THE INCORPORATION OF GEMCITABINE AND CYTARABINE INTO DNA BY DNA POLYMERASE β AND LIGASE

A Thesis in
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by
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ABSTRACT

2'-Deoxy-2',2'-difluorocytidine (gemcitabine, dFdC) and 1-β-D-arabinofuranosylcytosine (cytarabine, araC) are nucleoside analogues used in the treatment of a variety of malignancies. The active drug metabolites of both araC and dFdC inhibit DNA synthesis. Inhibition by araC occurs immediately after its incorporation, while dFdC inhibits via a masked termination mechanism in which after dFdC incorporation, an additional nucleotide is incorporated prior to the inhibition of DNA synthesis. However, these mechanisms of action were determined using high fidelity replicative polymerases and have not been examined in the context of DNA synthesis associated with DNA repair. We hypothesize that the base excision repair (BER) enzymes, DNA polymerase β (pol β) and ligase, may be capable of incorporating araC and dFdC into the DNA, bypassing the traditional and masked termination mechanisms associated with each of these drugs and subsequently causing cytotoxicity. Through the use of kinetic studies, we investigated the ability of DNA polymerase β and ligase to incorporate and subsequently ligate araC and dFdC into a gapped DNA substrate. Our results demonstrate that pol β efficiently can incorporate araC and dFdC metabolites into a gapped substrate, but subsequent ligation by ligase III/XRCC1 is poor. The unrepaired single strand break left in the DNA may be fatal and may provide a means by which these drugs can cause cytotoxicity via the BER pathway.
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ABBREVIATIONS

BER - base excision repair
Pol β - DNA polymerase β
Pol α - DNA polymerase α
Pol ε - DNA polymerase ε
Pol γ - DNA polymerase γ
Pol δ - DNA polymerase δ
dNTP - deoxynucleotide triphosphate
dC - 2'-deoxycytidine
dCMP - 2'-deoxycytidine monophosphate
dCTP - 2'-deoxycytidine triphosphate
dFdC - 2'-deoxy-2',2'-difluorocytidine or gemcitabine
dFdCMP - 2'-deoxy-2',2'-difluorocytidine 5'-monophosphate
dFdCDP - 2'-deoxy-2',2'-difluorocytidine 5'-diphosphate
dFdCTP - 2'-deoxy-2',2'-difluorocytidine 5'-triphosphate
araC - 1-β-D-arabinofuranosylcytosine or cytarabine
araCMP - 1-β-D-arabinofuranosylcytosine monophosphate
araCTP - 1-β-D-arabinofuranosylcytosine 5'-triphosphate
2'FdC - 2'-deoxy-2'-fluorocytidine
2'FdCMP - 2'-deoxy-2'-fluorocytidine 5'-monophosphate
2'FdCTP - 2'-deoxy-2'-fluorocytidine 5'-triphosphate
rC - cytidine
rCMP – cytidine 5'-monophosphate
rCTP – cytidine 5'-triphosphate
ROS - reactive oxygen species
ATP – adenosine 5'-triphosphate
DTT - dithiothreitol
BSA - bovine serum albumin
EDTA - ethylenediamine tetraacetic acid
Tris - tris(hydroxymethyl)aminomethane
NMR - nuclear magnetic resonance
PAGE - polyacrylamide gel electrophoresis
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Chapter 1

INTRODUCTION

Nucleoside analogues are an important class of cancer chemotherapeutics that are used for the treatment of various malignancies. The biological activity of most nucleoside antimetabolites is due to their ability to inhibit DNA synthesis needed for cell division and proliferation.

Two drugs of this class, 1-β-D-arabinofuranosylcytosine (cytarabine, araC) and 2'-deoxy-2',2'-difluorocytidine (gemcitabine, dFdC), are analogs of 2'-deoxycytidine (dC) (Figure 1).

AraC is successful in the treatment of hematological malignances (1). Gemcitabine is successfully used as a single agent or in combination chemotherapy in the treatment of non-small cell lung cancer (2), pancreatic (3, 4), ovarian (5), and breast cancer (6, 7), as well as in the

![Figure 1. Structures of 2'-deoxycytidine (dC), 1-β-D-arabinofuranosylcytosine (araC), Cytidine (rC), 2'-deoxy-2'-fluorocytidine (2'FdC), and 2'-deoxy-2',2'-difluorocytidine (dFdC). ](image)
treatment of hematological malignancies (8). dFdC has also shown promising efficacy for the
treatment of other malignancies (9-16) suggesting it will see more wide-spread use in the future.
In addition to its use as a monotherapy, gemcitabine is often most effective when used as part of a
combination therapy, frequently with platinum-based and topoisomerase-targeted
chemotherapeutic agents (17-19).

The mechanism of action of each drug is multifaceted but primarily involves inhibiting
DNA synthesis, although in slightly different ways. Both araC and dFdC are transported into the
cell where they are activated by phosphorylation (20, 21). Studies with intact cells have indicated
that inhibition of DNA synthesis is the primary mechanism of cytotoxicity (20, 22, 23). dFCDP
is a potent inhibitor of ribonucleotide reductase (24), which will lower dNTP pools and lead to
inhibition of DNA synthesis. AraC, when incorporated into the DNA, is an inhibitor of
topoisomerase I (25).

*In vitro* primer extension reactions with DNA polymerases α (pol α) and ε (pol ε) have
shown that araCTP and dFdCTP inhibit DNA synthesis by becoming incorporated into the DNA
and then inhibiting DNA synthesis (20, 22, 26). Inhibition by araC occurs immediately after araC
incorporation, while dFdC inhibits via a masked termination mechanism in which after dFdC
incorporation, an additional nucleotide is incorporated prior to inhibition of DNA synthesis. It is
thought that the addition of a correct base pair prevents the proof-reading exonuclease activity of
DNA pol ε to remove dFdCMP once incorporated into DNA (22).

