Synthesis and restriction enzyme analysis of oligodeoxyribonucleotides containing the anti-cancer drug 2',2'-difluoro-2'-deoxycytidine

Frank C. Richardson, Katherine K. Richardson, Julian S. Kroin and Larry W. Hertel

Toxicology Research Laboratories, Eli Lilly and Company, Greenfield, IN 46140 and 1Cancer Research Division, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285, USA

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ABSTRACT

The anti-cancer drug 2',2'-difluoro-2'-deoxycytidine (dFdC) is internally incorporated into DNA in vitro. To determine the effects of this incorporation on DNA structure and function, the β-cyanoethyl phosphoramidite of dFdC was synthesized and oligodeoxyribonucleotides containing dFdC were made using automated solid phase DNA synthesis techniques. Extension of the coupling time was required to achieve high coupling efficiency, suggesting a significant reduction in the rate of phosphotriester formation. Insertion of dFdC 5' into the recognition sequence of restriction enzymes HpalI and KpnI reduced the rate of cutting by 4% and 14% over 60 minutes. This reduction is similar to the effects seen with arabinofuranosylcytidine (ara-C) but small compared to the reductions caused by base analogues and phosphothioates. Insertion of dFdC into the BamHI recognition sequence, but not 5' to the cut site, did not alter the rate of cutting/recognition. The presence of a single dFdC reduced the Tm's of oligomers by 2–4°C, depending on sequence and location. These results demonstrate that, once incorporated into DNA, dFdC does not greatly alter recognition between DNA and restriction enzymes; however, it does significantly alter duplex stability.

INTRODUCTION

2',2'-difluoro-2'-deoxycytidine (dFdC) (Gemcitabine) (1) is an antimetabolite that has shown efficacy in leukemia as well as solid tumor xenograph models (2,3,4) and a therapeutic response in adenocarcinomas of lung and colon (5,6). The cytotoxic action of dFdC appears to be dependent on phosphorylation by deoxycytidine kinase to dFdCTP (7). The cytotoxic action of dFdC also correlates with its incorporation into DNA (8). DfdC is also internally incorporated into DNA (8); however, the consequences of this internally incorporated dFdC on DNA structure and function, including DNA replication, have not been determined.

This paper reports the synthesis of dFdC-containing oligodeoxyribonucleotides that can be used to study the effects of internally incorporated dFdC on DNA structure and function. Using these oligomers, the effects of dFdC incorporation on restriction enzyme digestion and thermal denaturation have been examined.

MATERIALS AND METHODS

Synthesis of 5'-O-(4,4'-dimethoxytrityl)-N4-benzoyl-2'-deoxy-2'-difluorocytidine-3'-O-(2-cyanoethyl-N,N-diisopropylamino)-phosphoramidate-(dFdC phosphoramidite)

Reagents and Instruments. Pyridine (EM Science-Omnisolve) was stored over 3A molecular sieves. Commercially available benzoyl chloride 99.2% (J.T. Baker), 4,4'-dimethoxytrityl chloride 98%, 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, N,N-diisopropylethylamine 99%, chlorotrimethylsilane 98%, and anhydrous tetrahydrofuran 99.9%, (Aldrich Chem. Co. Milwaukee, Wis.) were used without further purification. All procedures were performed to the exclusion of moisture and under nitrogen atmosphere. NMR spectra were measured with a General Electric QE300 using Me4Si as an internal standard when appropriate and are reported in 6. 31P NMR were measured with a Bruker AC 250 using H3PO4 as a reference standard. Mass spectra were measured on a Varian MAT731 or V.G. ZAB-3F. Analyses were performed by MC525, Lilly Research Laboratories, Indianapolis, IN.

N4-Benzoyl-2'-deoxy-2',2'-difluorocytidine (2). To a suspension of dFdC-HCl (0.9 g) (1) in anhydrous pyridine (30 ml) at 0°C were added 2.0 ml of chlorotrimethylsilane. After 30 minutes of stirring, benzoyl chloride (1.75 ml) was added and the solution was stirred for an additional 75 minutes. The chilled reaction was quenched with H2O (6 ml) and stirred for 15 minutes. Concentrated NH4OH (7.2 ml) was added and stirred at 4°C for 30 minutes. The reaction was concentrated in vacuo at 45°C to a residue (5.1 g). The reaction was slurried in ethyl acetate (20
ml) and the insolubles (ammonium benzoate) were filtered off. The filtrate was concentrated in vacuo at 45°C to a residue (2.26 g). The residue was slurried with diethyl ether (50 ml) and the solid was filtered and dried to yield 0.94 g (83.3%) of 2. 1H NMR (300 MHz, CD3OD) δ 3.78-4.00 (m, 3H, H-4', H-5'), 4.3 (m, 1H, H-3'); 6.26 (t, 1H, H-1'), 7.38-8.0 (m, 6H, H-5' and arom H), 8.4 (d, 1H, H-6). MS m/e 367 (M+).

