Cytarabine-induced destabilization of a model Okazaki fragment

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ABSTRACT

Cytarabine is a potent anticancer drug that interferes with elongation of the lagging strand at the replication fork during DNA synthesis. The effects of cytarabine substitution on the structural and thermodynamic properties of a model Okazaki fragment were investigated using UV hyperchromicity and ¹H NMR spectroscopy to determine how cytarabine alters the physicochemical properties of Okazaki fragments that are intermediates during DNA replication. Two model Okazaki fragments were prepared corresponding to a primary initiation site for DNA replication in the SV40 viral genome. One model Okazaki fragment consisted of five ribo- and seven deoxyribonucleotides on the hybrid strand, together with its complementary (DNA) strand. The second model Okazaki fragment was identical to the first with the exception of cytarabine substitution for deoxycytidine at the third DNA nucleotide of the hybrid strand. Thermodynamic parameters for the duplex to single strand transition for each model Okazaki fragment were calculated from the concentration dependence of the *T_m* at 260 nm. Cytarabine significantly decreased the stability of this model Okazaki fragment, decreasing the melting temperature from 46.8 to 42.4°C at a concentration of 1.33×10^{-5} M. The free energy for the duplex to single strand transition was 1.2 kcal/mol less favorable for the cytarabine-substituted Okazaki fragment relative to the control at 37°C. Analysis of the temperature dependence of the imino ¹H resonances for the two duplexes demonstrated that cytarabine specifically destabilized the DNA:DNA duplex portion of the model Okazaki fragment. These results are consistent with inhibition of lagging strand DNA synthesis by cytarabine substitution resulting from destabilization of the DNA:DNA duplex portion of Okazaki fragments *in vivo*.

INTRODUCTION

Frequency of nuclear DNA replication concomitant with cell division is one of the principal distinguishing characteristics between normal and malignant cells (1). For this reason DNA structures that

occur only during the course of DNA replication, such as Okazaki fragments, provide appealing targets for the design of anticancer drugs (2). Interference with the machinery required for DNA replication is, in fact, the mechanistic basis for several anticancer agents that are currently widely used for the treatment of numerous malignancies. Cytarabine (1-β-D-arabinofuranosylcytosine; Fig. 1), for instance, is an analog of deoxycytidine that is effective in the treatment of leukemia as a consequence of its facile uptake by transformed cells and its subsequent metabolism to the nucleoside triphosphate form that inhibits elongation of the lagging strand during DNA replication (3). Arabinosyl nucleosides are among the most potent nucleoside analogs available for the treatment of viral infections and cancer.

Synthesis of the lagging DNA strand at the replication fork occurs discontinuously and requires unwinding of the parental DNA strands, *de novo* synthesis of an RNA primer and elongation of the nascent DNA chain (4; Fig. 1). Completion of the replication process requires excision of the RNA primer by RNase H and ligation of the DNA chains. Okazaki fragments occur as intermediates during replication of the lagging strand and contain the RNA primer and the nascently synthesized DNA associated with the lagging DNA strand through complementary base pairing. Cytarabine substitution for deoxycytidine inhibits extension of Okazaki fragments by DNA polymerase α. Cytarabine, however, does not immediately terminate chain elongation (5–7). Analysis of the physical properties of nascent DNA from cells exposed to cytarabine revealed a reduction in the percentage of fragments between 0.5 and 40 kb long and an increased percentage of fragments that were ~100 nt long (8). Enzymatic digestion of nascent DNA obtained from pH-step alkaline elution of intact cells suggests that cytarabine inhibits chain elongation and leads to the production of very small nascent DNA fragments, although it is not usually the terminal nucleotide in these short fragments (8).

Understanding how cytarabine interferes with elongation of the lagging strand during DNA synthesis requires information about the structures and stabilities of model Okazaki fragments with and without cytarabine substitution. Relatively few structures of Okazaki fragments have been reported and virtually no physicochemical data concerning how cytarabine substitution affects Okazaki fragment structure and stability have been previously reported (9,10). We report here that, based on UV hyperchromicity and NMR spectroscopic data, cytarabine destabilizes a model

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Figure 1. Depiction of Okazaki fragments that occur as intermediates during replication of DNA. Cytarabine (top) is misincorporated for deoxycytidine during replication. Arabinosyl nucleosides such as cytarabine are analogs of 2'-deoxynucleosides with the 2'-hydroxyl *trans* to the 3'-hydroxyl of the sugar. (Bottom) Sequences for the model Okazaki fragments considered in the present study. Deoxynucleotides are written in upper case and ribonucleotides in lower case in the figure and throughout the text. The site of cytarabine substitution is designated by an X in the figure and is referred to as aC20 in the text.