These types of inhibition are not universal among polymerases. AraC and dFdC inhibit
pol α and pol ε more than DNA polymerase δ (pol δ) (27). Among the A-family polymerases,
dFdC is a weak inhibitor of DNA polymerase γ (pol γ) and does not follow the proposed masked
termination mechanism, but is able to extend past an internal dFdCMP residue (28).
The mechanism of action of araC and dFdCTP were determined using high fidelity replicative polymerases (22) and have not been examined in the context of DNA synthesis associated with DNA repair. In the base excision repair pathway (BER), DNA damage is recognized and excised by DNA glycosylases and/or endonucleases leaving behind a gap in the DNA that is filled by DNA polymerase β (pol β) and then sealed by a DNA ligase (29). Human DNA ligase III has been shown to partner with XRCC1 and is implicated in completion of short patch BER (30).

The BER pathway is the main pathway by which oxidative damage to the cell is repaired (31, 32). There has been surmounting evidence that indicates cancer cells are under increased oxidative stress (33, 34). Compared to normal cells, they have been shown to produce elevated levels of reactive oxygen species (ROS), which are known to react with pyrimidine and purine bases to form oxidative damage to the DNA (33). The oxidation of cytosine residues by ROS primarily leads to the formation of cytosine N-oxide derivatives (35) and damage by nitric oxide derivatives has been shown to lead to the deamination of cytosine (36), which occurs more frequently in cells under oxidative stress (31).

Additional damage to cytosine residues in the DNA as a result of the increased oxidative stress of cancer cells may provide opportunity for the incorporation of araC and gemcitabine residues via the BER pathway. It has been shown that the BER pathway is up-regulated in cells treated with an oxidative stress inducing agent (32). It is possible that this response extends to cancer cells as well since they are under high levels of oxidative stress and may further the opportunity for repair via base excision. Several tumor types, including kidney, breast, prostate, uterus, ovary, colon, lung, stomach, and rectum have even been shown to have increased expression of pol β (37).

Chemotherapeutic agents themselves have been known to cause oxidative stress. Treatment of cells with araC increases the production of ROS (38). The DNA alkylating agents,
cisplatin and carboplatin, which are commonly administered in combination therapy with
gemcitabine in the treatment of non-small cell lung cancer (2) and ovarian cancer (5)
respectively, have also been shown to induce oxidative stress (39). In addition, taxanes, such as
paclitaxel, which are given with gemcitabine in the treatment of breast cancer, generate low levels
of oxidative stress (39). The oxidative stress associated with cancer and the chemotherapy agents
used to treat it could result in damage of cytosine residues, providing an increased opportunity for
the incorporation of cytarabine and gemcitabine into the DNA via the BER pathway.

It is our hypothesis that araC and dFdC can be incorporated by pol β and subsequently
ligated by DNA ligase into a DNA substrate, thereby bypassing the traditional and masked chain
termination mechanisms of these drugs associated with pol α and pol ε. We investigated the
ability of DNA polymerase β and ligase to incorporate and subsequently ligate araC, dFdC, as
well as the structurally similar 2′-deoxy-2′-fluorocytidine (2′FdC) and cytidine (rC) into a gapped
DNA substrate relative to the natural cytidine (dC) substrate.
Chapter 2

MATERIALS AND METHODS

2.1. Reagents.

T4 polynucleotide kinase was purchased from Epicentre (Lexington, KY) and \([\gamma^{32}\text{P}]\) ATP (6000 Ci/mmoll) from Perkin-Elmer (Waltham, MA). Human ligase III and XRCC1 were purchased from Enzymax (Lexington, KY). DNA oligomers were purchased from IDT (San Jose, CA). The nucleotide triphosphates, dCTP and rCTP, were purchased from Promega (Madison, WI). Modified nucleotide triphosphates, araCTP and 2'FdCTP, were purchased from Trilink Biotechnologies (San Diego, CA). dFdCTP was a gift of Eli Lilly (Eli Lilly and Company, Indianapolis, IN) and was also synthesized from Gemzar® (Eli Lilly and Company, Indianapolis, IN) purchased from the Outpatient Pharmacy at the Penn State Hershey Medical Center (Hershey, PA). The concentration of each dNTP was determined by UV absorbance (40).

2.2. Synthesis of 2'-deoxy-2'-2'-difluorocytidine 5'-triphosphate (dFdCTP).

dFdC was separated from mannose and acetate present in Gemzar® (Eli Lilly and Company, Indianapolis, IN) using a G15 Sephadex (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) column. The dFdC was then dried by pyridine evaporation followed by high vacuum over phosphorus pentoxide overnight. One equivalent of dFdC (1) and 1.5 equivalents of 1,8-bis(dimethylamino)naphthalene were dissolved in the appropriate volume of trimethyl phosphate to achieve a dFdC concentration of 0.3 M. After cooling to -10°C, 1.1 equivalents of phosphorus oxychloride (POCl₃) were added dropwise, and then the solution was stirred for 2
hours at 0°C. Four equivalents of tributlyamine (Bu$_3$N) and 2.5 equivalents of tributylammonium pyrophosphate were cooled to -10°C in the appropriate amount of dry N-N-dimethylformamide to achieve a pyrophosphate concentration 0.15 M. This mixture was added to the nucleotide mixture (2) and warmed to room temperature (Figure 2).

Figure 2. Synthesis of 2’-deoxy-2’-2’-difluorocytidine 5’-triphosphate (dFdCTP)

\[ \text{Reagents and conditions: a} \text{ POCl}_3, 0^\circ \text{C} \text{ b} \text{ Bu}_3\text{N, tributylammonium pyrophosphate, -10°C} \]

The reaction was quenched with 10 volumes of 1 M triethylammonium bicarbonate (pH 7.5) for 20 minutes after which it was concentrated en vacuo. The crude triphosphate was purified by anion-exchange chromatography on a DEAE-cellulose column at 4°C. The triphosphate was eluted with a linear gradient of 100 mM to 1M triethylammonium bicarbonate (pH 7.5). The appropriate fractions were converted to the sodium salt and NMR was used to confirm dFdCTP (3). $^1$H NMR (D$_2$O) 7.83(1H, d, J=7.5 Hz), 6.16 (1H, t J=7.25 Hz), 6.08(1H, d, J=7.5 Hz), 4.45 (1H, m), 4.30 (1H, m), 4.21 (1H, m) 4.10 (1H, m). $^{31}$P NMR (D$_2$O) -10.9, -11.4, -23.2.

