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

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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 HpaII 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).

Difluorodeoxycytitine is a strong inhibitor of DNA synthesis (2,8), a process most likely critical to the cytotoxic action of the drug. DFdCTP may inhibit DNA synthesis by inhibiting the activity of ribonucleotide reductase causing a lowering of intracellular nucleotide pool concentrations (9). Primer-template experiments using DNA pol α and ε showed that insertions of dFdCTP may also inhibit DNA synthesis (8). Specifically, insertion of dFdCTP results in pausing of both polymerase α and ε one base following its insertion 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)-N_4-benzoyl-2′-deoxy-2′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 Me_4Si as an internal standard when appropriate and are reported in δ. ^{31}P NMR were measured with a Bruker AC 250 using H_3PO_4 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.

N_4-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 H_2O (6 ml) and stirred for 15 minutes. Concentrated NH_4OH (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* (85.3%) of **2.** ^{1}H NMR (300 MHz, CH_3OD) δ 3.78−4.0 (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)-N_4-benzoyl-2′deoxy-2′,2′-difluorocytidine (3). To a solution of **2** (0.56 g) in anhydrous pyridine (11 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* at 40°C. The residue was mixed with toluene and concentrated *in vacuo* at 40°C. This procedure was repeated one time. The residue was dissolved in ethyl acetate and washed five times with water. The organic layer was dried over anhydrous sodium sulfate and concentrated *in vacuo* to a foam (1.47 g). Further purification was accomplished using flash chromatography on silica gel, eluting with ethyl acetate-hexane (2−1) to yield 0.68 g of **3** (55%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.83 (s, 6H, OCH_3), 3.53−4.13 (m, 3H, H-4′, 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* 670 (M^+).

5′-O-(4,4′-dimethoxytrityl)-3′-O-(2-cyanoethyl-N,N-diisopropyl-phosphoramidite-N_4-benzoyl-2′-deoxy-2′,2′-difluorocytidine (4). To a solution of **3** (1.06 g) in anhydrous tetrahydrofuran (14 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 the filtrate was concentrated *in vacuo* at ambient temperature to a residue. The residue was dissolved in argon-saturated ethyl acetate and washed two times in a cold solution of saturated sodium bicarbonate. The organic layer was dried over anhydrous sodium sulfate and concentrated *in vacuo* with minimal heating to a yellow oil (1.74 g) which was further purified by flash chromatography on silica gel using ethyl acetate-hexane (2−1) as the eluent to obtain 1.1 g (80%) of the phosphoramidite **4.** ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.05−1.33 (m, 12H, $4CH_3$), 2.49 and 2.62 (two t, 2H, CH_2CN), 3.78 (s, 6H, 2-OCH_3), 3.44−4.23 (m, 7H, H-4′, H-5′, 2NCH, $POCH_2$), 4.52−4.82 (m, 1H, H-3′), 6.24−6.34 (m, 1H, H-1′) 6.96−7.98 (m, 19H, H-5, arom-H), 8.15 and 8.21 (two d, 1H, H-6); ^{31}P NMR (CD_3CN) δ 153.25 and 154.25 (2×t, $J \approx 7$ Hz); MS *m/e* 870 (M^+).

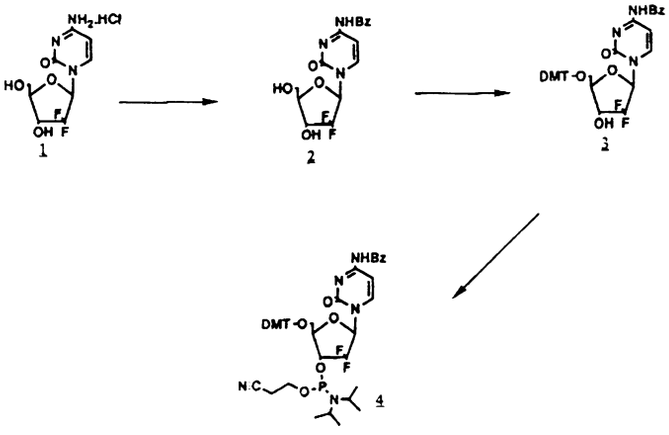

Figure 1. Synthetic scheme for dFdC phosphoramidite.

