Cytosine Arabinoside Substitution Decreases Transcription Factor–DNA Binding Element Complex Formation

Ximbo Zhang, MD, PhD; Frederick L. Kiechle, MD, PhD

Context.—The pyrimidine nucleoside analog, cytosine arabinoside (ara-C), is an effective therapeutic agent for acute leukemia. The phosphorylated triphosphate, cytosine arabinoside triphosphate, competes with deoxycytidine triphosphate as a substrate for incorporation into DNA. Once incorporated into DNA, it inhibits DNA polymerase and topoisomerase I and modifies the tertiary structure of DNA.

Objective.—To determine if the substitution of ara-C for cytosine in double-stranded oligonucleotides that contain 4 specific transcription factor binding sites (TATA, GATA, C/EBP, and AP-2α) alters transcription factor binding to their respective DNA binding elements.

Design.—Transcription factors were obtained from nuclear extracts from human promyelocytic leukemia HL-60 cells. [32P]-end-labeled double-stranded oligonucleotides that contained 1 or 2 specific transcription factor binding sites with or without ara-C substitution for cytosine were used to assess transcription factor binding by electrophoretic mobility shift assay.

Results.—The substitution of ara-C for cytosine within and outside the transcription factor binding element (AP-2α, C/EBP), outside the binding element only (GATA, TATA), or within the binding element only (AP-2α) all result in a reduction in transcription factor binding to their respective DNA binding element.

Conclusion.—The reduction of the binding capacity of transcription factors with their respective DNA binding elements may depend on structural changes within oligonucleotides induced by ara-C incorporation. This altered binding capacity of transcription factors to their DNA binding elements may represent one mechanism for ara-C cytotoxicity secondary to inhibition of transcription of new messenger RNAs and, subsequently, translation of new proteins.

(Arch Pathol Lab Med. 2004;128:1364–1371)
be reduced (approximately 100-fold) by the incorporation of Ara-C into DNA, and this reduction in transcription was dependent on where Ara-C was incorporated, indicating that there are vulnerable areas in the DNA sequence. The active metabolite of Ara-C, Ara-CTP, is a competitor of DNA polymerase α and, to a lesser extent, polymerase β. A more recent study shows that trapping of topoisomerase I cleavage complexes at Ara-C incorporation sites leads to an inhibition of the re-pair step of the topoisomerase I reaction. Evaluation of a 3.1 resolution crystal structure of human topoisomerase I in covalent complex with a 22-base pair oligonucleotide containing Ara-C at the +1 position of the noncissile DNA strand demonstrates that numerous subtle structural changes introduced by Ara-C contribute to a new positioning of the free 5′-sulfhydryl away from the 3′-phosphotyrosine linkage, which decreases the single-strand re-ligation reaction and produces a stabilized covalent topoisomerase I–DNA complex.

To further determine the effect of Ara-C incorporation into DNA on DNA replication and RNA transcription, both in vitro and in vivo studies are needed. For example, the structure of a DNA duplex containing Ara-C determined by x-ray diffraction or nuclear magnetic resonance demonstrates that incorporation of Ara-C into DNA can cause localized alteration in the DNA structure, including sugar pucker, backbone torsion angles, base stacking, and other helical parameters. Additional in vitro studies using Ara-C–substituted oligonucleotides demonstrate that the localized alterations in the DNA duplex are associated with topoisomerase I inhibition. These results support the in vivo finding that cAMP-dependent protein kinase A and protein kinase C inhibit the transcriptional activity of Ara-C.

Preparation of Radiolabeled Oligonucleotides

Double-stranded oligonucleotides were end-labeled with [32P] adenosine triphosphate (NEN Life Science Products Inc) using T4 polynucleotide kinase (Roche) and were purified with ProbeQuant G-50 Micro Columns (Pharmacia Biotech).