2.3. Generation and Purification of Histidine-tagged Polymerase β Enzymes.

Dr. Joann Sweasy (Yale University, New Haven, CT) supplied the cDNA of WT polymerase β which was subcloned behind an N-terminal hexahistidine tag in the pET28a vector and transformed into BL21-λDE3 E. coli cells. Protein expression and purification were performed as previously described (41). Pol β expression was induced with 2 mM IPTG for 3 h
at 30 °C. Cell extracts were prepared and the protein composition was analyzed by SDS PAGE. Figure 3, lane 4 shows that pol β was overexpressed in the cell extracts. The cell extracts were mixed with Ni–NTA resin (Qiagen, Valencia, CA) prior to being loaded on a column. The column was washed and figure 3, lane 3 shows the protein composition of the wash. After the washing, the protein was eluted and figure 3, lane 2 shows the protein after elution from the column. Fractions containing protein were exchanged into 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, and 15% glycerol buffer using a Centricon-10 device (Millipore, Bedford, MA). The sample was loaded on a column containing single-stranded DNA–cellulose (Sigma) and washed with 250 mM NaCl, and protein was eluted with 500 mM NaCl. Protein-containing fractions were concentrated and exchanged into 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, and 15% glycerol buffer (42) and the final pure protein can be seen in lane 5. Final protein purity was visualized with Commassie Brilliant Blue (Figure 3).

![Figure 3. Representative gel of the purification of DNA polymerase β.](image)

1 0.5 ml protein standard (Fermentas) 2 Ni-NTA fraction 3 Ni-NTA column wash 4 total cell extract 5 DNA cellulose fractions
2.4. DNA Substrates.

A 17-mer primer, a 33-mer template strand, and a 15-mer downstream oligonucleotide were used to construct a single nucleotide gapped substrate. The primer strand was 5'-end-labelled with $^{32}$P using T4 polynucleotide kinase and [$\gamma^{32}$P] ATP (6000 Ci/mmol) as previously described (42). The 15-mer downstream oligonucleotide was phosphorylated on the 5'-end with nonradioactive ATP. The unreacted [$\gamma^{32}$P] ATP and ATP were removed using a G-25 spin column. The oligodeoxynucleotides were annealed at a primer:downstream:template ratios of 1:3:1. The sequences of the primer-downstream oligonucleotide/template DNA for the gapped substrate is shown in Figure 4.

![Single Nucleotide Gapped Substrate](image)

Figure 4. Single nucleotide gapped DNA substrate for DNA polymerase $\beta$. The single Basepair gap is underlined and the templating base (G) is shown in boldface.

2.5. Single Turnover Kinetic Experiments.

A KinTek-3 rapid quench apparatus was used to perform experiments ranging in time from 2 milliseconds to 60 seconds at 37°C in 50 mM Tris-HCl (pH 7.4) containing 20 mM NaCl, 1 mM DTT, 10 mM MgCl$_2$, and various concentrations of dNTP. The reactions were initiated by the addition of dNTP and MgCl$_2$ to DNA polymerase $\beta$ and a radiolabelled single nucleotide gapped DNA substrate. The final concentrations of DNA and polymerase were 25 nM and 100 nM respectively. Reactions were quenched with 300 mM EDTA (pH 8.0). Experiments were repeated 3 times for each dNTP.

Ligase III, XRCC1, and ATP were pre-incubated for 15 min at 37°C in 50 mM Tris-HCl (pH 7.4), 20 mM NaCl, 1 mM DTT, and 10 mM MgCl₂. A nicked DNA substrate was created by incubating a 5'-32P labeled single nucleotide gapped DNA substrate with DNA polymerase β and 200 µM dNTP at 37°C in 50 mM Tris-HCl (pH 7.4), 20 mM NaCl, 1 mM DTT, and 10 mM MgCl₂ for at least 15 minutes. Reactions were initiated by the addition of the ligase III, XRCC1, and ATP mixture to the newly formed nicked DNA substrate and allowed to incubate at 37°C for various times in 50 mM Tris-HCl (pH 7.4), 20 mM NaCl, 1 mM DTT, and 4 mM MgCl₂. The final concentrations of ligase III and XRCC1 were 10 nM each. The final ATP concentration was 2 mM and the final DNA concentration was 10 nM. Reactions were quenched with 10% 300 mM EDTA pH 8.0 and 90% formamide containing 0.025% (w/v) bromophenol blue and 0.025% (w/v) xylene cyanol. Experiments were repeated 2-4 times for each dNTP.


T4 ligase and ATP were pre-incubated for 15 min at 37°C. A nicked DNA substrate was created by incubating a 5'-32P labeled single nucleotide gapped DNA substrate with DNA polymerase β and 200 µM dNTP at 37°C in 50 mM Tris-HCl (pH 7.4), 20 mM NaCl, 1 mM DTT, and 10 mM MgCl₂ for at least 15 minutes. The newly formed nicked DNA substrate was subsequently reacted with the T4 ligase reaction mixture in 33 mM Tris-acetate (pH 7.8) containing 66 mM potassium acetate, 10 mM magnesium acetate and 0.5 mM DTT over time using a KinTek-3 rapid quench apparatus. The final concentrations were as follows: 50 nM DNA, 750 nM T4 ligase, and 2 mM ATP. The reactions were quenched with 10% 300 mM
EDTA pH 8.0 and 90% formamide containing 0.025% (w/v) bromophenol blue and 0.025% (w/v) xylene cyanol. Experiments were repeated 2-4 times for each dNTP.

2.8. Product Analysis by polyacrylamide gel electrophoresis (PAGE).

The reaction products were separated on a denaturing PAG consisting of 20% acrylamide (19:1, acrylamide-\(N,N'\)-methylene bisacrylamide) with 7M urea in 89 mM Tris, 89 mM boric acid, and 2 mM Na\(_2\)EDTA (pH 8.0). The size of the gel was 40 cm × 33 cm × 0.4 cm and was run at 2000 V for 2-2.5 h. The gel was visualized and quantified using a Typhoon 9200 Phosphoimager with ImageQuant software. The progress of the reaction was quantitated by dividing the total radioactivity of the product band by the radioactivity of the product and reactant bands.