Syntheses of Oligonucleotides Containing dFdC at a Predetermined Site

The dFdC-2-cyanoethyl-phosphoramidite **4** was dissolved in anhydrous acetonitrile to a concentration of 0.1 g/ml and subsequently filtered through a 0.2 μm nylon membrane into an amber colored vial compatible with the ABI Model 381A DNA synthesizer. Standard 0.2 μM trityl-on syntheses were performed using a Model 381A DNA synthesis protocol with one modification. The coupling time was extended from 30 to 900 seconds during the addition of the dFdC phosphoramidite. The coupling efficiency was determined from the trityl yield for each coupling step (11). Full-length trityl-on oligomers were purified using OPC™ columns (Applied Biosystems, Foster City, CA). Base composition analysis (11) and either gel electrophoresis of ^{32}P-labelled oligomer (10) or trityl-off HPLC (10) were used to evaluate the purity of the oligomers. Oligomers synthesized using this procedure are listed in Figure 2. These oligomers were constructed to contain recognition sequences for the restriction endonucleases KpnI, BamHI, HpaII, or MspI and are listed in Figure 2.

Restriction Enzyme Digestion

Oligomeric duplexes listed in Figure 2 were formed by mixing complementary oligomers (100 pmol each) in 0.5 ml of distilled deionized water, placing in boiling water and allowing to cool overnight. Ten pmol of duplex oligomer was then end-labelled with phosphorus-32 (^{32}P) using methods reported in the DNA Synthesis Users Manual (10) with modifications previously

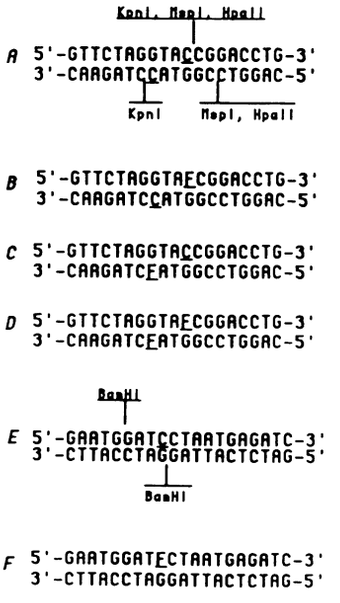

Figure 2. Oligomeric duplexes used in restriction enzyme digestion or thermal denaturation experiments (E = dFdC). Cleavage sites for restriction endonucleases are indicated.

described (12). Briefly, 6.5 pmol of duplex oligomer were kinased for 2 hours in 1×kinase buffer (70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.1 mM KCl, 5 mM dithiothreitol), 20 μCi [^{32}P]-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 H_2O to a final volume of 20 μl. Labelled duplexes were separated from unincorporated [^{32}P]-ATP using G-25 spin columns (Boerhinger 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 (Boerhinger Mannheim Corp.) using 1×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 KpnI, (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) (resulting in a final volume of 15 μl) followed by placement in a dry ice-isopropanol bath.

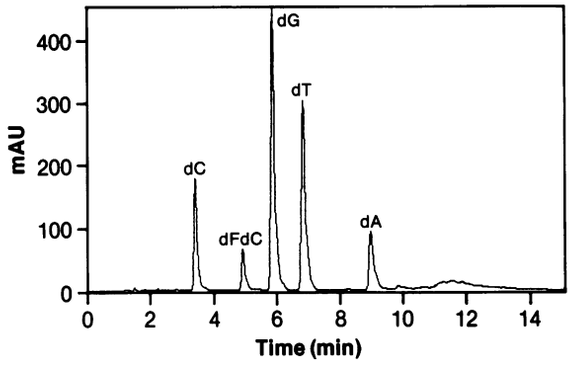

Figure 3. HPLC Chromatograph of enzyme digest of dFdC-containing oligomer. (HPLC conditions as described: Supelco 5 μm RP-C_{18}, 4 mm×25 cm; A = 50 mM $NaPO_4$, 10% MeOH, pH 6.5, B = 90/10 MeOH/H_2O; gradient = 90/10 to 80/20 A/B over 20 minutes; flow rate = 1.8 ml/min; monitor at 280 nm.)

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 KpnI, 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 T_m 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 $NaPO_4$, 25 mM NaCl, pH 7.2 resulting in a sodium concentration of 75 mM. The T_m 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 T_m Analysis System (Beckman Instruments, Inc. Fullerton, CA). The effect of dFdC on thermal stability was determined by comparing the T_m and the melting curve profiles of the oligomeric duplexes containing dFdC to the corresponding control duplexes.

