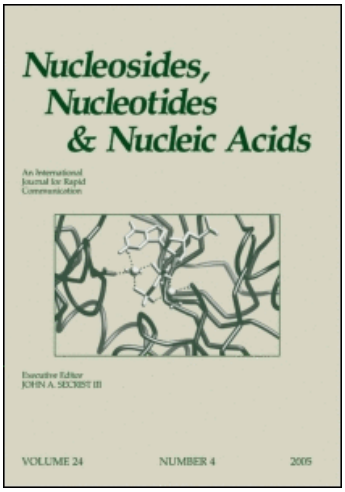

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

2'-*C*-Cyano-2'-deoxy-1-β-D-arabinofuranosylcytosine (CNDAC): A Mechanism-Based DNA-Strand-Breaking Antitumor Nucleoside[1]

Akira Matsuda[a]; Atsushi Azuma[a]
[a] Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

To cite this Article Matsuda, Akira and Azuma, Atsushi(1995) '2'-*C*-Cyano-2'-deoxy-1-β-D-arabinofuranosylcytosine (CNDAC): A Mechanism-Based DNA-Strand-Breaking Antitumor Nucleoside[1]', Nucleosides, Nucleotides and Nucleic Acids, 14: 3, 461 — 471
To link to this Article: DOI: 10.1080/15257779508012407
URL: http://dx.doi.org/10.1080/15257779508012407

NUCLEOSIDES & NUCLEOTIDES, 14(3-5), 461-471 (1995)

2'-*C*-CYANO-2'-DEOXY-1-β-D-ARABINOFURANOSYL-CYTOSINE (CNDAC): A MECHANISM-BASED DNA-STRAND-BREAKING ANTITUMOR NUCLEOSIDE[1]

Akira MATSUDA* and Atsushi AZUMA

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan.

Abstract: The antitumor mechanism of action of 2'-*C*-cyano-2'-deoxy-1-β-D-arabinofuranosylcytosine (CNDAC) has been examined. CNDAC was designed as a potentially DNA-self-strand-breaking nucleoside. It had potent antitumor effects against various solid tumors *in vitro* as well as *in vivo*. Using a chain-extension method with Vent (exo$^-$) DNA polymerase and a short primer/template system, we found that 5'-triphosphate of CNDAC (CNDACTP) was incorporated into the primer at a site opposite a guanine residue in the template. After further chain-extension reaction of the primer containing CNDAC at the 3'-terminus, chain elongation was not observed. Therefore, CNDACTP appeared to act as a chain-terminator. Analyses of the structure of the 3'-terminus in the primer revealed 2'-*C*-cyano-2',3'-didehydro-2',3'-dideoxycytidine (ddCNC) together with CNDAC and 2'-*C*-cyano-2'-deoxy-1-β-D-ribofuranosylcytosine (CNDC). The existence of ddCNC in the 3'-end of the primer would be due to the self-strand-break by the nucleotide incorporated next to CNDAC. We also found that CNDAC was epimerized to CNDC in near-neutral to alkaline media. Therefore, CNDC found in the primer was epimerized after incorporation of CNDACTP into the primer. We also described the metabolism of CNDAC.

INTRODUCTION

Since 1-β-D-arabinofuranosylcytosine (*ara*-C) has been introduced as an antileukemic agent for the treatment of adult acute myeloblastic leukemia,[2] a number of analogues of *ara*-C and its pro-drugs have been synthesized. However, *ara*-C and its pro-drugs are hardly effective against solid tumors.[3] On the other hand, introduction of certain substituents, such as halogeno,[4] amino,[5a] azido,[5a,b] methyl,[6] and fluoromethyl[7] groups, but not a hydroxyl group into the 2'β position of 2'-deoxycytidine, causes tumor cell growth inhibition. Additionally, 2'-difluoro (gemcitabine),[8] 2'-methylidene (DMDC),[9] and 2'-fluoromethylidene[10] derivatives of 2'-deoxycytidine have also potent antitumor activity and the former two nucleosides are now being used in clinical trials against solid tumors.

In addition to these nucleoside antimetabolites, we have designed and synthesized a new type of antimetabolite, 2'-*C*-cyano-2'-deoxy-1-β-D-arabinofuranosylcytosine (CNDAC) as a potential DNA-self-strand-breaking nucleoside.[11] Introduction of a cyano group into the 2'β position would increase the acidity of the 2'α proton. If such a nucleoside is enzymatically phosphorylated to its 5'-triphosphate and then incorporated into DNA, the cyano group becomes β to the phosphodiester bond at the 3' position in the newly synthesized DNA. In this case, a β-elimination reaction would produce DNA self-strand breaks as illustrated in Figure 1. Since strand breaks in DNA by radiation therapy have been hypothesized to cause tumor cell death, it is worth examining whether CNDAC inhibits tumor cell growth or not.

CNDAC inhibited cell growth of various human tumor cells including sarcomas, osteosarcomas, fibroblastomas, and carcinoma *in vitro* with a unique spectrum different from that of *ara*-C.[11] CNDAC had a prominent antitumor activity toward P388 mouse leukemia, with a T/C value of >600%, and M5076 mouse reticulum cell sarcoma *in vivo*.[11] CNDAC also showed excellent antitumor activity against HT-1080 human fibrosarcoma, which is refractory to *ara*-C, in chick embryos or athymic mice.[12] Whether such potent antitumor efficacy of CNDAC comes as in our hypothesis or not is interesting to find out. In this report, we deal with the chemical stability of CNDAC, the inhibitory mode of CNDACTP toward DNA polymerase, and its metabolism.

