RNA synthesis (Krett et al. 2004). Two such compounds utilized in this research are 8-chloro adenosine and 8-amino-adenosine. 8-Chloro-adenosine decreases RNA synthesis while 8-amino-adenosine decreases both RNA and DNA synthesis (Gandhi et al. 2001). This results in a decrease in the production of a variety of proteins. The cytotoxic effects of 8-chloro-adenosine are manifested in decreased expression of MET. MET is the receptor for hepatocyte growth factor in myeloma cells (Stellrecht et al. 2007). 8-Chloro-adenosine is also implicated in decreased cyclin-E expression in breast cancer cells. Due to these properties, this compound is presently being clinically investigated in a Phase I clinical trial (Stellrecht et al. 2010; Jordheim et al. 2013).

And yet, these compounds display inconsistency in terms of impact, largely due to patient variability. For example, cytarabine, as well as fludarabine, are functional in a few hematological malignancies, but not in solid tumors. This inconsistency may rise from a matter of patient variability or a mechanism of action. The compounds are specific for reasons not yet understood. The effects of gemcitabine (Figure 2.3) are presently the most consistently predictable in the field of anticancer nucleoside analogues; it displays activity in both hematological malignancies and several solid tumors (Song et al. 2013).
These studies have shaped the direction of current cancer therapeutics research—research has evolved from the notion of an overlying “cancer treatment”, due to tumor specificity; rather, current efforts aim to treat each tumor type as a separate disease.

4’-Thioaracytidine is a nucleoside in which the sugar has been modified, and demonstrates antitumor activity in several tumor subtypes. 4’-thioaracytidine exhibits a unique ability to retain a tri-phosphorylated state, unlike cytarabine and fludarabine. A tri-phosphorylated state is more difficult to degrade intracellularly, thus making it a more stable compound. 4’-Thioaracytidine is currently being evaluated in Phase I clinical trials (Someya et al. 2006).

Many anticancer compounds can also target viruses, the most prominent of which is cytarabine (Figure 2.1). Cytarabine can target herpesvirus infection (Renis 1973; Hryniuk et al. 1972). The compounds toxacitabine and 2-amino-9-beta-D-arabinosyl-6-methoxy-9H-guanine, both of which are anticancer compounds, display promising attributes in their pre-clinical trials (Jordheim et al. 2013).

Troxacitabine (Figure 2.4) is a synthetic nucleotide analogue (Galmarini et al. 2001) that, in pre-clinical trials, appeared to behave as an antitumor agent (Grove et al. 1995; Kadhim et al. 1997; Parker 2013).

It has a nontraditional stereochemical configuration, and is the first of its kind to be developed clinically. Being a nucleoside analogue, troxacitabine requires phosphorylation in vitro by cellular kinases, after which its incorporation into the DNA strand causes inhibition of the polymerase. This compound does not have inhibitory effects on ribonucleotide reductase nor mitochondrial DNA synthesis; in the case of gemcitabine, the inhibition of ribonucleotide reductase may enhance its efficacy.

![Figure 2.3: Chemical structure of gemcitabine (Song et al. 2013).](image)

![Figure 2.4: Chemical structure of troxacitabine (Galmarini et al. 2001).](image)
Troxacitabine is hence in phase II testing due to its breadth: it is cytotoxic against both leukemic and epithelial malignancies, and may have effects on acute leukemia (Giles et al. 1999; Jordheim et al. 2013).

The compound 9-beta-arabinofuranosylguanine, also referred to as ara-G (Figure 2.5), is a distinctive anticancer nucleoside analogue (Galmarini et al. 2001) that exhibits resistance to cleavage by purine nucleoside phosphorylase (PNP) (Mahmoudian et al. 1999).

Pre-clinical trials revealed that ara-G was toxic to T-lymphocytes, and therefore represented a powerful antitumor candidate, but development of this compound was halted due to problems with insolubility in water (Aguayo et al. 1999). This led researchers to the discovery of the compound 2-amino-9-beta-D-arabinosyl-6-methoxy-9H-guanine, also referred to as GW506U78, which is a derivative of ara-G, but with improved solubility, allowing the compound the access the cell (Aguayo et al. 1999). After accessing the cell, GW506U78 can then be converted to ara-G via plasma adenosine deaminase (Kurtzberg et al. 1999; Jordheim et al. 2013).