Table 1

Base Composition of Oligomers Used in Restriction Enzyme and Thermal Denaturation Experiments

	Observed/expected percentage base composition (%/%)						
Base	Oligo 1*	Oligo 2*	Oligo 3*	Oligo 4*	Oligo 5*	Oligo 6*	Oligo 7*
A	15.8/15.7	15.3/15.7	25.5/26.3	24.4/26.3	37.0/35.0	36.1/35.0	26.6/25.0
C	26.4/26.3	20.6/21.0	32.4/31.6	28.3/26.3	15.6/15.0	10.3/10.0	26.0/25.0
G	33.4/31.6	33.7/31.6	28.1/26.3	27.2/26.3	25.7/25.0	27.2/26.3	16.5/15.0
T	24.3/26.3	24.7/26.3	14.0/15.7	13.6/15.7	21.5/25.0	21.4/20.0	30.8/35.0
dFdC	0/0	5.6/5.3	0/0	6.4/5.2	0/0	5.3/5.0	0/0

*Oligomer 1 = 5'-GTTCTAGGTACCGGACCTG-3', Oligo 2 = 5'-GTTCTAGGTAECGGACCTG-3', Oligo 3 = 5'-CAGGTCCGGTACCTAGAAC-3', Oligo 4 = 5'-CAGGTECGGTACCTAGAAC-3', Oligo 5 = 5'-GAATGGATCCTATTGAGATC-3', Oligo 6 = 5'-GAATGGATECTATTGAGATC-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′-dimethoxytrityl)-N_4-benzoyl dFdC in a two step procedure (14) (Figure 1). The crude N_4-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 100×4.6 mm, 5 μm, Astec C_{18} column using a gradient of 70% A (1.0 M triethylammonium acetate, pH = 7.0) to 70% B (CH_3CN) over 20 min with a flow rate of 1.2 ml/min. Similar results could be obtained with solution A = H_2O. The 1.3:1 ratio of diastereomers was verified by integration in both proton and ^{31}P NMR.

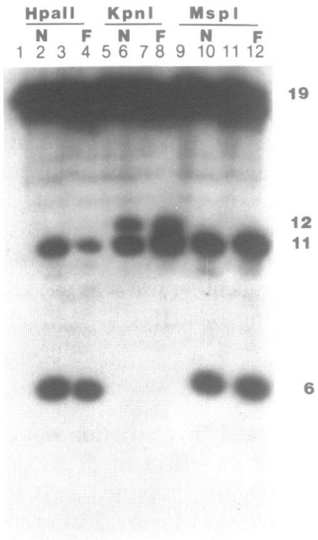

Figure 4. Representative autoradiograph of restriction enzyme digestions of oligomeric duplexes *A* & *B* (Figure 2) by HpaII, KpnI, or MspI. Odd numbered lanes contain either normal (*A*) or dFdC-containing (*B*) duplexes without enzyme. Even numbered lanes contain indicated enzyme and either dFdC-containing (F) or normal (N) duplexes. (For radioactive quantitation gels were run with each sample lane separated by two-empty lanes to prevent cross contamination of radioactive bands.)

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 ^{32}P end-labelled oligomers used in the enzyme digestion studies (Figure 4, control lanes) demonstrated that full-length oligomers containing dFdC could be recovered following NH_4OH deprotection and OPC™ clean-up.

Enzymatic Digestion Using KpnI, MspI, or HpaII

A representative autoradiograph of oligomeric duplexes containing dFdC digested by KpnI, MspI and HpaII is presented in Figure 4. 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