Okazaki fragment having a sequence derived from the genome of simian virus 40 (11). Replication of this viral genome has been well characterized previously and the sites of initiation for polymerase α 2 and polymerase α primase are known (12,13). The results obtained are consistent with inhibition of lagging strand DNA synthesis by cytarabine substitution resulting from destabilization of the DNA:DNA duplex portion of Okazaki fragments *in vivo*.

MATERIALS AND METHODS

Synthesis of model Okazaki fragments

The RNA:DNA hybrid strand of the model Okazaki fragment consists of five ribo- and seven deoxyribonucleotides. The sequence for the model Okazaki fragment consisting only of native nucleosides is shown in Figure 1 and is referred to as [OKA] throughout the text. Synthesis of the model Okazaki fragment substituted with cytarabine (Fig. 1, referred to as [ARAC] throughout the text) was prepared using similar methodology. 1- β -D-Arabinofuranosylcytosine (cytarabine) was purchased from Sigma and was converted to a suitably protected 3'-O-phosphoramidite for incorporation into the RNA:DNA hybrid strand of [ARAC] using methods similar to those previously described (14). Briefly, the approach involves bis-protection of the 3'-OH and 5'-OH groups with a disiloxane reagent followed by acetylation at the 2'-OH and N4 amino group. The 3'-OH and 5'-OH groups were then deprotected with tetrabutylammonium fluoride. Tritylation with the bulky reagent

4,4'-dimethoxytrityl chloride occurred selectively at the 5'-OH and was followed by phosphoramidation at the 3'-OH. The resulting 5'-O-dimethoxytrityl 3'-O-phosphoramidite was purified by column chromatography, characterized by ^1H and ^{31}P NMR (5:4 ratio of diastereomers 155.6:155.1 referenced to 85% H_3PO_4 at 0.0 p.p.m.) and FAB MS ($M+1 = 830.3$) and incorporated in place of one deoxycytidine (aC20; Fig. 1) during chemical synthesis of the RNA:DNA hybrid strand of [ARAC].

Automated solid phase synthesis was performed on a Perkin-Elmer/Applied Biosystems Division 394 DNA synthesizer with eight base positions. Phosphoramidites were used as 0.1 M acetonitrile solutions with the araC, rA and rG reagents installed on the extra base positions. The entire synthesis was performed using the RNA 10 micromole scale synthesis cycle with trityl-off/manual ending. The CPG support was removed from the synthesis column, transferred to a glass screw-capped tube, and anhydrous methanol (3–4 ml) was added. The solution was saturated with ammonia, sealed and heated overnight at 50°C. The methanol solution was carefully removed from the support and evaporated to dryness. The residue was redissolved in neat triethylamine trihydrofluoride (750 ml) and left to stir overnight at room temperature. Water (2 ml) was added to dissolve the white suspension and the solution was evaporated to a thick oil (~100 ml). The oil was redissolved in water and aliquots (~250 A_{260} units) were purified by anion exchange HPLC using a Waters DEAE-5PW column (22.5 $\times$ 150 mm) and an aqueous sodium perchlorate gradient. Product-containing fractions were pooled, concentrated by evaporation and desalted on Sephadex G-25.

UV hyperchromicity studies

Thermodynamic analyses for cytarabine-substituted [ARAC] and control Okazaki fragments [OKA] were conducted as previously described using a CARY-1 UV spectrophotometer (15,16). Six melting curves were acquired for each of eight concentrations (10^{-4} – 10^{-6} M) for both samples. The buffer used consisted of 0.1 M NaCl and 2 mM sodium cacodylate, pH 7.3. All the initial and final absorbance values were between 0.25 and 2.2 OD. Six measurements were made for each sample at each concentration. Numerical values for the thermodynamic parameters ΔG° , ΔH° and ΔS° associated with the duplex to single strand transition for both the model Okazaki fragments were obtained using the software written by Petersheim and Turner (17). Melting temperatures, T_m , were calculated from the first derivative of the absorbance versus temperature data for each model Okazaki fragment at each concentration. Numerical values for ΔH° and ΔS° were obtained from plots of $1/T_m$ versus $\ln(c_T/4)$, with regression yielding correlation coefficients in excess of 0.99 (18).