Preparation of Nuclear Extracts

HL-60 cell nuclear extracts used for gel mobility shift assay were prepared by a procedure previously described. Untreated HL-60 cells were washed twice in ice-cold phosphate-buffered saline. The cells were then resuspended in buffer A (10mM HEPES, pH 7.9, 1.5mM MgCl2, 10mM potassium, 0.5mM dithiotheriol [DTT], 0.5mM phenylmethylsulfonyl fluoride, 0.05% Non-det P-40, 1% aprotinin, 1mM EDTA, and 1mM of leupeptin) and homogenized briefly on ice. After incubation on ice for 15 minutes, homogenate was centrifuged at 14 000 rpm in a microcentrifuge at 4°C for 10 minutes. The pellet was washed twice in buffer A and resuspended in buffer B (20mM HEPES, pH 7.9, 0.42M NaCl, 1.5mM MgCl2, 0.2mM EDTA, 0.5mM DTT, and 6.25% glycerol), which contained protease inhibitors. After incubation on ice for 40 minutes, the nuclear suspension was centrifuged at 14 000 rpm in a microcentrifuge at 4°C for 15 minutes. The protein concentrations of collected supernatant were determined by bichoninic acid protein assay reagent (Pierce).

Electrophoretic Mobility Shift Assay

The binding reaction mixture contained 20mM Tris hydrochloride, pH 7.5; 10mM sodium acetate; 0.5mM EDTA; 5% glycerol; 0.5 to 6 μg of total proteins of nuclear extracts; and 1 × 106 cpm of [32P]-labeled oligonucleotides. The incubation was performed for 15 minutes at room temperature. DNA-protein complexes were separated by electrophoresis on 4% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer and visualized by autoradiography.

RESULTS

Effect of All Oligonucleotide Cytosines Substituted by Ara-C on Their Respective Transcription Factor Binding Capacity

To determine the effect of Ara-C incorporation into the original oligonucleotides on the binding capacity to their respective DNA binding elements, the following oligonucleotides were used. Double-stranded 5′-TCAGCCCTTATTAAAGC GGGGGGGGCGGCCC-3′ (TATAAA or TATA box element), 5′-TTTATAAAAGCATATTATTT-3′, 5′-TTTATAAAACATATTTAA TTT-3′, 5′-TTTATAAAATCTTTAATT-3′, 5′-TTTATTTAAATTATATT TAAATCTTTAT-3′, 5′-GATGCAAAGTGGCCTCGGGCGGG-3′ (GGCCGGG or AP-2α element). 5′-AAATTTAATTATTTCCCGGG CCGGCTTAT-3′, 5′-TCGAGAGTGGCCTCTGGCA-3′ (TGCCG AAA or C/EBP element). In the present study, our results demonstrate that trapping of topoisomerase I cleavage complexes at Ara-C incorporation sites leads to an inhibition of the re-pair step of the topoisomerase I reaction. Evaluation of a 3.1 resolution crystal structure of human topoisomerase I in covalent complex with a 22-base pair oligonucleotide containing Ara-C at the +1 position of the noncissile DNA strand demonstrates that numerous subtle structural changes introduced by Ara-C contribute to a new positioning of the free 5′-sulfhydryl away from the 3′-phosphotyrosine linkage, which decreases the single-strand re-ligation reaction and produces a stabilized covalent topoisomerase I–DNA complex.