Troxacitabine and GW506U78 are excellent examples of the progression of anticancer nucleoside and nucleotides analogue research. Developmental research on troxacitabine has helped researchers understand the selectivity of its stereochemistry in regards to specific types of DNA. This enables researchers to develop selective drug targets. The logical development of GW506U78 from ara-G demonstrates the ability of researchers to examine analogues structurally, and make appropriate changes based on specific properties of that compound. This is the direction of current nucleoside and nucleotide analogue research— trial and error, slowly molding the ideal compound for particular cancer or viral infection types. This will yield new agents with enhanced solubility and bioavailability, as well as the ability to overcome resistance mechanisms (Jordheim et al. 2013).
Research on anticancer nucleoside and nucleotide analogues has brought about the development of many successful candidates for therapeutics. The breakthroughs with cytarabine and other analogues brought about a profound interest in nucleoside and nucleotide analogues for anticancer therapeutics. Studies on the chemical structure of novel compounds, however, have brought about candidates for specificity and retention. Thusly, there is significant effort being made to improve their utility. These novel analogues exhibit the potential to transform the position of nucleoside and nucleotide analogues in cancer therapy.
III. Antiviral Nucleotide and Nucleoside Analogues

Antiviral nucleoside analogues emerged in 1969, the first of which was edoxudine. Since the development of edoxudine, 24 additional antiviral nucleoside and nucleotide analogues have been created and approved by the FDA. Antiviral analogues can target several viruses, including HIV, HBV, CMV and HSV infections (De Clercq and Holy 2005). HIV, HBV and HCV infections are life threatening and chronic, and represent the largest class of antiviral research; these infections affect about 600 million people throughout the world (Hurwitz and Schinazi 2013). Another classification is acute infections: independent viruses that cycle through the population with minor impact for the majority of people, but dangerous to some individuals, such as pregnant and feeding women, or the elderly (De Clerq and Holy 2005). The final classification of viral infections is those that are common, such as a cold (caused by rhinoviruses), but do pose an economic impact in the form of sick employees.

The ability of nucleoside and nucleotide analogues to treat a variety of viral diseases is both a benefit and a potential roadblock. It is beneficial because it allows one simple mechanism to be applied across the board to many diseases without extensive time used and research to be conducted. It can be problematic, however, because each virus is specific, variable and irregular, making it difficult to predict the outcome of clinical application of a given antiviral agent (Jordheim et al. 2013). There are four main antiviral targets: HBV, HCV nucleosides, HIV nucleosides, and herpes.

Anti-HCV nucleoside analogues largely revolve around the compound 2’-deoxy-2’fluorocytidine (FdC) (Figure 3.1), and chemically altered derivatives (Stuyver et al. 2004). One such derivative is valopicitabine, which was the first clinically used anti-HCV nucleoside analogue. Valopicitabine can function by itself, but is often combined with pegylated interferon or ribavirin, to decrease chance of resistance. The success of this compound prompted the

![Figure 3.1: Chemical structure of FdC (Stuyver et al. 2004).]
development of more anti-HCV compounds, notably mericitabine. Mericitabine is another FdC derivative that is currently in Phase II clinical trials, and is presently on the forefront of anti-HCV nucleoside development (Le Pogam et al. 2010; Wedemeyer et al. 2013).

Anti-HIV nucleosides are dideoxyribose derivatives. One particularly potent derivative is apricitabine, which has elevated genetic resistance, making it an attractive agent. Apricitabine functions best against HIV-1 strains and HIV strains with mutations in reverse transcriptase. In 2005, tested over a 48-week treatment period, apricitabine was highly effective and did not show signs of resistance (Bethall et al. 2005). Despite anticipation of its arrival on the market in 2010, however, apricitabine was suddenly withdrawn, seemingly due to legal and commercial complications. One year later, it was reinstituted as an anti-HIV candidate, and is currently in Phase III clinical trials (Cahn and Wainberg 2010). Apricitabine represents the progression of anti-HIV analogues, as well as that which needs to be addressed in future compounds: apricitabine is a worthy candidate due to its resistance to mutation; this effect ought to be taken into consideration upon further development of more anti-HIV nucleosides. Studies since the development of apricitabine have led to the generation of the compound festinavir. Festinavir, which is a derivative of an early anti-HIV nucleoside analogue names stavudine but with an additional 4’ position ethynyl group on the ribose ring, has been found have reduced toxicity in patients, as well as increased potencies. Festinavir, then, expresses properties better than its forefathers, and is presently being evaluated in Phase II clinical trials. It has a prolonged antiviral effect, and is an attractive model for development of differing antiviral drug resistance profiles.