2.9. Data Analysis.

Data were fitted by nonlinear regression using Prism version 4 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com). Data from the single turnover kinetics were fitted to eq 1 where \([P]\) is the amount of product formed, \([DNA]\) is the total amount of DNA reacted, and \(k\) is the first-order rate constant for the incorporation of dNTP.

\[
[P] = [DNA](1-e^{-kt})
\]

(eq 1)

The \(k\) determined for each concentration was then fit to a hyperbolic equation (eq 2) where \(k_{pol}\) is the maximum \(k\) of dNTP incorporation and \(K_d\) is the dissociation constant for the dNTP with the polymerase-DNA complex.

\[
k = (k_{pol}[dNTP])/(K_d+[dNTP])
\]

(eq 2)
The observed rates (k) for the Ligase III/XRCC1 Assay and the T4 Ligase Assay were calculated in the same fashion as for the single turnover kinetics using the first order equation (eq 1).
Chapter 3

RESULTS


The ability of DNA polymerase β to incorporate dCTP and its analogs into a gapped DNA substrate was characterized using single turnover kinetic studies in which enzyme was used in excess over DNA (100 nM polymerase and 25 nM DNA). Reactions went to completion and the observed rates \( k \) were determined at different concentrations of dNTP by fitting the data to a first order equation (eq 1). A representative plot for the incorporation of each substrate examined at a concentration of 30 µM as a function of time is shown in Figure 5A. The first-order rate constants \( k \) were plotted versus the dNTP concentration (Figure 5B), and the \( k_{\text{pol}} \) and \( K_d \)

![Graph A](image1)

![Graph B](image2)

Figure 5. Single turnover kinetics for the incorporation of dCTP and its analogs by pol β into a single nucleotide gapped substrate. (A) Time course for the reaction between DNA (25 nM), pol β (100 nM), and 30 µM dCTP (■), araCTP (▼), 2’FdCTP (♦), dFdCTP (▲), or rCTP (●). Reactions were quenched with 300 mM EDTA. The solid line represents the best fit to eq 1. (B) Plot of \( k \) versus [dNTP]. The \( k \) determined for each concentration was fit to a hyperbolic equation (eq 2) where \( k_{\text{pol}} \) is the maximum \( k \) of dNTP incorporation and \( K_d \) is the dissociation
parameters were determined by fitting the data to eq 2. The single turnover parameters are summarized in Table 1.

Table 1. Kinetic parameters for the DNA polymerase β catalyzed incorporation of dCTP and analogs into DNA

<table>
<thead>
<tr>
<th>Single-turnover Parameters</th>
<th>dNTP</th>
<th>$k_{pol}$ (s$^{-1}$)</th>
<th>$K_d$ (µM)</th>
<th>$k_{pol}/K_d$ (µM$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCTP</td>
<td>4.6 ± 1.9</td>
<td>18 ± 6</td>
<td>0.25 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>dFdCTP</td>
<td>0.7 ± 0.6</td>
<td>20 ± 8</td>
<td>0.033 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>araCTP</td>
<td>4.3 ± 0.8</td>
<td>18 ± 5</td>
<td>0.25 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>2’FdCTP</td>
<td>1.4 ± 0.07</td>
<td>7.3 ± 0.2</td>
<td>0.19 ± 0.008</td>
<td></td>
</tr>
<tr>
<td>rCTP</td>
<td>0.3 ± 0.2</td>
<td>49 ± 9</td>
<td>0.006 ± 0.003</td>
<td></td>
</tr>
</tbody>
</table>

aData represent the mean ± standard deviation of 3 independent experiments.

Previously it was found that pol β incorporates dCTP opposite a G residue into a single gapped nucleotide substrate with a $k_{pol}/K_d$ value of 6.6 µM$^{-1}$s$^{-1}$ where the $k_{pol}$ was 12.5 s$^{-1}$ and the $K_d$ was 1.9 µM (43). Here, we report a $k_{pol}/K_d$ value of 0.25 µM$^{-1}$s$^{-1}$ where the $k_{pol}$ was 4.6 s$^{-1}$ and the $K_d$ was 18 ± 6 µM at a pH of 7.7. Dissimilar results may be indicative of the different DNA sequences of the substrates.

DNA polymerase β was able to incorporate araCTP with the same efficiency as dCTP (0.25 µM$^{-1}$s$^{-1}$), with almost identical $k_{pol}$ and $K_d$ values. The structure of rCTP is similar to araCTP and differs only in the orientation of the 2’-OH group; however, incorporation of rCTP was the most severely hindered with a $k_{pol}/K_d$ value of 0.006 µM$^{-1}$s$^{-1}$, demonstrating that pol β incorporates rCTP 42-fold less efficiently than dCTP. The $K_d$ value for rCTP (49 ± 9 µM) was 2-3-fold higher than that of dCTP and araCTP (18 µM) suggesting that there is some discrimination by the polymerase at the level of nucleotide binding. Furthermore, a 15-fold decrease in $k_{pol}$ was seen for rCTP relative to dCTP (0.30 s$^{-1}$ vs 4.6 s$^{-1}$) implying that the rate of phosphodiester bond
formation was also altered by rCTP. 2'FdCTP was also found to be efficiently incorporated (0.19 µM⁻¹s⁻¹).

In the case of dFdCTP, the efficiency of incorporation was decreased only 8-fold relative to dCTP (0.033 µM⁻¹s⁻¹ vs 0.25 µM⁻¹s⁻¹) indicating that pol β effectively incorporated the analog into a single gapped DNA substrate. Comparable $K_d$ values were observed for dCTP and dFdCTP (18 µM and 20 µM). The diminished $k_{pol}/K_d$ can be attributed to the observed decrease in $k_{pol}$, which was lower for dFdCTP than dCTP (0.7 s⁻¹ vs 4.6 s⁻¹), implying that the formation of the phosphodiester between the incoming dFdCTP and the primer nucleotide was hindered.