5'-O-(4,4'-dimethoxytrityl)-N6-benzoyl-2'-deoxy-2',2'-difluorocytidine (3). To a solution of 2 (0.56 g) in anhydrous pyridine (11 ml) was added 4-dimethylaminopyridine (0.02 g), triethylamine (0.46 ml), and 4,4'-dimethoxytritylchloride (0.922 g). To a solution of 3 (1.06 g) in anhydrous tetrahydrofuran (14 ml) were added 4-dimethylaminopyridine (0.02 g), triethylamine (0.46 ml), and 4,4'-dimethoxytritylchloride (0.922 g). After stirring overnight at ambient temperature, the reaction was concentrated in vacuo to a yellow oil (1.74 g) which was further purified by flash chromatography on silica gel, eluting with ethyl acetate-hexane (2:1) to yield 0.68 g of 3 (55%). 1H NMR (300 MHz, CDCl3) δ 3.83 (s, 6H, OCH3), 3.53-4.13 (m, 3H, H-5'), 4.48 (m, 1H, H-3'), 6.42 (m, 1H, H-1'), 6.8-7.9 (m, 19H, H-5', arom-H), 8.2 (d, 1H, H-6); MS m/e 367 (M+).

5'-O-(4,4'-dimethoxytrityl)-3'-O-(2-cyanoethyl-N,N-diisopropyl-phosphoramidite-N6-benzoyl-2'-deoxy-2',2'-difluorocytidine (4). To a solution of 3 (1.06 g) in anhydrous pyridine (11 ml) was added diisopropylethylamine (1.1 ml) followed by 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (0.71 ml) over 5 minutes. After 3 hours, the precipitate (diisopropylethylamine-HCl) was filtered and washed five times with water. The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was dissolved in argon-saturated ethyl acetate and washed two times with minimal heating to a yellow oil (1.74 g) which was further purified by flash chromatography on silica gel, eluting with ethyl acetate-hexane (2:1) to yield 0.68 g of 3 (55%). 1H NMR (300 MHz, CDCl3) δ 3.83 (s, 6H, OCH3), 3.53-4.13 (m, 3H, H-5'), 4.48 (m, 1H, H-3'), 6.42 (m, 1H, H-1'), 6.8-7.9 (m, 19H, H-5', arom-H), 8.2 (d, 1H, H-6); MS m/e 367 (M+).

Figure 1. Synthetic scheme for dFdC phosphoramidite.

Figure 2. Oligomeric duplexes used in restriction enzyme digestion or thermal denaturation experiments (F = dFdC). Cleavage sites for restriction endonucleases are indicated.
described (12). Briefly, 6.5 pmol of duplex oligomer were 
kinased for 2 hours in 1 x kinase buffer (70 mM Tris-HCl, pH 
7.6, 10 mM MgCl₂, 0.1 mM KCl, 5 mM dithiothreitol), 20 μCi 
{³²P}-ATP (spec. act. 3000 Ci/mmol; Amersham Corp., 
Arlington Heights, IL), 1 mM spermidine, 1.6 U T4 kinase (New 
England Biolabs, Beverly, MA) and H₂O to a final volume of 
20 μl. Labelled duplexes were separated from unincorporated 
{³²P}-ATP using G-25 spin columns (Boehringer Mannheim 
Corp., Indianapolis, IN).

Approximately, 0.33 pmol (30,000 cpm) of labelled duplex 
E or F was digested for 60 min at 37 °C with 1 U of BamHI 
(Boehringer Mannheim Corp.) using 1 x Stratagene Universal 
buffer (Stratagene, La Jolla, CA) in a total volume of 10 μl. 
Approximately 0.33 pmol of labelled duplex A or B (Figure 2) 
was digested in 10 μl with 1 U of either Kpnl, (New England 
Biolabs), MspI, (Pharmacia, Piscataway, NJ) or HpaII (Gibco/ 
BRL, Gaithersburg, MD) using the appropriate dilution of 
Stratagene Universal buffer. Digestions were allowed to proceed 
for 0.5, 1, 3, 10, 30, and 60 min. All digestion reactions were 
stopped by the addition of 5 μl stop solution (Gibco/BRL).