Anti-herpes nucleosides are acyclic sugars. The most famous anti-herpes nucleoside on the market is acyclovir, from which stemmed several other compounds: ganciclovir, penciclovir, and most recently, cyclopropavir. Cyclopropavir (Figure 3.2) is a derivative of the traditional nucleoside guanosine, but with a

Figure 3.2: Chemical structure of cyclopropavir (Chou et al. 2012).
methylenecyclopropane group as a side chain (Chou et al. 2012). Cyclopropavir is interesting because it combats multiple strains of herpesvirus, including human herpesviruses 6A, 6B and 8, and human cytomegalovirus (Price and Prichard 2011). It exhibits many unique properties not present in currently approved anti-herpes agents, including the ability to inhibit replication of human cytomegalovirus, and the ability to remain continuously active against ganciclovir-resistant strains (Chou and Bowlin 2011).

Antiviral nucleosides and nucleotides have seen an influx of development over the past ten years, making them attractive subjects for future studies. Future research, however, needs to focus on ensuring the analogues’ incorporation into the viral genome, and not the genomic and mitochondrial DNA of the host cells. In addition, it is important that a variety of compounds continue to be developed, to stave off resistance to particular compounds by viruses being treated solely by a few compounds (Jordheim et al. 2013). Effective treatment will most likely require the use of many compounds, for which research ought to be continued.

The success and failures of current therapeutics, as well as the number of studies elucidating DNA polymerase mechanisms, have helped develop a greater understanding of the way in which polymerases accurately and successfully incorporate a given nucleotide. This research has lead to the amelioration of current nucleotide analogues, and still leave quite a bit of room for development of further systems.
IV. tC, tC(O), and dinitro-tC(O)

tC AND tC(O)

A study conducted in 2009 investigated the compound 1,3-diaza-2-oxo-phenothiazine (tC) and its oxo-homolog, 1,3-diaza-2-oxo-phenoxazine (tC(O)) (Figure 4.1) (Stengel et al.). These compounds are unique cytosine analogues with fluorescent properties. Unlike traditional cytosine, tC contains substantial extra mass in the major groove, but remains similar to cytosine in its hydrogen bonding abilities (Stengel et al. 2009). Extensive studies characterizing these compounds in relation to all four natural nucleobases using the KF of DNA Pol I helped elucidate their incorporation properties.

Stengel et al. discovered many unique characteristics of these compounds. KF inserts dtCTP and dtC(O)TP efficiently opposite both G and A. KF inserts both analogues opposite G with the same efficiency (defined by \(V_{\text{max}}/K_M\)) with which dCTP is inserted. KF incorporated the analogues approximately 4- to 11-fold less efficiently opposite template A than incorporation of dTTP. The KF is able to distinguish the structure of the template, thereby not generating dtC(O)-TP-pyrimidine mismatches. KF polymerizes dtCTP and dtC(O)TP 200- and 1000-fold less effectively opposite T, respectively, than it does dATP (Stengel et al. 2009).

Many N4-substituted cytosine analogues prefer to exist in the imino tautomer (Harris et al. 2003). The analogues tC and tC(O), however, have an unclear tautomeric equilibrium- and one cannot determine the tautomeric equilibrium base solely on structure. The compounds likely base pair with G in the amino tautomer, and in the imino tautomer they base pair with A (Figure 4.1). Polymerization favored the amino tautomer.
10:1 because the amino tautomer is 10-fold more stable; this helps to explain why multiple polymerases are able to insert the compound more efficiently opposite G than A (Table 4.1).