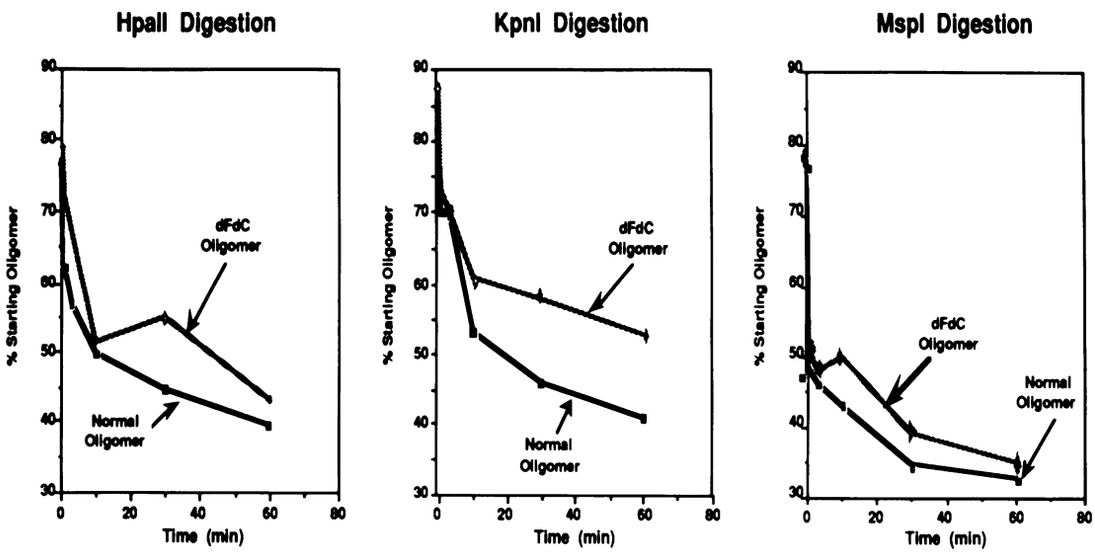

Figure 5. Representative autoradiograph of BamHI or control (no enzyme) restriction digestion: lane 1 = oligomeric duplex *E* ; lane 2 = oligomer duplex *F* ; lane 3 = an oligomeric duplex containing a G·T mismatch in the restriction recognition sequence.

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 KpnI by approximately 10% and by HpaII by approximately 4%. Although a slower rate of digestion was also apparent with MspI, 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).

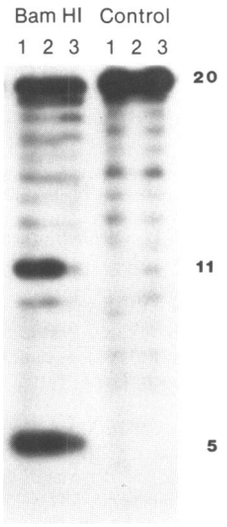

Figure 6. Restriction digestion of dFdC oligomeric duplex *B* or normal oligomeric duplex *A* (Figure 1). Each data point represents the average of three independent experiments. Two-way ANOVA demonstrated a significant difference between dFdC and normal duplex oligomers when digested with HpaII or KpnI.

Table 2

BamHI Digestion of Normal and dFdC-Containing Oligomeric Duplexes

Oligomeric Duplex*	% Decrease in Full Length Duplex
E	53
F	53

* Study was run in triplicate. Difference between % at $t = 0$ and $t = 60$ minutes was determined. See Figure 1 for duplex sequence.

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 Tm 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

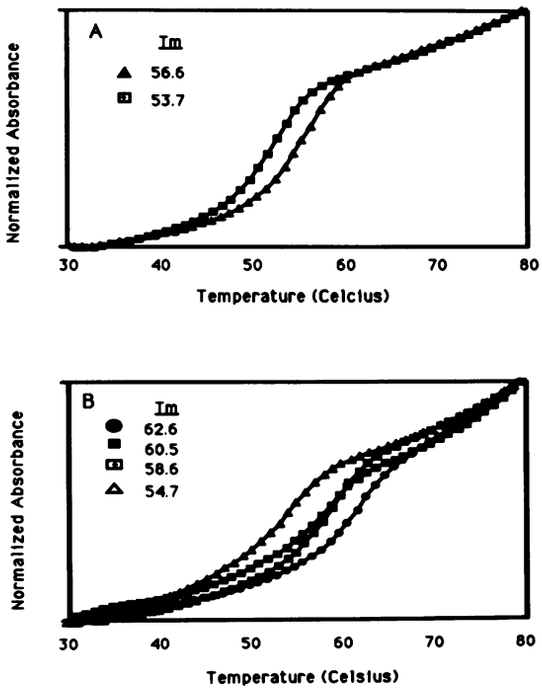

Figure 7. Thermal melting curves for oligomeric duplexes listed in Figure 1. Graph A - - = duplex *E*, - - = duplex *F*, Graph B - - = duplex *A*, - - duplex *B*, - - = duplex *C*, - - = duplex *D*. Absorbance at 260 nm was normalized to absorbance at 30°C = 0% and absorbance at 80°C = 100%.

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 surprizing 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 phosphothioate 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 cleavageThe 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 a total of 8°C, as did the simultaneous placement of dFdT and dFdC in the same strand (data not shown). Thus, although direct comparisons could not be done, dFdC appears to be more destablizing than ara-C and much less destabilizing than a base pair mismatch.

Difluorodeoxycytidine 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.

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