NMR spectroscopy

All NMR experiments were performed using a Varian UNITY 500 NMR spectrometer equipped with a 3 mm $^1\text{H}\{^{13}\text{C},^{31}\text{P}\}$ PFG probe. 1D ^1H NMR spectra in 90% H_2O were obtained at 26, 29, 32, 35 and 38°C using a 1–3–3–1 binomial pulse for water suppression (19,20). 2D NOESY spectra in 90% H_2O solution were acquired using a 1–1 echo pulse sequence for water suppression. All spectra in H_2O were acquired with an 11 kHz spectral window centered about the ^1HDO resonance. NOESY spectra in D_2O were acquired for mixing times of 100, 150 and 200 ms using the standard three pulse sequence with States'

Figure 2. Plots of the temperature dependence of the melting temperatures ($1/T_m$, in $K \times 10^3$) for [ARAC] (top) and [OKA] (bottom). The T_m for [OKA] is 46.8°C, while the T_m for [ARAC] is 42.4°C, both at a concentration of 1.33×10^{-5} M. Correlation coefficients for regression of the data exceeded 0.99 for both [OKA] and [ARAC].

method of phase cycling (21). Four hundred free induction decays (FID), 16 scans each, with alternating block acquisition, were collected in the t_1 dimension. 2K data points over a spectral width of 5000 Hz were collected in the t_2 dimension with the carrier frequency set at the ^1HDO resonance. A relaxation delay of 10 s was included between scans to allow adequate relaxation for cross-peak quantitation. The 2D NMR data were apodized in both dimensions using Gaussian filter functions and were extended using linear prediction in the t_1 dimension to a final matrix size of $2k \times 2k$ points using VNMR 5.3B from Varian. Transformed spectra were analyzed using SPARKY (UCSF).

RESULTS

UV hyperchromicity studies

The effects of cytarabine substitution on the stabilities of the model Okazaki fragments were determined by both parametric fitting of individual UV hyperchromicity curves and from the concentration dependence of the T_m (Fig. 2). The results are summarized in Table 1. The sequences for [OKA] and [ARAC] are shown in Figure 1. Cytarabine substitution significantly decreased the stability of the model Okazaki fragment, with [ARAC] having a melting temperature of 42.4°C, compared with 46.8°C for [OKA], both at a concentration of 1.33×10^{-5} M. The decreased melting temperature of [ARAC] relative to [OKA] corresponds to a less favorable change in free energy for the melting transition of ~ 2 kcal/mol at 37°C (Table 1).

The change in free energy associated with the melting transitions for both [OKA] and [ARAC] calculated from either the concentration dependence of the melting temperature or from fitting the individual melting curves are identical. The relative enthalpic and entropic contributions to the overall change in free energy for the two methods are also within the experimental uncertainties. The enthalpic and entropic contributions to the free energy of duplex melting for [OKA] and [ARAC] measured from the temperature dependence of the T_m are statistically distinguishable and indicate that the more favorable free energy change associated with [OKA] melting is due to a more favorable

enthalpic term. The more favorable enthalpy associated with the melting transition is largely offset by a less favorable entropy change associated with the transition (Table 1). The relative enthalpic and entropic contributions derived from analysis of individual melting curves is consistent with destabilization of [ARAC] relative to [OKA] resulting from less favorable enthalpic factors, although uncertainties in measurement do not permit this conclusion to be drawn from these data alone. These results demonstrate that cytarabine substitution can destabilize Okazaki fragments. The structural basis for this destabilization was investigated in greater detail using ^1H NMR spectroscopy.

Table 1. Summary of the thermodynamic data for the model Okazaki fragments

	T_m	ΔH (kcal/mol)	$T\Delta S_{37}$ (kcal/mol)	ΔG°_{37} (kcal/mol)
Average of fitted parameters ^a				
[OKA]	46.8 ± 0.5	-85 ± 9	-75 ± 8	-11 ± 1
[ARAC]	42.4 ± 0.5	-87 ± 9	-78 ± 8	-9 ± 0.9
Concentration dependence of T_m ^b				
[OKA]	46.8 ± 0.5	-96 ± 10	-85 ± 9	-11 ± 1
[ARAC]	42.4 ± 0.5	-73 ± 7	-64 ± 7	-9 ± 1

^aCalculated at 1.33×10^{-5} M for [OKA] and [ARAC].