The studies by Stengel et al. investigated incorporation of dtCTP and dtC(O)TP by both KF and pol α, as well as some studies using BF pol and HSV pol, and found that despite differing substrate specificities, they polymerize the analogues with strikingly similar efficiency. The formation of base pairs occurs nearly identically regardless of placement of the analogue in the primer or the template strand, thereby showing that the imino tautomer of tC(O) is the main proponent of mutagenic pairing opposite A. Calculation of $V_{\text{max}}/K_M$ found that the tautomeric equilibrium of tC and tC(O) is relatively the same; this demonstrates that the tautomeric equilibrium plays a significant role in defining incorporation ability (Stengel et al. 2009).

<table>
<thead>
<tr>
<th>dNTP</th>
<th>DNA</th>
<th>$V_{\text{max}}$</th>
<th>$K_M$ [μM]</th>
<th>$V_{\text{max}}/K_M$</th>
<th>Discrimination</th>
</tr>
</thead>
<tbody>
<tr>
<td>dTTP</td>
<td>DNA_A</td>
<td>4.1</td>
<td>0.6</td>
<td>6.5</td>
<td>1</td>
</tr>
<tr>
<td>dtCTP</td>
<td>DNA_A</td>
<td>1.6</td>
<td>2.7</td>
<td>0.6</td>
<td>11</td>
</tr>
<tr>
<td>dtC(O)TP</td>
<td>DNA_A</td>
<td>2.2</td>
<td>2.4</td>
<td>0.9</td>
<td>7</td>
</tr>
<tr>
<td>dCTP</td>
<td>DNA_G</td>
<td>3.63</td>
<td>0.23</td>
<td>15.8</td>
<td>1</td>
</tr>
<tr>
<td>dtCTP</td>
<td>DNA_G</td>
<td>4.5</td>
<td>0.18</td>
<td>25</td>
<td>0.6</td>
</tr>
<tr>
<td>dtC(O)TP</td>
<td>DNA_G</td>
<td>3.4</td>
<td>0.13</td>
<td>26.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 4.1: Kinetic parameters for incorporation of dtCTP and dtC(O)TP analogues into DNA_A and DNA_G by KF (Stengel et al. 2009).

**d(dinitro-tC(O))TP: A NOVEL NUCLEOTIDE ANALOGUE**

The nucleotide analogue d(dinitro-tC(O))TP (Figure 4.2) was synthesized in December 2011 by Brittney Rodgers and Byron Purse of the Purse Laboratory at the University of San Diego.

Due to the structural similarities of dinitro-tC(O) to tC and tC(O), I founded my assays on the studies of these compounds. Whereas tC and tC(O) are derived from...
cytosine via an expansion of the carbon ring at the N\textsuperscript{4} and C\textsuperscript{5} positions (Stengel et al. 2009), d(dinitro-tC(O))TP has an additional two nitro groups at the top of the tricyclic portion of the structure. I examined the incorporation of a triphosphorylated dinitro-tC(O) opposite natural bases, characterizing the structural and electronic properties of the compound to understand its incorporation preferences using the Klenow Fragment of DNA polymerase I. Nitro groups are extremely electron withdrawing. My studies aim to address how the two nitro groups affected the tautomeric equilibrium between imino and amino forms.

ASSAY METHODOLOGY

All reagents were of highest quality commercially available. Unlabeled dNTPs were purchased from Invitrogen. d(dinitro-tC(O))TP was synthesized in the Purse Laboratory at University of San Diego. \textit{E. coli} DNA Polymerase I Klenow fragment (exo-) was purchased from New England Biolabs.

5’-labeling of primer strands

DNA primers were 5’-\textsuperscript{32}P-labeled using T4 polynucleotide kinase (purchased from New England Biolabs) and [\gamma - \textsuperscript{32}P]ATP. The labeled primer was annealed to the appropriate template strands. The sequences of the used primer-templates are given in Chart 1.

<table>
<thead>
<tr>
<th>DNA\textsubscript{A}</th>
<th>5’ - TCCATATCACAT</th>
<th>3’ - AGGTATAGTGTAATTCTATCATCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA\textsubscript{G}</td>
<td>5’ - CCATATCACAT</td>
<td>3’ - GGTATAGTGTTCTTATCATCT</td>
</tr>
</tbody>
</table>

Chart 1: Sequences of Primer-Templates. The letter after "DNA" designates the template base being replicated.

Polymerization assays

All kinetic data were determined under steady-state conditions.