Prior to electrophoresis, samples were denatured by heating 
to 100°C for three minutes. To separate the restriction fragments 
from the full-length oligomer, 5 μl of each sample were 
electrophoresed on a 20% denaturing polyacrylamide gel as 
described (12). The gels were subsequently autoradiographed for 
4–8 hours (Kodak X-Omat, Sigma Chem. Co., St. Louis, MO). 
Radiolabelled restriction fragments as well as the uncut oligomeric 
duplex were separately excised from the gel using the 
autoradiographs as overlays, and the amount of radioactivity in 
each band was determined by liquid scintillation counting. The 
cpm was used as an index of the amount of DNA present. 
Radioactivity associated with each full length oligomer was 
divided by the total cpm in the respective lane (which included 
full-length oligomer and the two restriction fragments) and was 
expressed as a percentage. Percentage changes were used to 
determine rates of enzyme digestion in control and dFdC 
containing oligomers.

Data from digestions using either Kpnl, MspI or HpaII were 
analyzed using a two-way ANOVA (SAS, SAS Institute, Cary, 
NC). A non-paired Student's-t test was used in analysis of data 
from experiments using BamHI.

**Thermal Denaturation**

Duplexes that were used in restriction enzyme studies were 
analyzed to determine the effect of dFdC on Tₘ following a 
modification of previously reported procedures (13). The 
oligomeric duplexes listed in Figure 2 were made by annealing 
1200 pmol of respective complementary oligomers in 1 ml of 
50 mM NaPO₄, 25 mM NaCl, pH 7.2 resulting in a sodium 
concentration of 75 mM. The Tₘ was established by 
determining the maximum change in absorbance at 260 nm as 
a function of increasing temperature (1 °C/min) using a Beckman 
DU-8 Tₘ Analysis System (Beckman Instruments, Inc. 
Fullerton, CA). The effect of dFdC on thermal stability was 
determined by comparing the Tₘ and the melting curve profiles 
of the oligomeric duplexes containing dFdC to the corresponding 
control duplexes.

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**Table 1**

| Base Composition of Oligomers Used in Restriction Enzyme and 
| Thermal Denaturation Experiments
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<td>Base</td>
<td>Oligo 1*</td>
<td>Oligo 2*</td>
<td>Oligo 3*</td>
<td>Oligo 4*</td>
<td>Oligo 5*</td>
<td>Oligo 6*</td>
<td>Oligo 7*</td>
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</tr>
<tr>
<td>A</td>
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<td>25.5/26.3</td>
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<td>10.3/10.0</td>
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</tr>
<tr>
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<td>33.7/31.6</td>
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<td>T</td>
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<td>21.4/20.0</td>
<td>30.8/35.0</td>
</tr>
<tr>
<td>dFdC</td>
<td>0/0</td>
<td>5.6/5.3</td>
<td>0/0</td>
<td>6.4/5.2</td>
<td>0/0</td>
<td>5.3/5.0</td>
<td>0/0</td>
</tr>
</tbody>
</table>

*Oligomer 1 = 5'-GTTCTAGGTATCCCGGACGCTG-3'; Oligo 2 = 5'-GTTCTAGGTATACCGGACGCTG-3'; 
Oligo 3 = 5'-CACGCTCCGTATCTGACGACT-3'; Oligo 4 = 5'-CACGCTCCGTATCTGACGACT-3'; 
Oligo 5 = 5'-GAATGGATCTATTTGATGAC-3'; Oligo 6 = 5'-GAATGGATCTATTTGATGAC-3'; 
Oligo 7 = 5'-GATCTCATTAGGATCCATTC-3'.
RESULTS

Synthesis of dFdC phosphoramidite

The β-anomer of 2'-2'-difluoro-2'-deoxycytidine.HCl (99.3% purity) was converted to the bis blocked 5'-O-(4,4'-dimethoxy-trityl)-N4-benzoyl dFdC in a two step procedure (14) (Figure 1). The crude N4-benzoylated dFdC was purified by selective solubility using ethyl acetate and ether in a two step procedure (85.3% yield). dFdC phosphoramidite was prepared using fresh phosphoramidite reagent (80% yield) following methods previously reported (15,16) with modifications as described in Materials and Methods. The crude product was purified by flash chromatography on silica gel to obtain dFdC phosphoramidite as a 1.3:1 mixture of diastereomers. The diastereomers were separable by TLC (Rf = 0.35) using ethyl acetate-hexane 2:1, and HPLC with retention times of 26.19 and 26.83 minutes on a 100x4.6 mm, 5 μm, Astec C18 column using a gradient of 70% A (1.0 M triethylammonium acetate, pH = 7.0) to 70% B (CH3CN) over 20 min with a flow rate of 1.2 ml/min. Similar results could be obtained with solution A = H2O. The 1.3:1 ratio of diastereomers was verified by integration in both proton and 31P NMR.