3.2. Ligation by the human Ligase III/XRCC1 complex.

Incorporation of the nucleotides into genomic DNA by the BER pathway requires ligase to seal the nick. Human DNA ligase III has been shown to partner with XRCC1 and is implicated in completion of short patch BER (30). To gain a better understanding of what may be occurring in vivo, we examined the ability of the ligase III/XRCC1 complex to seal a nicked DNA substrate containing a dFdCMP residue at the primer terminus and complete bypass of the masked termination mechanism. Ligation of the natural substrate, dCMP, as well as the other 2'-ribose modified nucleotides (araCMP, 2'FdCMP, and rCMP) located at the primer terminus of a nicked DNA substrate was also examined. The nicked substrates were created by incubating pol β and dNTP with the single nucleotide gapped substrate. Nearly all of the 17-mer primer was converted to an 18-mer after 15 minutes to form a nicked DNA substrate (Figure 6A, lanes 2, 4-8, and 14-18), with the exception of dFdCMP, in which only approximately 50% of the 17-mer was converted (Figure 6A, lanes 9-13).

Subsequent ligation by the ligase III/XRCC1 complex was performed and the time course of the reactions followed first order kinetics. The ligation reaction neared completion by 960
seconds for dCMP (Figure 6A, lane 7) and rCMP (data not shown) and was approximately 50% complete for 2′FdCMP (data not shown). Less than 20% ligation was observed for dFdCMP and araCMP even after 3840 seconds (Figure 6A, lanes 13 and 18 respectively).

The formation of ligated product (33-mer) as a function of time was determined by fitting the data to eq 1. A representative plot is shown in Figure 6B. The observed rates (k) are shown in Table 2.

![Figure 6](image)

Figure 6. Ligation by the ligase III/XRCC1 complex and T4 ligase. (A) A representative gel of the ligation of dCMP, dFdCTP, and araCTP by ligase III and XRCC1. A single gapped nucleotide DNA substrate was incubated with each nucleotide triphosphate and pol β to form a nicked DNA substrate. The DNA (10 nM) was then incubated with 10 nM of the ligase III/XRCC1 complex and 2 mM ATP. The 17-mer primer is shown in lane 1. The reaction product (newly formed 18-mer) after a 15 min incubation of the single nucleotide gapped substrate with pol β and dCTP is shown is lane 2. Lane 3 is the 33-mer ligated product. Reactions were quenched with EDTA at 10s, 60s, 240s and 960s, 3840s for dCMP (lanes 4-8), dFdCMP (lanes 9-13), and araCMP (lanes 14-18). (B) The data for dCMP (■), rCMP (●), 2′FdCMP (♦), araCMP (▼), and dFdCMP (▲) were fit to the first-order equation (eq 1) and the k determined.
Our results using ligase III/XRCC1 demonstrated that 2'FdCMP and rCMP with first order rate constants of 0.008 s\(^{-1}\) and 0.007 s\(^{-1}\), respectively could be ligated just as successfully as the natural substrate dCMP (0.01 s\(^{-1}\)). The ligation of araCMP was approximately 20-fold slower than the natural substrate with a rate of 5\(\times\)10\(^{-4}\) s\(^{-1}\), while dFdCMP was severely inhibited with a 300-fold slower rate of 3.4\(\times\)10\(^{-5}\) s\(^{-1}\).

### 3.3. Ligation by T4 Ligase.

In addition to the ligase III/XRCC1 complex, we also examined T4 ligase, which like ligase III/XRCC1, is capable of sealing a nicked DNA substrate (44). We examined the ligation of the natural substrate, dCMP, as well as the other 2'-ribose modified nucleotides (dFdCMP, araCMP, 2'FdCTP, and rCMP) located at the primer terminus of a nicked DNA substrate. The nicked substrate was created in the same fashion as for ligase III/XRCC1. Nearly all of the 17-mer primer was converted to an 18-mer after 15 minutes to form a nicked DNA substrate.

Subsequent ligation by T4 ligase was performed using enzyme in excess over DNA (750 nM T4 ligase and 50 nM DNA) and the time course of the reactions followed first order kinetics. The ligation reaction neared completion by 30 seconds when approximately 75% of the 18-mer was ligated to form a 33-mer strand. A representative plot showing the ligation of dCMP by T4 ligase is shown in Figure 6C.
The formation of ligated product (33-mer) as a function of time was determined by fitting the data to eq 1. The observed rates ($k$) are shown in Table 2. While T4 ligase sealed a dFdCMP residue into a nicked DNA substrate, it did so 13-fold more slowly than it did dCMP ($0.03 \pm 0.01 \text{ s}^{-1}$ and $0.42 \pm 0.13 \text{ s}^{-1}$). The other nucleotide analogs, araCMP ($0.20 \pm 0.08 \text{ s}^{-1}$), 2’FdCMP ($0.14 \pm 0.03 \text{ s}^{-1}$), and rCMP ($0.17 \pm 0.05 \text{ s}^{-1}$) were each ligated approximately 2-fold more slowly than dCMP ($0.42 \pm 0.13 \text{ s}^{-1}$).

Figure 6. (C) A single gapped nucleotide DNA substrate was incubated with each dNTP and pol β to form a nicked DNA substrate. The DNA (50 nM) was then incubated with 0.02 units of T4 ligase and 2 mM ATP. The data for dCMP (■), araCMP (▼), 2’FdCMP (♦), rCMP (●), and dFdCMP (▲) were fit to the first-order equation (eq1) and the $k$ determined.

The formation of ligated product (33-mer) as a function of time was determined by fitting the data to eq 1. The observed rates ($k$) are shown in Table 2.