^bErrors in thermodynamic parameters are estimated to be 10%.

NMR of [OKA] exchangeable ^1H

^1H NMR spectroscopy was used to identify changes in structure and stability of individual base pairs in [ARAC] relative to [OKA] that were responsible for the global destabilization of [ARAC] observed in UV hyperchromicity experiments. Assignment of ^1H resonances was accomplished using 2D NOESY spectra in both H_2O and D_2O solution. The temperature dependence of chemical shifts and line widths for the imino ^1H resonances of the AT and GC base pairs was used to assign resonances for which NOE information was ambiguous. Temperature-dependent behavior of resonance intensities and NOEs was also used to determine the relative stabilities of specific regions in the duplexes.

The 1D ^1H NMR spectrum for [OKA] in H_2O is shown in Figure 3. [OKA] consists of five GC and seven AT base pairs. The ^1H NMR spectrum indicates that three imino ^1H resonate in the 12–13 p.p.m. region characteristic of GC base pairs and six imino ^1H resonate in the 13–14 p.p.m. region characteristic of AT base pairs. The two terminal GC base pairs and the penultimate AT base pair of the DNA:DNA duplex portion of [OKA] do not appear in the spectrum due to fraying at the ends of the duplex at this temperature (26°C). The three GC imino protons were assigned on the basis of cross-peaks in the NOESY spectra and the temperature dependence of the imino proton resonance intensities. G5 is flanked by two AT base pairs and cross-peaks between the imino ^1H resonances of both T19 and T21 with the imino proton of G5 are observed in the NOESY spectrum (see Fig. 2 for residue numbering). g15 and g16 (ribonucleotides designated lower case; Fig. 1) are adjacent to one another in the interior of the duplex, however, no cross-peak between the imino ^1H resonances of these base pairs was observed in the NOESY spectrum nor was a cross-peak observed to T11 or T8, the AT base pairs that flank g15 and g16, respectively. Assignment of the g15

Figure 3. Temperature dependence of the 1D ^1H NMR spectrum in 90% H_2O for the region characteristic of imino hydrogens from AT base pairs. The top two spectra are of [OKA] and the bottom two spectra are of [ARAC]. Spectra shown were acquired at 26 and 38°C. Solvent suppression was achieved using a 1–3–3–1 binomial pulse sequence.

and g16 imino protons was therefore made on the basis of the temperature dependence of the imino proton resonance intensities. The more downfield resonance decreased in intensity at elevated temperature more rapidly than the upfield resonance. The downfield resonance was therefore assigned to g15, the resonance nearer the terminus of the duplex and thus more susceptible to end fraying at elevated temperature.

This observed temperature dependence of the G imino protons of [OKA] is also consistent with the temperature behavior of the neighboring AT imino protons. The imino proton resonance of T11, which flanks g15, is barely observable at 38°C due to exchange broadening, while T8, adjacent to g16, shows considerably less exchange broadening at elevated temperatures. The amino ^1H resonances of the GC base pairs in [OKA] were also assigned based on NOE cross-peaks. The resonance assignments for the exchangeable protons of [OKA] are summarized in Table 2.

The imino protons of AT base pairs in [OKA] were also assigned based on cross-peaks in the NOESY spectrum and the temperature dependence of the resonance frequencies and line widths (Fig. 3). T19 and T21 each show an NOE cross-peak to G5. The assignment of T19 is further established by an NOE cross-peak to T7, which is slightly downfield of T19. T7 in turn shows an NOE cross-peak to neighboring T8, which resonates furthest downfield of all the imino protons. T21 shows an NOE cross-peak to its neighbor T22. Each of the T imino protons also shows an NOE cross-peak to a single adenosine H2 resonance which is assigned to its base pairing partner (see below). In addition, T19 and T21 show NOE cross-peaks to G5 NH_2 .

Table 2. Assignment of exchangeable ^1H resonances for [OKA]

Base pair ^a	AH2/GNH ₂ ^b	T/G NH ^c	C NH ₁ /NH ₂ ^d
A3-T22	7.24	14.04	
A4-T21	7.36	14.07	
G5-C20	6.08	12.55	8.24/6.86
A6-T19	7.83	13.60	
T7-A18	7.33	13.65	
T8-A17	7.88	14.08	
C9-G16	5.88	12.16	8.23/7.41
C10-G15	6.12	12.73	8.42/6.80
T11-A14	7.77	13.86	

^aResidue numbering given in Figure 1.