Synthesis of dFdC Containing Oligomers

Oligomers containing dFdC at preselected sites were successfully synthesized. Standard procedures for synthesis with normal nucleotides had to be altered by extending the coupling time from 30 to 900 seconds during the addition of dFdC. Coupling efficiencies rose from < 10% with a 30 second coupling time to >95% when coupling times were extended to 900 seconds (data not shown). Full-length dFdC containing oligomers were obtained in high purity using simple trityl-on OPC™ clean-up. Base composition analysis of selected oligomers demonstrated that both dFdC and the normal nucleosides were in expected proportions (Figure 3, Table 1). Trityl-off HPLC (data not shown), as well as gel electrophoresis of 32P end-labelled oligomers used in the enzyme digestion studies (Figure 4, control lanes) demonstrated that full-length oligomers containing dFdC could be recovered following NH4OH deprotection and OPC™ clean-up.

Enzymatic Digestion Using Kpnl, Mspl, or HpalII

A representative autoradiograph of oligomeric duplexes containing dFdC digested by Kpnl, Mspl and HpalII is presented in Figure 5. All enzymes digested both normal and dFdC-containing duplexes. The sizes of the fragments generated were consistent with sizes expected as indicated in Figure 2. Figure 4
demonstrates that dFdC does not greatly alter recognition or cutting by these restriction enzymes. Since a more detailed characterization of these enzyme digestions was desired, time-course studies were conducted and are presented in Figure 5. These studies demonstrated that the presence of dFdC in the duplex significantly \( p < 0.01 \) reduced the rate of digestion by Kpnl by approximately 10% and by HpaII by approximately 4%. Although a slower rate of digestion was also apparent with Mspl, the difference between the dFdC containing duplex and the control was not significant \( p < 0.06 \). Therefore, these results demonstrate that dFdC does reduce the rate of digestion by two of these three restriction enzymes.

Enzymatic Digestion Using BamHI
A representative autoradiograph of the digestion of duplex \( F \) containing dFdC with BamHI is presented in Figure 6. This autoradiograph demonstrates that dFdC does not significantly alter digestion by BamHI as compared to a G·T mismatch. BamHI generated 5-base and 11-base fragments as expected (Figure 6). DfDc had no effect on the rate of BamHI digestion over the 60 minutes (Table 2) examined. Examination at earlier time points also revealed no difference in rates of digestion between the two duplexes (data not shown).

Thermal Denaturation
Thermal melting curve profiles and \( T_m \)'s are presented in Figure 7. Incorporation of one dFdC reduced the \( T_m \)'s of all duplexes. The net decrease varied from 2–4°C and appeared to be dependent on the location of the dFdC. Incorporation of dFdC into both oligomers reduced the \( T_m \) by 8°C, indicating that the effect on \( T_m \) may be more than additive.

**DISCUSSION**

The purpose of these experiments was to synthesize oligomers containing dFdC at unique locations in a DNA sequence and to begin characterization of the biological and physical effects of dFdC on DNA. Synthesis of the dFdC phosphoramidite precursor was achieved using procedures developed for the synthesis of the phosphoramidite of 2′-deoxycytidine. Using automated DNA synthesis procedures it was necessary to increase the coupling time approximately thirty-fold when coupling the dFdC phosphoramidite to achieve a coupling efficiency that was equivalent to dC phosphoramidite at equimolar concentrations. The extension of the coupling time indicated that the rate of phosphite triester bond formation was slower than the rate of bond formation during addition of normal nucleosides. It seems probable that the 2′-fluorines are responsible for altering the rate of phosphotriester formation. The mechanism for this alteration is not clear. Direct steric interactions seem unlikely based on the similarity of the van der Waals radii of hydrogen (1.2 Å) and fluorine (1.35 Å). A mechanism based on electronic interactions seems more plausible. 2′-fluorine substitutions have been shown to change deoxyribose conformation, a change based on electronegativity rather than size (17). It remains to be determined if the effect of dFdC on \( T_m \) is due to loss of electron density at the 5′ end of double-stranded DNA.