Table 2. The $k$ values calculated for T4 ligase and ligase III/XRCC1 catalyzed nick sealing of dCMP and anologs

<table>
<thead>
<tr>
<th>dNTP</th>
<th>$k_{obs}$ (s$^{-1}$)</th>
<th>$k_{obs}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 Ligase</td>
<td>Ligase III/XRCC1</td>
<td></td>
</tr>
<tr>
<td>dCMP</td>
<td>0.42 ± 0.13</td>
<td>0.01 ± 0.004</td>
</tr>
<tr>
<td>dFdCMP</td>
<td>0.03 ± 0.01</td>
<td>3.4x10$^{-5}$ ± 2.9x10$^{-5}$</td>
</tr>
<tr>
<td>araCMP</td>
<td>0.20 ± 0.08</td>
<td>5x10$^{-4}$ ± 3x10$^{-4}$</td>
</tr>
<tr>
<td>2’FdCMP</td>
<td>0.14 ± 0.03</td>
<td>0.008 ± 0.003</td>
</tr>
<tr>
<td>rCMP</td>
<td>0.17 ± 0.05</td>
<td>0.007 ± 0.001</td>
</tr>
</tbody>
</table>

aData represent mean ± standard deviation of 3-4 independent experiments.
Chapter 4

DISCUSSION

In the BER pathway, DNA damage is recognized and excised by DNA glycosylases and/or endonucleases leaving behind a gap in the DNA. This gap is filled by pol β and sealed by a DNA ligase to complete the pathway (29). We proposed that araC and dFdC could be incorporated by pol β and subsequently ligated by DNA ligase into a DNA substrate, thereby bypassing the traditional and masked chain termination mechanisms. We investigated the ability of DNA polymerase β and ligase to incorporate and subsequently ligate araC, dFdC, as well as the structurally similar 2′FdC and rC into a gapped DNA substrate relative to the natural dC substrate. Our results demonstrate that pol β can efficiently incorporate araCTP and dFdCTP into a gapped substrate, but subsequent ligation by ligase III/XRCC1 is poor. The unrepaired single strand break left in the DNA may be potentially fatal and may provide a means by which these drugs can cause cytotoxicity via the BER pathway.

Single turnover kinetics showed that pol β can incorporate both araCTP and dFdCTP into a gapped DNA substrate. AraCTP was found to be incorporated with similar effectiveness in comparison to the natural substrate, while dFdCTP was incorporated approximately 8-fold more slowly. Previous steady state studies found that pol ε and pol α incorporate dFdCTP 35- and 40-fold less efficiently than dCTP (22). In another study, araCTP and dFdCTP were incorporated 24- and 81-fold less efficiently than dCTP by pol α (26). Additionally it was found using pre-steady state kinetics that pol γ incorporated dFdCTP 430-fold less efficiently than dCTP (28). Variations between these results as well as our results may be explained by the differences in the DNA sequences of the substrates used.
Subsequent ligation of both araCMP and dFdCMP by the ligase III/XRCC1 complex were found to be poor with 20-fold and 300-fold decreases in ligation efficiency respectively. T4 ligase also had difficulty ligating dFdCMP doing so 13-fold slower than the natural dCMP substrate. We are the first to demonstrate the inability of a DNA ligase to ligate either araCMP or dFdCMP into a nicked DNA substrate.

4.1. Steric interactions, electrostatic interactions, and/or conformational changes at the 2’-position of the ribose moiety.

The five nucleotides that we investigated differ in the substituents on the 2’-position of the ribose moiety. The different substituents may affect the reactivity of the nucleotide in the polymerase and ligase reactions through steric interactions, electrostatic interactions and/or conformational changes.

The most obvious change between the dCTP analogs is the difference in the size of the substituents at the 2’-position. The change from hydrogen (H) to fluorine (F) to a hydroxyl (OH) successively increases the volume of the molecule. In addition, the different stereochemistry of the 2’-OH of rCTP and of araCTP would affect the size of the molecule on different faces of the ribose ring. The differing size of the substituent would be expected to alter the reactivity of each reaction in an enzyme-specific manner.

The different substituents have different electron withdrawing potential. The two electron-withdrawing fluorine atoms located at the 2’-ribose position of dFdCTP have been shown to significantly alter the electrostatic surface of the nucleoside analog (Figure 7b) (45). The electron withdrawing potential of the fluorines should decrease the electron density of the oxygen on the 3’-position. This is the atom that would act as the nucleophile in the ligase reaction in which the dFdCMP is in the primer terminus of the DNA. During these reactions, the 3’-OH is
coordinated to a Mg$^{2+}$ which enhances reactivity by lowering the pK$_a$ of the oxygen so that the oxyanion can be formed in the active site and act as the nucleophile (29). The fluorine substituent can have two effects. It will either lower the pK$_a$ of the 3'-OH to allow more oxyanion to form thereby increasing reactivity or it will decrease the negative charge on the oxygen thereby decreasing reactivity.

The differing substitutions at the 2'-position induce the ribose ring to adopt different conformations. Using NMR, the structure of an oligodeoxynucleotide duplex with an internal araC or dFdC residue was determined (45, 46, 47). The dFdC residue was determined to preferentially adopt a C3'-endo sugar pucker rather than the C2'-endo sugar pucker adopted by araC and dC in B-form DNA (Figure 7c) (45, 46, 47). Differences in the puckering will alter the positions of the 5'- and 3'-positions of the ribose. Small changes of either the nucleophile or leaving group in a reaction can have large effects on the rate of reaction.

![Figure 7. Electrostatic potentials and adapted conformations for dC and dFdC](image-url)

a Structures of 2'-deoxycytidine (dC) and 2'-deoxy-2'-2'-difluorocytidine (dFdC)

b Electrostatic surface
4.2. Incorporation of dNTP into DNA by DNA polymerase β

The substitution of the sugar on the 2'-position can affect reactivity via several possible mechanisms including steric interactions between that position and the protein, electrostatic interactions that affect the reactivity of the triphosphate and conformational changes of the ribose ring that would alter the position of the reactive moieties.

4.2.1. Steric Interactions

The change from H to F to OH successively increases the volume of the molecule thereby impacting reactivity. While araCTP was incorporated as efficiently as the natural substrate by pol β, the structurally similar rCTP was the most severely hindered. The two structures differ only in the orientation of the 2'-OH. A tyrosine residue (Y271) located in the catalytic active site of the polymerase domain and positioned between the terminal primer nucleotide and the incoming dNTP has been suggested to play a role in nucleotide discrimination (48, 49). Previous studies have shown that the pol β variant, Y271A has decreased $k_{pol}$ and $K_d$ values, relative to wild type, suggesting that loss of the aromatic ring reduces the enzyme’s ability to discriminate against the incorrect nucleotide (49). It has been proposed that Y271 prevents the incorporation of rCTP by sterically excluding ribose from the active site of polymerase β (48, 49). However, in the case of araCTP, the opposing orientation of the hydroxyl group may allow it to evade discrimination by

the polymerase and could explain why a change in the efficiency of incorporation by pol β was not observed. We can attribute the 40-fold decrease in the efficiency of pol β to incorporate rCTP to discrimination by the active site residue Y271. However, an earlier study reported that pol β incorporates rCTP 2000-fold less effectively than the natural substrate (50). Our single turnover kinetic results demonstrated only a 40-fold decrease and did not fall in agreement with previously reported results. This result suggests that sequence of the DNA substrate also affects the ability of the polymerase to select against the incorporation of ribonucleotides.