The reactions with KF contained 2 \mu M 5’-\textsuperscript{32}P-primer-template, 5 mM MgCl\textsubscript{2}, 50 mM Tris-HCl pH 7.5, and varying concentrations of natural or analogue dNTPs in a total volume of 10 \mu l. Reactions that served to derive kinetic parameters contained 0.5 nM KF.
(-exo). Polymerization was initiated by mixing equal volumes of reaction mixture and enzyme followed by incubation at 37 °C. The reactions were stopped by addition of two volumes gel loading buffer (90% formamide with 50 mM EDTA) after 1—10 minutes. Extension products were separated by denaturing gel electrophoresis (20% polyacrylamide, 8 M urea) and analyzed by phosphor imaging (Typhoon scanner, Molecular Dynamics). The parameters $K_M$ and $V_{max}$ were obtained by nonlinear curve fitting.
V. Results

KF inserts d(dinitro-tC^{(O)})TP efficiently opposite G and A

The single nucleotide insertion assays evaluated the incorporation of d(dinitro-tC^{(O)})TP opposite guanosine and adenosine; G and A were positioned on synthetic oligonucleotides (Chart 1); these assays allowed for the determination of the $K_M$ and $V_{max}$.

Preliminary experiments indicated poor to nonexistent incorporation of d(dinitro-tC^{(O)})TP opposite T and C.

Knowing that d(dinitro-tC^{(O)})TP is derived from the canonical pyrimidine dCTP, I wanted to investigate its ability to be incorporated across from a templating guanosine. Figure 5.1 shows the result.

![Figure 5.1: Primer elongation by KF after incorporation of d(dinitro-tC^{(O)})TP. The primer/template sequence is shown above the figure. Assay contained: 2 µM DNAc/standard primer; 2, 5, 10, 20, 40 and 500 µM dCTP. The enzyme concentration was 0.5 nM KF. The time points recorded were 2.5 and 10 minutes.](image)

Knowing the ambivalence with which dtCTP and dtC^{(O)}TP were incorporated opposite both a templating guanosine and adenosine, I investigated the incorporation of d(dinitro-tC^{(O)})TP across from a templating adenosine. Figure 5.2 shows the result.
Graph 5.1 visually depicts the Michaelis-Menten kinetics with which d(dinitro-tC(O)TP is incorporated opposite a templating adenosine.

Figure 5.2: Primer elongation by KF after incorporation of d(dinitro-tC(O)TP. The primer/template sequence is shown above the figure. Assay contained: 2 µM DNA_A/standard primer; 2, 5, 10, 20, 40 and 500 µM d(dinitro-tC(O)TP; 0.5, 0.75, 1, 2, 4 and 6 µM dTTP. The enzyme concentration was 0.5 nM KF. The time points recorded were 2.5 and 10 minutes.

Graph 5.1: Michaelis-Menten parameters for incorporation of d(dinitro-tC(O)TP opposite template adenosine.
Graph 5.2 visually depicts the Michaelis-Menten kinetics with which dTTP, the correct nucleotide, is incorporated opposite a templating adenosine.

![Graph 5.2: Michaelis-Menten parameters for incorporation of dTTP opposite template adenosine.](image)

Table 5.1 shows the quantitative data of how the rates of incorporation compared.

<table>
<thead>
<tr>
<th>dNTP</th>
<th>DNA</th>
<th>$V_{\text{max}}$</th>
<th>$K_M$ [$\mu$M]</th>
<th>$V_{\text{max}}/K_M$</th>
<th>Discrimination</th>
</tr>
</thead>
<tbody>
<tr>
<td>dTTP</td>
<td>DNA_A</td>
<td>2.47</td>
<td>0.32</td>
<td>7.70</td>
<td>1</td>
</tr>
<tr>
<td>d(dinitro-tC(0))TP</td>
<td>DNA_A</td>
<td>2.38</td>
<td>0.25</td>
<td>9.48</td>
<td>1.23</td>
</tr>
<tr>
<td>dCTP</td>
<td>DNA_G</td>
<td>0.69</td>
<td>0.19</td>
<td>3.59</td>
<td>1</td>
</tr>
<tr>
<td>d(dinitro-tC(0))TP</td>
<td>DNA_G</td>
<td>2.93</td>
<td>0.38</td>
<td>7.70</td>
<td>2.15</td>
</tr>
</tbody>
</table>

Table 5.1: Kinetic parameters for incorporation of d(dinitro-tC(0))TP analogues and their correct counterparts into DNA by KF.