MATERIALS AND METHODS

Cells

Human promyelocytic leukemia HL-60 cell line was cultured in RPMI-1640 medium (Hyclone, Logan, Utah) with 10% fetal bovine serum. It was maintained in a 37°C, 5% carbon dioxide, fully humidified incubator and prepared for experimental procedures when in log-phase growth.
Figure 1. Effect of cytosine arabinoside (Ara-C) substitution for cytosines in an oligonucleotide that contained the C/EBP DNA binding element on the binding capacity of transcription factor C/EBP to its respective DNA binding element. Following C/EBP binding to its element forms, 3 different molecular weight complexes or bands I, II, and III are formed; the band densities of Ara-C-substituted element–C/EBP complexes are decreased when compared with those of non–Ara-C-substituted element–C/EBP complexes, suggesting that the element substituted by Ara-C causes a decrease in the binding capacity of C/EBP to its DNA binding element. Electrophoretic mobility shift assay is a useful method to identify intracellular concentration of a specific transcription factor, the binding capacity of a specific transcription factor to its DNA binding element, and whether or not a transcription factor binds to its DNA binding domain but also has protein binding domains that bind with other proteins to form more complicated complexes. Since binding of transcription factor to its DNA binding element is the most important step in the initiation of RNA transcription, disruption of this complex will lead to inhibition of messenger RNA synthesis and subsequent protein synthesis.

TAAAA) within this oligonucleotide has no cytosine, but there are 19 cytosines in this double-stranded oligonucleotide. There were 19 cytosines substituted with Ara-C in Ara-C–incorporated oligonucleotide. Double-stranded 5’-CACTTTGATACAGAAATGATAACTCT-3’ is an oligonucleotide that contains 2 GATA elements. The GATA elements (TGATAA) within this oligonucleotide have 2 cytosines, and the total cytosines are 9 in this double-stranded oligonucleotide. Nine cytosines were substituted by Ara-C in Ara-C–incorporated oligonucleotide. A double-stranded 5’-TGCAGACGTTGCGGACTGTGCA-3’ oligonucleotide contains a C/EBP element. The C/EBP element (TTGCGGCA) within this oligonucleotide has 4 cytosines, and the total cytosines are 10 in this double-stranded oligonucleotide. Ten cytosines were substituted by Ara-C in Ara-C–incorporated oligonucleotide. Double-stranded 5’-GATCGAATGTACGCGGCGGGCCCGT-3’ oligonucleotide contained an AP-2α element. The AP-2α element (GCCCGCGG) within this oligonucleotide has 8 cytosines, and there are a total of 19 cytosines in this double-stranded oligonucleotide. Nineteen cytosines were substituted by Ara-C in the Ara-C–incorporated oligonucleotide. As shown in Figures 1 through 4, 4 Ara-C–incorporated oligonucleotides decreased the binding capacities with their specific DNA binding proteins to form DNA–transcription factor complexes (see band I), and the concentrations of high-molecular DNA–transcription factor complexes (see bands II and III) were decreased by Ara-C incorporation. These results demonstrate that the decrease in formation of DNA–transcription factor complexes after Ara-C incorporation into oligonucleotides is independent of the amount of Ara-C–incorporated into transcription factor elements, since 4 Ara-C–incorporated oligonucleotides including double-stranded 5’-TCAGCCTTATACAGAAATCAGGCGGCGCCGCG-3’ oligonucleotides without cytosine in TATA element have the same effect on transcription factor–DNA binding.
Effect of Ara-C Substitution for Cytosines Only Within the AP-2α DNA Binding Element

To determine whether the formation of DNA–transcription factor complexes would be altered if only the cytosines within a transcription factor DNA binding element were substituted by Ara-C, we modified the double-stranded 5′-AATTTAATTAATTGCCGCGTGTATAT-3′ oligonucleotide into 5′-AATTTAATTAATTTGXXGCGTTATAT-3′ (X = Ara-C) to substitute Ara-C for cytosines in only the portion of the AP-2α DNA binding (Figure 5, A) element. As shown in Figure 5, B, Ara-C–incorporated AP-2α oligonucleotide has the same effect on reducing the formation of DNA–AP-2α complex, as noted in Figures 3 and 4 (TATA element result). These results further support the conclusion that the Ara-C incorporation–induced decrease in the formation of DNA–transcription factor complex is independent of location of substitution.