^bResonance frequency for adenosine H2 or guanosine NH_2 of the indicated base pair.

^cResonance frequency of thymidine or guanosine imino ^1H .

^dResonance frequencies of cytidine amino ^1H .

[OKA] ^1H NMR temperature effects

The resonance assignments based on NOE cross-peaks were further substantiated by the temperature dependence of the 1D ^1H NMR spectrum of the imino region (Fig. 3). The spectrum shows six discrete resonances at 26°C. The imino proton of T23, the penultimate AT base pair in the DNA:DNA duplex portion of [OKA], is not observed at 26°C due to exchange broadening. As the temperature is elevated above 26°C the imino proton resonance from T19, the furthest upfield of the AT imino protons, displays the most minimal changes in frequency and intensity. All other AT resonances show substantial chemical shift changes and line broadening as the temperature is elevated. The greatest diminution in signal intensity occurs for T11, the penultimate AT base pair for the RNA:DNA hybrid portion of [OKA]. T21 and T22, the third and fourth base pairs from the terminus of the DNA:DNA duplex portion of [OKA], show the next greatest diminution in intensity at elevated temperatures, while T7 and T8, the deoxynucleotides at the (DNA:DNA)–(DNA:RNA) junction, show somewhat less exchange broadening at elevated temperatures. The temperature dependence of the resonance intensities for the T imino protons is indicative of end fraying of the [OKA] duplex, with fraying more severe in the DNA:DNA duplex portion than in the hybrid portion. The interior of the duplex, consisting of nucleotides G5–C10 in the DNA strand and g15–C20 in the hybrid strand, shows little evidence of strand dissociation based on the characteristics of the imino ^1H resonances at 38°C, a value approaching the melting temperature for [OKA] determined from the first derivative of the UV hyperchromicity profile at 260 nm (see above).

Base stacking in [OKA]

The assignments of the adenosine H2 resonances were further investigated using 2D NOESY spectra of [OKA] in D_2O solution at 26°C (Fig. 4A). Each of the adenosine H2 resonances shows NOE cross-peaks to either the H1' resonance of the 3' neighbor (intrastrand) or to the H2 resonance of an adjacent adenosine. The DNA:DNA portion of [OKA] contains three consecutive AT base pairs with all three of the adenines consecutive in the DNA strand. NOE cross-peaks were observed from A3 H2 to both A2

A

B

Figure 4. 2D NOESY spectra acquired in D₂O solution with a 200 ms mix time for (A) [OKA] and (B) [ARAC]. The region of the spectra containing NOE cross-peaks between adenosine H2 resonances and intra- and inter-strand H2-H1' resonances is shown. Major differences between the spectra are reduced intensities for H2-H2 resonances in [ARAC] relative to [OKA] and changes in chemical shift for aC20 H6-H5 relative to C20 H6-H5. Cytidine H6-H5 NOEs are indicated only with the residue number.

and A4 H2 (Fig. 4A). A4 H2 was further characterized by NOE cross-peaks to T22 H1', the 3' neighbor of its base pair partner, and G5 H1', its own 3' neighbor. a17 is a ribonucleotide and is flanked by A18, a deoxyribonucleotide at the DNA:RNA junction. A third AT base pair involving A6 concludes a second stretch of three consecutive AT or TA base pairs. A particularly strong H2-H2 NOE cross-peak is observed between A18 and A6, adenosines on opposite strands of [OKA] and participants in the first two DNA:DNA base pairs flanking the RNA:DNA hybrid portion of [OKA]. The intensity of this NOE indicates that efficient stacking occurs between these two purine bases in

[OKA]. The assignment of A18 H2 is further established by an NOE to H1' of its 3' neighbor T19, while A6 H2 shows NOE cross-peaks to both T7 H1' and C20 H1', the 3' neighbor of itself and the 3' neighbor of its base pair partner, T19. Finally, a14 shows an NOE cross-peak to H1' of its 3' neighbor g15 (Fig. 4A).