KF polymerizes d(dinitro-tC(0))TP opposite A with approximately the same efficiency, $V_{\text{max}}/K_M$, with which it inserts the correct nucleotide, dTTP. KF polymerizes d(dinitro-tC(0))TP opposite G with approximately 2-fold more efficiency than it does the correct nucleotide, dCTP (Table 5.1).
VI. Discussion and Conclusions

d(dinitro-C^tO)TP was derived in part from the studies conducted by Stengel et al. using tC and tC^tO. Whereas tC and tC^tO are derived from cytosine via an expansion of the carbon ring at the N^4 and C^5 positions (Stengel et al. 2009), dinitro-tC^tO expands on the structure of tC^tO through the addition of two nitro groups at the top of the tricyclic portion of the structure. Studies of dtCTP and dtC^tOTP found that these compounds hydrogen bond similarly to their parental structure cytosine, but extend their bulky tricyclic rings into the major groove of the DNA; despite this addition, KF is able to polymerize the compounds equally as efficiently as a dCTP analogue, and only 4-11 times less efficiently as a dTTP analogue (Table 1). Thus, KF ambivalently incorporates dtCTP and dtC^tOTP opposite both A and G, which appears remarkable in light of the natural ability of KF to recognize mismatches (Stengel et al. 2009).

KF incorporates d(dinitro-tC^tO)TP efficiently opposite both A and G (Table 5.1), but with varying degrees. KF incorporates d(dinitro-tC^tO)TP as efficiently as dTTP opposite A, and about twice as efficiently as dCTP opposite G (Table 5.1). A higher \( \frac{V_{\text{max}}}{K_m} \) indicates a higher affinity for the substrate; this would indicate that KF has the greatest affinity for d(dinitro-tC^tO)TP as a T analogue. KF appears to have an equal affinity for dTTP upon incorporation opposite A as it does d(dinitro-tC^tO)TP upon incorporation opposite G. Of all the substrates investigated, KF has the least affinity for incorporation of dCTP opposite G. Acting as a T and C analogue, this indicates that d(dinitro-tC^tO)TP behaves as a pyrimidine analogue.

Stengel et al. found that a dtC^tOTP-G base pair has a higher melting temperature than the canonical C-G base pair, leading to the conclusion that the novel compound lends a stabilizing effect to the duplex. This effect may be derived from \( \pi-\pi \) interactions between the aromatic tricycle and the heterocycles of the traditional bases. This is most likely true of d(dinitro-C^tO)TP.
I propose that d(dinitro-tC(O))TP incorporates opposite A in the imino tautomer (Figure 6.1); in the same manner, d(dinitro-tC(O))TP most likely incorporates opposite G via the amino tautomer (Figure 6.2). This is largely because the hydrogen bonding ability of d(dinitro-tC(O))TP should not differ from that of tC and tC(O); the nitro groups do not influence the compounds ability to pair with other bases.

The cytosine analogue dinitro-tC(O) and its sister compounds tC and tC(O) are a largely uncharted category of traditional nucleotide analogues; usually, nucleotide analogues are not quite as sizable as these compounds, but the size difference appears to have minimal effect on incorporation, particularly because hydrogen bonding patterns remain unaffected. A number of studies examine compounds in which the hydrogen bond donors and acceptors are substituted with differing chemical groups comparable in size to that which is being replaced, leading to the conclusion that hydrogen bonding is not the key component in correct polymerization—rather, concluded that size appears to be the deciding factor. (Kool 2002; Henry and Romesberg 2003).

My studies on d(dinitro-tC(O))TP further supports the idea that selection for the correct nucleotide based on size is only true for analogues
closely related to the canonical bases. The size of d(dinitro-tC(O))TP is radically different from traditional nucleotides, and polymerization assays show that it can be incorporated efficiently but unselectively via KF, much like dtCTP and dtC(O)TP (Stengel et al. 2009). Studies of these compounds indicate that KF uses different mechanisms to select for the incoming nucleotide- and that both the size and hydrogen bonding patterns of the incoming nucleotide are equally important.