As for dFdCTP, the presence of two slightly larger fluorine atoms may contribute to the slowed phosphodiester bond formation observed. In the case of 2'dFdCTP, although the single fluorine atom is slightly larger than a hydrogen atom and is orientated in the 2'-position in the same manner as the hydroxyl group of rCTP, it seems capable of escaping discrimination and was effectively incorporated by pol β.

4.2.2. Electrostatic Interactions

In phosphodiester bond formation by a DNA polymerase, the 3'-OH of the primer terminus residue acts as a nucleophile in an attack on the α-phosphate of the incoming nucleotide. Due to the two fluorine atoms located at its 2'-position, the triphosphate group of the incoming dFdCTP in the pol β reaction has been shown to have decreased electron density compared to dCTP, thus reducing reactivity at this site during phosphodiester bond formation (45). This result has also been observed in the case of dFdCTP with other polymerases as well, including pol α, pol ε, and pol γ (22, 27, 28).

In the case of 2'FdCTP, the single fluorine atom may explain the small decrease we observed in kₚₒᵢ, however, it was not a dramatic enough effect to influence the overall pol β catalyzed incorporation of 2'FdCTP into DNA. AraCTP was incorporated as effectively as dCTP, therefore, the 2'-OH of araCTP does not appear to influence the electron density of its
triphasate. Since, rCTP is structurally similar to araCTP, its 2'-OH also probably does not affect the electron density of its triphosphate and may not be a contributing factor to the large loss of efficiency of its incorporation by pol β.

4.2.3 Conformational Changes

It is possible that dFdCTP adopts a C3'-endo sugar pucker conformation in the active site of pol β causing improper alignment of the α–phosphate of dFdCTP and the 3'-OH of the primer nucleotide thus hindering phosphodiester formation. The observed 8-fold decrease in efficiency suggests that the altered nucleotide conformation and charge distribution of dFdC minimally affects the ability of pol β to catalyze the incorporation of this analog into a gapped DNA substrate.

In contrast to dFdC, araC was shown to adopt the C2'-endo sugar pucker similarly to dCMP in B-form DNA (46, 47), which may explain why araCTP was observed to be incorporated by pol β with a similar efficiency to the natural substrate. This conformation may extend to rCTP as well and is likely not a contributing factor to its poor incorporation efficiency by pol β. No information is available about the conformational preference of 2'dFdC; however, it is likely that its conformation plays little to no role based on the minimal changes we observed in the efficiency of 2'dFdC incorporation by pol β.

4.3. Ligation

The inhibition of a DNA polymerase by araC occurs immediately after araCTP incorporation, while dFdC inhibits via a masked termination mechanism in which after dFdCTP incorporation, an additional nucleotide is incorporated prior to inhibition of DNA synthesis (20, 22, 26). Since pol α and pol ε are capable of incorporating dFdCTP into a DNA substrate (20,
22, 26), and then subsequently incorporating an additional nucleotide, despite the possible C3'-endo sugar pucker of dFdCMP, we expected ligase III/XRCC1 to be capable of sealing dFdCMP into the DNA. Since pol α and pol ε are capable of incorporating araCTP into a DNA substrate, but subsequent polymerization is inhibited, we expect ligase III/XRCC1 to not be capable of sealing an araCMP residue into the DNA.

We made these assumptions based on the fundamental similarities between the DNA polymerase and ligase reactions (29, 51). In both reactions a 3'-OH acts as a nucleophile in an attack on a phosphate (29, 51). In the case of DNA polymerase the 3'-OH of the primer terminus residue acts as a nucleophile in an attack on the α-phosphate of the incoming nucleotide (29), while in the case of DNA ligase the 3'-OH of the primer terminus residue acts as a nucleophile on the 5'-phosphate of the adjacent nucleotide (51). We therefore predicted that the ligase reactions would perform similarly to the polymerization reactions observed for DNA polymerases α and ε.

Our results, however, did not correspond with our expectations. T4 ligase was fairly efficient at ligating both araCMP and dFCMP residues. The inability of ligase III/XRCC1 to ligate an araCMP residue into the DNA fell in agreement with the classic termination mechanism found to occur with pol α and pol ε. Conversely, ligase III/XRCC1 was extremely ineffective at ligating a dFdCMP residue, which does not coincide with the masked termination mechanism found to occur with pol α and pol ε.

4.3.1 Steric Interactions

The fidelity of DNA ligases relies heavily on the correct base pairing, particularly, at the 3'-OH end of a nicked DNA substrate (51, 52, 53). Mismatches are better tolerated at the 5'-end of the nick, whose orientation mainly relies on the interaction of its adenyl group with several active site residues (51, 52, 53). On the other hand, the 3'-end of the DNA does not have many
interactions with the protein and relies solely on the formation of correct Watson-Crick base pairs for proper alignment (51, 52, 53). Thus, the steric volume of the moieties at the 2’-position should not affect reactivity. Therefore, the differing substituents of each substrate located at the 2’-position likely do not contribute to the variations we observed in ligation efficiency by ligase III/XRCC1.

**4.3.2 Electrostatic Interactions**

The two fluorine atoms may decrease the electron density of the ribose 3’-OH of dFdCMP active in phosphodiester formation impeding nick closure. The 3’-OH is the atom that would act as the nucleophile in the ligase reaction in which the dFdCMP is in the primer terminus of the DNA. During these reactions, the 3’-OH is coordinated to a Mg$^{2+}$ which enhances reactivity by lowering the pK$_a$ of the oxygen so that the oxyanion can be formed in the active site and act as the nucleophile (29). The fluorine substituent will either lower the pK$_a$ of the 3’-OH to allow more oxyanion to form thereby increasing reactivity or it will decrease the negative charge on the oxygen thereby decreasing reactivity.