NMR of [ARAC] exchangeable ¹H

The exchangeable ¹H resonances for [ARAC] were assigned by methods similar to those described for [OKA]. In particular, the region of the imino proton spectrum of [ARAC] that is

characteristic of GC base pairs is essentially identical to that observed for [OKA] (Fig. 3). G5 of [ARAC] also shows NOE cross-peaks to T19 and T21, although they are reduced in intensity relative to [OKA]. G5 also shows slightly greater diminution in intensity at elevated temperatures in [ARAC] relative to [OKA] (Fig. 3). Increased temperature sensitivity for G5 in [ARAC] relative to [OKA] is consistent with cytarabine destabilizing the duplex near the site of substitution (see Discussion). G5, however, shows essentially no change in chemical shift in [ARAC] relative to [OKA], despite being the base pairing partner of cytarabine in [ARAC]. g15 and g16 show similar temperature dependencies in [ARAC] as in [OKA]. The imino proton resonances of AT base pairs in [ARAC] also show similar NOE and temperature dependence characteristics as those observed for [OKA]. T19, the furthest upfield T imino proton, displays an NOE cross-peak to T8 and changes only slightly in intensity at elevated temperatures (Fig. 3). T19 is, however, displaced ~0.1 p.p.m. upfield in [ARAC] relative to [OKA] as a consequence of being adjacent to the site of cytarabine substitution. T7 and T8 show little change in chemical shift, NOE characteristics or temperature susceptibility in [ARAC] relative to [OKA]. Both resonances remain substantially unaltered in intensity at 38°C, a temperature approaching the melting temperature calculated from the UV hyperchromicity studies. The largest changes in chemical shift, NOE cross-peak intensities and temperature susceptibility in [ARAC] relative to [OKA] occur for T21 and T22. T21 and T22 are shifted upfield and broadened at 26°C and both resonances show a more marked diminution in intensity and a broadening at elevated temperatures in [ARAC] relative to [OKA]. In fact, T21, which is four bases into the interior of the DNA:DNA duplex portion of [ARAC] but is adjacent to cytarabine, shows greater diminution in intensity at 38°C than does T11, the penultimate base pair in the RNA:DNA portion of [ARAC]. The temperature dependence of the imino proton resonances clearly indicates that cytarabine substitution destabilizes the DNA:DNA duplex portion of [ARAC].

Base stacking in [ARAC]

The assignment of the adenosine H2 resonances for the AT base pairs of [ARAC] was also investigated using 2D NOESY spectra of [ARAC] in D₂O at 26°C. The results are shown in Figure 4B. Many of the same H2–H2 and H2–H1' interactions that were observed for [OKA] were also observed for [ARAC]. For instance, A18 H2 shows an NOE cross-peak to T19 H1', however, the A18 H2–A6 H2 cross-peak that is readily observed in the NOESY spectrum of [OKA] is reduced in intensity for [ARAC], suggesting that cytarabine substitution alters base stacking among nearby base pairs. A6 H2 continues to show NOE cross-peaks to T7 H1' and to aC20 H1'. The NOE from a14 H2 to g15 H1' is not reduced in intensity in [ARAC] relative to [OKA]. The most significant change in NOE intensities involving adenosine H2 resonances between [ARAC] and [OKA] occurs for A2, A3 and A4. Significant NOEs are observed between these three consecutive adenosines for [OKA], however, only the H2–H2 interaction between A3 and A4 is readily apparent in the NOESY spectra for [ARAC]. The A4 H2–T22 H1' interaction that was weakly observed for [OKA] is also absent for [ARAC]. These changes in NOE intensities for adenosine H2 involving the duplex DNA portion of [ARAC], particularly interstrand H2–H1' NOEs, are consistent with destabilization of the DNA:DNA duplex portion

of [ARAC] as a consequence of cytarabine substitution. This interpretation is also consistent with the increased susceptibility of the imino ¹H resonances for the T nucleotides complementary to A2, A3 and A4 to increases in temperature (see above). The resonance assignments for the exchangeable protons of [ARAC] are summarized in Table 3.

Table 3. Assignment of exchangeable ¹H resonances for [ARAC]

	AH2/GNH ₂	T/G NH	C NH ₁ /NH ₂
A3-T22	7.27	14.04	
A4-T21	7.47	14.00	
G5-aC20	6.26	12.56	8.23/6.71
A6-T19	7.88	13.58	
T7-A18	7.36	13.71	
T8-A17	7.92	14.16	
C9-G16	5.92	12.18	8.27/7.35
C10-G15	5.76	12.76	8.41/6.76
T11-A14	7.77	13.90	