Effect of Location of Ara-C Incorporation Into Oligonucleotides Outside TATA Element on DNA–Transcription Factor Complex Formation

To determine if Ara-C incorporation into different locations outside the TATA DNA binding element could alter DNA–transcription factor complex formation, we modified 4 double-stranded oligonucleotides, each containing only one cytosine either 1, 2, 5, or 10 base pairs from the TATA element: TTTATACAAACGTTAAATT-3′, 5′-TTTATACAAACGTTAAATT-3′, 5′-TTTATAAAATATTTGTTATAT-3′, and 5′-TTTATAAAATATTTGTTATAT-3′ (X = Ara-C). As shown in Figures 6 through 9, whether the Ara-C is located 1, 2, 5, or 10 base pairs away from the TATA DNA binding element, Ara-C–incorporated oligonucleotides demonstrated decreased binding capacity with their transcription factors. This finding indicates that changes in DNA–transcription factor binding capacity induced by Ara-C incorporation into oligonucleotide outside the TATA DNA binding element is independent of the location of Ara-C incorporation into oligonucleotides outside the TATA element (at least 10 base pairs away from the DNA binding element; Figure 9).

COMMENT

In the present study, we demonstrate that incorporation of Ara-C into oligonucleotides with the respective DNA binding elements for 4 transcription factors (C/EBP, GATA, TATA, and AP-2α) can significantly reduce the binding capacity of transcription factors with their respective DNA binding elements. This reduction in DNA–transcription factors complex formation is independent of
the numbers of cytosines within or outside the element or the total number of cytosines in the oligonucleotides. The results suggest that the sites and numbers of Ara-C molecules incorporated into the oligonucleotides do not play an important role in the reduction in binding capacity of transcription factors to their respective DNA binding elements. An early study indicates that substitutions of Ara-C for cytosine in the central promoter region have no observable effect on RNA polymerase binding, initiation rate, or transcriptional output, suggesting that substitution of Ara-C itself does not alter gene transcription. However, transcription factors might be much more sensitive to changes in DNA structure induced by Ara-C incorporation compared with RNA polymerase. For example, a bend in DNA is induced by the TATA binding protein on binding to its TATA box DNA binding element. The extent to which the DNA is bent in the TATA binding protein–TATA DNA binding element complexes with proteins to form more complicated complexes. A, Ara-C–substituted oligonucleotide sequence with TATA element; B, electrophoretic mobility shift result.

Figure 5. Effect of cytosine arabinoside (Ara-C) substitution for cytosines only within the C/EBP DNA binding element on the binding capacity of transcription factor AP-2α to its respective DNA binding element. This figure demonstrates that the band densities (bands I and II) of Ara-C–substituted element–AP-2α complexes are decreased when compared with those of non–Ara-C–substituted element–AP-2α complexes, suggesting that the element that contained Ara-C has a decreased binding capacity of AP-2α for its DNA binding element. A, Ara-C–substituted oligonucleotide sequence with AP-2α element; B, electrophoretic mobility shift result.

Figure 6. Effect of cytosine arabinoside (Ara-C) incorporation into the first cytosine outside the TATA DNA binding element on the binding capacity of transcription factor TATA to its DNA binding element. The band densities of Ara-C–substituted element–TATA complexes are decreased when compared with those of non–Ara-C–substituted oligonucleotide–TATA complexes, suggesting that the TATA element Ara-C located on the base outside the element can bind with TATA protein (see band II) but fails to form the larger complexes (see band I). This result indicates that localized alteration in the oligonucleotide structure has a critical effect on further binding capacity of transcription factor–DNA binding element complexes with proteins to form more complicated complexes. A, Ara-C–substituted oligonucleotide sequence with TATA element; B, electrophoretic mobility shift result.
Figure 7. Effect of cytosine arabinoside (Ara-C) incorporation into the first 2 bases outside the TATA DNA binding element on the binding capacity of transcription factor TATA to its DNA binding element. The band densities (bands I and II) of Ara-C-substituted element–TATA complexes are decreased when compared with those of non-Ara-C-substituted oligonucleotide–TATA complexes, suggesting that the addition of an additional Ara-C adjacent to the TATA element decreased the formation of both large (band I) and small (band II) complexes (compare with Figure 6). A, Ara-C-substituted oligonucleotide sequence with TATA element; B, electrophoretic mobility shift result.