Our results demonstrated that ligase III/XRCC1 could not effectively ligate a dFdCMP residue at the primer terminus of a DNA substrate and facilitate nick sealing. This result indicates that the two fluorine atoms potentially could be responsible for decreasing the negative charge on the oxygen thereby explaining the observed decrease in reactivity. The single fluorine atom of 2’FdCMP did not seem to have an electron withdrawing effect on its 3’-OH. It was ligated as effectively as the natural substrate, dCMP. Also, rCTP was incorporated as efficiently as dCMP, therefore, no electrostatic interactions seem to be playing a role in its ligation.

However, this rationale does not fully explain the large differences observed for pol β and ligase III/XRCC1 in their efficiencies toward dFdC. Nor does it extend to araCMP, which is known to adopt the C2’-endo sugar pucker and can be effectively incorporated by pol β, but is poorly dealt with by ligase III/XRCC1.
4.3.3. Conformational Changes

Our results demonstrated that ligase III/XRCC1 was 300-fold worse at ligating a dFdCMP residue into a nicked DNA substrate than the natural substrate. Differences in the nucleotide conformation of dFdC may also provide insight into the delayed nick sealing of a dFdCMP residue by ligase III/XRCC1. Once incorporated into the DNA, the dFCMP residue, at the primer end of the nick site, may also adopt the C3'-endo sugar pucker conformation altering the positioning of the reactive groups necessary for phosphodiester formation, thus preventing nick sealing by ligase III/XRCC1.

As stated previously, ligase III/XRCC1 has been shown to be more sensitive and not as successful at ligating mismatches at the 3'-OH end (52). While base pairing is not altered, its inability to ligate mismatches at the 3'-OH may indicate that the ligase is sensitive to structural changes occurring at the terminal nucleotide. The ligase enzyme may not tolerate structural changes to the DNA caused by dFdCMP and araCMP thereby accounting for the large inability of ligase III/XRCC1 to deal with dFdCMP versus pol β. 2'FdCMP and rCTP probably do not influence the structural integrity of the DNA; hence, they do not alter ligation efficiency.

Additionally, T4 ligase, known to not be as specific in ligating mismatches (54), can ligate an araCMP residue. While T4 ligase also seems to be able to ligate dFdCMP; it did so slightly less efficiently.

4.4 Biological Implications

Our conclusions indicate that pol β can efficiently incorporate both araCTP and dFdCTP into a gapped substrate, but that ligation of the resulting DNA by ligase III/XRCC1 is poor. The unrepaired single strand break left in the DNA may provide a means by which these drugs can
cause cytotoxicity via the BER pathway. Oxidative damage incurred by cellular DNA is primarily repaired via BER (31, 32). Cancer cells are known to be under persistent oxidative stress compared to normal cells (33, 34), with increased production of ROS (33). Damage to DNA induced by ROS, such as oxidized cytosine residues, when repaired by the BER pathway, may provide additional opportunity for the incorporation of these drugs into the genome of cancer cells. Not only has the BER pathway been shown to be up-regulated in cells exposed to an oxidative stress inducing agent (32), several tumor types, including kidney, breast, prostate, uterus, ovary, colon, lung, stomach, and rectum have been shown to have increased expression of pol β (38).

Actually, many cells that overexpress pol β have been shown to be resistant to different chemotherapeutic agents including cisplatin (55). However, when given simultaneously with gemcitabine, the efficacy of cisplatin has been shown to be greatly improved (56, 57, 58). This fact could partially be attributed to the ability of chemotherapeutic drugs, like cisplatin, as well as carboplatin, to induce oxidative stress (39). Similarly, cytarabine has been known to increase the production of ROS (38). The oxidative stress associated with cancer and the chemotherapeutic agents used to treat it could result in damage of cytosine residues providing an increased opportunity for the incorporation of cytarabine and gemcitabine into the genome via the BER pathway leading to tumor cell death.

Our findings also provide further insight into the mechanisms by which these chemotherapeutic drugs cause tumor cell death. A more thorough evaluation of their mechanism of action will enable us to develop more effective chemotherapy strategies and to understand which patients would benefit most with the treatment. As previously mentioned, several tumor tissues have been shown to have increased expression of pol β (37), which may provide more opportunity for the incorporation of gemcitabine into the genome via the BER pathway and thus make it more cytotoxic in individuals with these types of tumors.
Additionally, several studies have shown that 30% of human tumors express a variant form of pol β (59). Depending on the variant expressed, tumor tissue may be more or less susceptible to treatment by gemcitabine. For example, the pol β deletion 208-236, identified in colorectal, breast, and lung adenocarcinomas, spans a region of the enzyme containing residues involved in contacts with the phosphate backbone of the DNA substrate (59). The deletion decreased the activity of the enzyme, as well as its ability to fill gaps in the DNA thereby reducing BER (59). These factors indicate that individuals with this variant of pol β potentially would not benefit as greatly from treatment with gemcitabine as those who possess an intact pol β.

We should also take into account that pol β does not solely participate in the BER pathway, but has several other cellular functions where it may be capable of incorporating gemcitabine into the genome and causing cytotoxicity. In addition to short patch BER, pol β functions in long patch BER (60) and has also been shown to participate in lesion bypass during DNA replication (61, 62). For example, pol β has demonstrated the ability to bypass d(GpG)-cisplatin adducts in vitro (62). As previously stated, gemcitabine improves the cytotoxicity of cisplatin when given concurrently (56, 57, 58). The ability of pol β to bypass lesions induced by cisplatin and continue DNA replication may provide yet another means by which the enzyme can incorporate gemcitabine into the DNA and cause cytotoxicity. It may also be one explanation for how gemcitabine enhances the cytotoxicity of cisplatin.

In addition to the ability of gemcitabine and cytarabine to inhibit DNA polymerases associated with DNA replication, we have demonstrated that pol β and ligase, which participate in the BER pathway, may provide another means by which gemcitabine and cytarabine inhibit DNA synthesis and exert their cytotoxic effects. By gaining a more thorough understanding of
their mechanisms of action, it should be possible to develop more effective chemotherapy strategies and gain an understanding of which patients would benefit the most from treatment.
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