DISCUSSION

Despite the widespread utilization of antimetabolites for cancer chemotherapy, few details are known concerning the structural basis for their antiproliferative and cytotoxic activities. Inhibition of DNA synthesis and disruption of DNA function are considered to be the major metabolic targets of cytarabine and other arabinosyl nucleosides, however, the molecular bases for these activities are not clear. The present study shows that cytarabine substitution for deoxycytidine significantly destabilizes a model Okazaki fragment and causes structural perturbations in the DNA:DNA duplex portion of the Okazaki fragment. These results provide one possible explanation for previous results, indicating that aphidicolin, a DNA polymerase inhibitor, significantly antagonizes both cytarabine incorporation into DNA and cell killing (22). Aphidicolin treatment of cells eliminates cytarabine misincorporation into DNA and thus cytarabine-mediated decreases in stability or changes in structure of Okazaki fragments cannot occur. Analysis of the physical properties of nascent DNA (nDNA) reveals that exposure of cells to cytarabine results in a reduction in the fraction of fragments between 0.5 and 40 kb long and an increased fraction of fragments that were ~100 nt long, a result consistent with Okazaki fragment destabilization being a principal mechanism for the biological activity of cytarabine (8). The ability of cytarabine to influence elongation of the lagging strand during DNA replication at points distal to its stable incorporation is consistent with induction of localized unfolding that inhibits completion of replication.

The UV hyperchromicity studies of [OKA] and [ARAC] presented here demonstrate that cytarabine substitution destabilizes Okazaki fragments. These observations provide a rationale for how cytarabine substitution inhibits elongation of the lagging strand at the replication fork during DNA synthesis by favoring localized unfolding in the nDNA from the Okazaki fragment. The magnitude and direction of the changes in thermodynamic parameters associated with the melting transition for these model Okazaki fragments are indicative of the energetics that may be

responsible for termination of DNA synthesis *in vivo*. A single cytarabine substitution destabilizes the 12mer model Okazaki fragment by slightly more than 1 kcal/mol free energy at 37°C and depresses the melting temperature of the 12mer duplex by ~5°C. Destabilization of the model Okazaki fragment in the present study is significantly greater than the destabilization in DNA or RNA duplexes upon substitution of 5-fluorouridine for uridine observed in previous studies (23,24). The magnitude of destabilization, however, is not so large as to reasonably account for the effectiveness of cytarabine at inducing chain termination by favoring complete dissociation of the nDNA from the lagging strand. These results, however, are consistent with local unfolding about the site of cytarabine substitution inhibiting completion of the chain elongation process.

NMR studies provide additional insight into the localization of structural changes that arise from misincorporation of cytarabine in place of deoxycytidine. Overall, the extent of deformation of the structure of the model Okazaki fragment due to cytarabine substitution is modest. This conclusion is based mainly on the chemical shifts of the exchangeable ¹H resonances that differ insignificantly between [OKA] and [ARAC] (see Tables 2 and 3). A previous study on the effect of cytarabine substitution on the structure of a DNA duplex also concluded that structural effects were slight and localized to near the site of cytarabine substitution (10). The effects of cytarabine substitution on the dynamics of the Okazaki fragment appear to be more significant. Line widths for the imino ¹H resonances of dA-dT base pairs near the site of cytarabine substitution show the largest increases in temperature-dependent line broadening for any resonances in the model Okazaki fragment. Cytarabine substitution thus alters the dynamic behavior of the DNA:DNA duplex portion of the model Okazaki fragment. These changes in rates of base pair opening downstream of the site of cytarabine misincorporation may be responsible for the loss of processivity for the DNA replication complex. Further evidence for localized disturbances in structure and dynamics of the DNA:DNA duplex portion of [ARAC] were evident in decreased intensities for NOESY cross-peaks between H2 ¹H resonances, which are indicative of stably stacked adjacent purines. Such stable stacking interactions were evident for [OKA], but were noticeably perturbed in [ARAC].

Rational control over replication of DNA in malignant tissues is an important objective in the design of new anticancer drugs. In the present study we have shown that cytarabine, a nucleoside analog that is effective in the treatment of leukemia and that inhibits elongation of the lagging strand at the replication fork during DNA synthesis, destabilizes a model Okazaki fragment. Understanding the structural and thermodynamic consequences of cytarabine substitution will allow more accurate and detailed rationalizations of its biological properties to be developed. A greater understanding of the molecular basis for anticancer drugs

that are currently useful clinically may lead to the design and development of more potent and selective inhibitors in the future.

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