With d(dinitro-tC(O))TP, then, it would appear that its ability to hydrogen bond in the same pattern as its parent compound, cytosine, is sufficient to allow KF to identify it as correct. The function of the bulky cyclic addition that juts into the major groove is less clear. Because it is readily incorporated into the DNA strand, it does not appear to be detrimental enough to disqualify the compound as incorrect. d(dinitro-tC(O))TP is an exciting compound because it retains traditional hydrogen bonding properties, thereby maintaining the identified prerequisite for selection of the correct incoming nucleotide, but expands upon the traditional cytosine structure by expanding into the major groove. d(dinitro-tC(O))TP represents an expansion of the studies conducted by Stengel et al. by the addition of the nitro groups. tC and tC(O) express tenfold preference for C, whereas dinitro-tC(O) appears to exist in closer tautomeric equilibrium. Thus, the addition of nitro groups causes d(dinitro-tC(O))TP to be recognized more like T. Electron-withdrawing groups therefore has an undesirable effect, causing the strand to be less mutagenic, and future compounds to be explored should evaluate the effect of adding electron-donating groups to the structure.

The development of nucleotide and nucleoside analogues over the past decade has resulted in the creation of a powerful tool against a variety of viruses and cancers. These compounds are structurally and mechanistically diverse, leading to a plethora of applications. As seen above, nucleotide and nucleoside analogues have progressed significantly and have a dramatic impact on cancer and viral therapeutics; the applications of these compounds clinically have led to the recognition of that which is beneficial and detrimental to the compounds therapeutically. Background research has worked to widen knowledge about the individual mechanisms of resistance to nucleotide and nucleoside analogues, such as cellular drug uptake, interaction with cellular targets, drug metabolism, and apoptosis. Researchers have taken this knowledge and applied it to
more compounds, expanding the library of agents available to patients (Jordheim et al. 2013). Researchers are currently developing many additional agents that are presently in clinical trials. However, viruses continue to mutate and emerge, presenting a need for constant development of new compounds. Several viral infections, both life-threatening and not, do not have viable therapeutics, and could be addressed by new or repositioned nucleoside and nucleotide analogues.

There are still fundamental flaws in the nucleoside and nucleotide analogue therapeutic system. Viral genomes demonstrate strong variation, and viruses have heterogenous resistance mechanisms (Hurwitz and Schinazi 2013). Patients have diverse genetic variability. Nucleoside and nucleotide analogues can attack host nucleic acid, leading to extremely dangerous circumstances for the patient. Moreover, due to the novelty of the system, the cost of such therapeutics is not yet a viable, low-cost option for diseases in low-income countries, which is one of the places that needs it the most (Veltkamp et al. 2008).

Nucleosides with ambivalent base pairing properties, like tC, tC(O) and dinitro-tC(O), have the potential to have huge impacts on cancer therapeutics. This is because they can be introduced to the genome and incorporated extensively as mutagens, allowing the cell to recognize the sequence as error catastrophe. Currently, however, d(dinitro-tC(O))TP is therapeutically unviable; it is unable to be phosphorylated in the cell, potentially due to inhibition of the kinases by the bulky cyclic structure. Further studies on d(dinitro-tC(O))TP could need to elucidate its mutagenic capabilities as the templating base, as the studies by Stengel et al. determined for tC and tC(O). If d(dinitro-tC(O))TP were to exhibit mutagenic capabilities in the templating strand, this would support multiple rounds of cell division, thereby making it a suitable candidate that would survive past the first few rounds of replication. Thus, it appears that the merit of d(dinitro-tC(O))TP is founded in kinetic studies, not actual therapeutic application. Pursuit of a different compound, with electron-donating groups rather than electron-withdrawing groups, would be an interesting avenue for future research. It appears as though the addition of nitro groups shifts the tautomeric equilibrium from the amino preference of tC(O) to a greater preference for the imino tautomer. Perhaps the addition of electron-donating groups would push the tC(O) compound significantly more towards the amino
tautomter. This would help to confirm the effects of adding electron-affective groups to nucleotide analogues.
VII. References


Van Rompay, A.R., M. Johansson, and A. Karlsson. “Substrate specificity and