<table>
<thead>
<tr>
<th>Oligomeric Duplex*</th>
<th>% Decrease in Full Length Duplex</th>
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<tr>
<td>( E )</td>
<td>53</td>
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<tr>
<td>( F )</td>
<td>53</td>
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* Study was run in triplicate. Difference between % at \( t = 0 \) and \( t = 60 \) minutes was determined. See Figure 1 for duplex sequence.

Figure 6. Restriction digestion of dFdC oligomeric duplex \( B \) or normal oligomeric duplex \( A \) (Figure 1). Each data point represents the average of three independent experiments. Two-way ANOVA demonstrated a significant difference between dFdC and normal duplex oligomers when digested with HpaII or Kpnl.

Figure 7. Thermal melting curves for oligomeric duplexes listed in Figure 1. Graph A - = duplex \( E \), - = duplex \( F \), Graph B - = duplex \( A \), - = duplex \( B \), - = duplex \( C \), - = duplex \( D \). Absorbance at 260 nm was normalized to absorbance at 30°C = 0% and absorbance at 80°C = 100%.

Table 2

<table>
<thead>
<tr>
<th>BamHI Digestion of Normal and dFdC-Containing Oligomeric Duplexes</th>
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<tr>
<td>Oligomeric Duplex*</td>
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<tr>
<td>( E )</td>
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<td>( F )</td>
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determined whether conformational changes affect the rate of phosphotriester bond formation and whether an electronic effect extends to the phosphorus. Although not necessarily mechanistically related, an increase in the coupling time to 300–720 seconds is also necessary for successful chemical RNA synthesis using 2'-O-silyl-3'-O-phosphoramidites (18). Once the phosphotriester bond and the resultant phosphodiester bond were formed, they appeared to be as resistant to chemical cleavage as equivalent bonds between normal nucleosides. In addition, base composition analysis indicated that snake venom phosphodiesterase (in excess) cleaved the phosphodiester bond 5' to dFdC as readily as the other phosphodiester bonds in the oligomer. This is not surprising since studies with ara-C have demonstrated similar results (19).

The construction of dFdC-containing oligomers at unique sites has allowed us to examine the effects of dFdC on restriction enzyme digestion. Previous work has demonstrated that base alterations in the recognition sequence including chemical ethylation and methylation (12,20), natural methylation of deoxycytosine (21,22), and the incorporation of nucleoside analogues can abolish restriction enzyme recognition in an enzyme-dependent manner (22,23) by altering the shape of the major groove. Furthermore, the presence of a phosphorylato linkage in a restriction site significantly reduces the rate of cleavage when located 5' to the cleavage site and results in the selective cleavage of the unmodified strand (24). These reports demonstrated that chemical modification of DNA in two distinct locations can alter restriction enzyme digestion by two separate mechanisms. Experiments described here demonstrated that modification of the sugar causes a small but significant decrease in restriction enzyme digestion; but, only when the modification was 5' to the cleavage site. The effects of dFdC on enzyme recognition have not been studied.

Thermal denaturation of dFdC-containing duplexes were conducted to determine the effects of dFdC on duplex stability. Previous studies have demonstrated that 2 ara-C molecules in a self-complementary 13-base oligonucleotide reduced $T_m$ by only 4°C (19), whereas a simple C:T mismatch reduced the $T_m$ of an A:T rich 9-base oligonucleotide by 24°C (25). These studies demonstrated that one dFdC decreased the $T_m$ 2–4°C and that placement of 2-dFdC’s in opposite strands decreased the $T_m$ by 10°C, as did the simultaneous placement of dFdT and dFc in the same strand (data not shown). Thus, although direct comparisons could not be done, dFdC appears to be more destabilizing than ara-C and much less destabilizing than a base pair mismatch.

Diffuorodeoxycytidine is an effective antimetabolite for the treatment of several forms of cancer. While the effects of dFdC-TP insertion on DNA synthesis have been evaluated (8), the effects of an internally incorporated dFdC on DNA structure and function had not been determined. These studies showed that an internally incorporated dFdC induces a small but significant change in duplex stability and a slight decrease in the rate of endonuclease digestion. It remains to be determined if dFdC, like ara-C (26,27), also alters oligomeric conformation and/or shape.

ACKNOWLEDGEMENTS

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