Figure 8. Effect of cytosine arabinoside (Ara-C) incorporation into the fifth base (cytosine) outside the TATA DNA binding element on binding capacity of transcription factor TATA to its element. The band densities (bands I and II) of Ara-C-substituted element–TATA complexes are decreased when compared with those of non-Ara-C-substituted oligonucleotide–TATA complexes. A, Ara-C-substituted oligonucleotide sequence with TATA element; B, electrophoretic mobility shift result.

The box complex differs among the various sequences tested and is correlated with the stability of the complex; that is, the greater the stability of the complex, the more the DNA appears to be bent by TATA binding protein.23,24 This result shows that change in conformation induced by transcription factor binding has a close association with the efficiency of transcription initiation. Incorporation of Ara-C into DNA causes localized alterations in the DNA duplex, including changes in sugar pucker, base stacking, backbone torsion angle, and other helical parameters.12,13 These localized changes in DNA structure reduce the flexibility within DNA, which may result in the decrease in binding capacity of transcription factors with their elements as observed in our electrophoretic mobility shift studies (Figures 1 through 9). These structural changes may also explain the decreased formation of high molecular weight into oligonucleotides (Figures 1 through 9).

In contrast to the promoter region, coding strand substitutions of Ara-C for cytosine defined an area of high sensitivity in the initiation region where miss start, primer slippage, and inability to escape from abortive cycling occur, depending on the position substituted.5 These results show that RNA polymerase II might be more sensitive to the substitution of Ara-C for cytosine. Although Ara-C incorporation can inhibit DNA polymerases6–9 and topoisomerase I10,11 and reduce the binding capacity of tran-
The band densities (bands I and II) of Ara-C-substituted element-TATA of the 1-valent complex with a 22-base pair DNA duplex that con-

servation factors with their elements (Figures 1 through 9), the mechanisms by which Ara-C incorporation into DNA causes dysfunction of nuclear proteins or enzymes are not fully understood. Chrencik et al. evaluated the 3.1Å res-

cription, and nuclear proteins or enzymes, leading to re-

terdation of the growth of malignant cells.

Although Ara-C is one of the most commonly used drugs for treatment of acute myeloid leukemia, more than half of the patients treated with Ara-C and daunorubicin are refractory or experience a relapse, suggesting that the leukemic cells of such patients acquire resistance to both drugs. Multidrug resistance is a major obstacle to suc-

cessful treatment. Furthermore, the exact mechanism by which leukemic cells develop cross-resistance to these agents has not been clearly elucidated. The elucidation of the primary intracellular targets for Ara-C will permit op-

timized selection of new anticancer regimens and the de-

development of new anticancer drugs. In the present study, our results suggest that reduction of binding capacity of transcription factors with their respective DNA binding elements may depend on structural changes of oligonucle-

otides induced by Ara-C incorporation. This mechanism differs from the mechanism by which Ara-C mediates topoisomerase I trapping. These data provide valuable information useful in developing chemotherapeutic treatment strategies.

This work was supported by grants from the William Beaumont Hospital Research Institute and Elsa U. Pradee Foundation. The authors thank Keya Wilson for typing the manuscript.

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Figure 9. Effect of cytosine arabinoside (Ara-C) incorporation into the tenth base (cytosine) outside the TATA DNA binding element on bind-

ing capacity of transcription factor TATA to its DNA binding element. The band densities (bands I and II) of Ara-C-substituted element–TATA complex

es are decreased when compared with those of non–Ara-C–substituted oligonucleotide–TATA complexes. A, Ara-C-substituted oligonucleotide sequence with TATA element; B, electrophoretic mobility shift result.