Chemical Stability of CNDAC

We have found that when N^4-acetyl-CNDAC was treated with NH_3/MeOH at room temperature there was a glycosyl bond cleavage.[11b] A β-elimination reaction was observed to produce a 2'-*C*-cyano-2',3'-didehydro-2',3'-dideoxycytidine derivative when a 5'-protected CNDAC was treated with *N,N*-thiocarbonyldiimidazole[13] in DMF at room temperature. Moreover, even on treatment with *N,N*-carbonyldiimidazole under the same conditions, the dehydrated product was also obtained.[11b] These reactions may proceed intramolecularly from the 3'-thiocarbonylimidazole ester or the 3'-carbonylimidazole ester. Although these reactions were model studies, it can be imagined that the 2'α proton of CNDAC could have enough acidity to break DNA strands intramolecularly and/or intermolecularly, when it is incorporated into DNA. Therefore, we tried to measure the pKa value of the 2'α proton of CNDAC.

CNDAC was incubated in 0.01 M Tris-HCl buffer (pH 9) at 37 °C. After a 2-hr incubation, a sample was analyzed on HPLC using a reverse phase column. As shown in Figure 2a, three new peaks along with the starting material were detected. Structures of these compounds were assigned as cytosine (peak 2), 1,4-anhydro-2-*C*-cyano-2-deoxy-D-*erythro*-pent-1-enitol (peak 3), and 2'-*C*-cyano-2'-deoxy-1-β-D-ribofuranosylcytosine

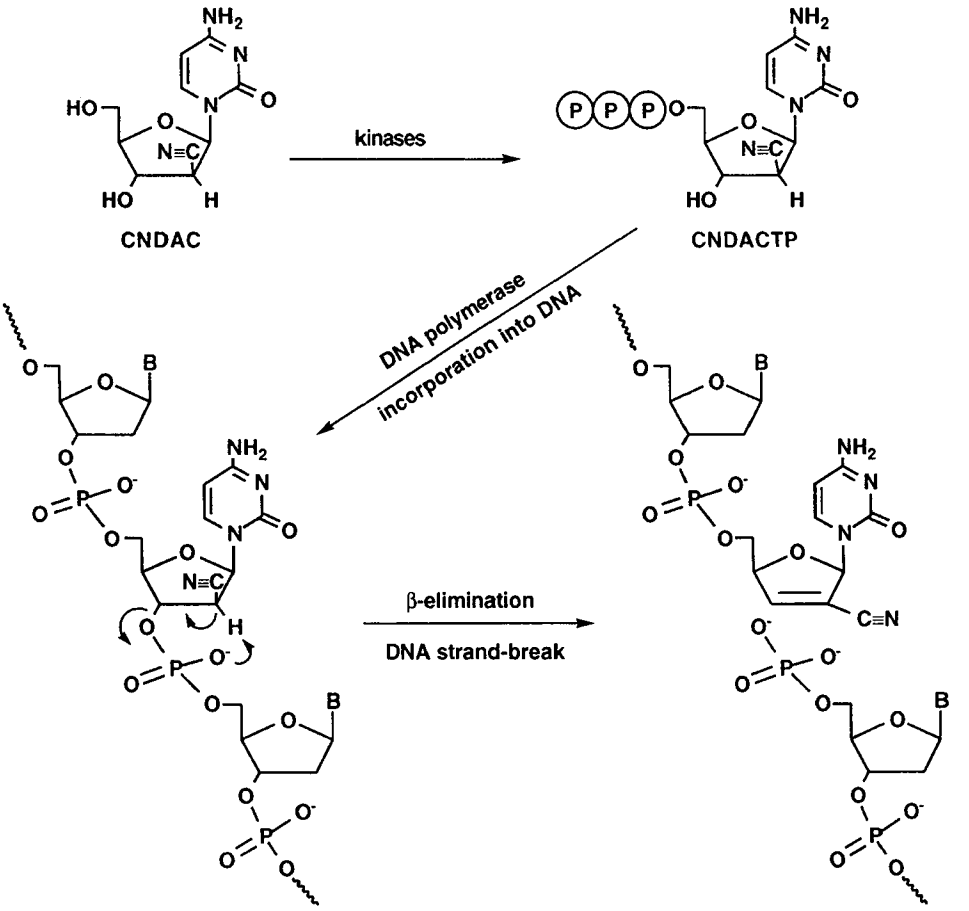

Fig. 1. A Possible Pathway of Degradation of a DNA-Strand Containing CNDAC

(CNDC, peak 4), respectively, by mass, NMR, and UV spectroscopies. The course of the reaction is shown in Figure 2b. When the isolated CNDC was treated under the same conditions, a similar chromatogram was obtained after 2 h. Therefore, CNDAC was in equilibrium with CNDC, and both nucleosides gradually decomposed to cytosine and the glycal (Figure 3). Since an admixture of cytosine and the glycal under the same conditions did not give nucleoside products, the decomposition processes are not reversible. This equilibrium was reached within about 2 h in this buffer at pH 9. Even at pH 7.5, either CNDAC or CNDC reached an equilibrium within about 38 h. In every case, the ratio of CNDAC and CNDC in the equilibrium was about 3:5, suggesting CNDC is more

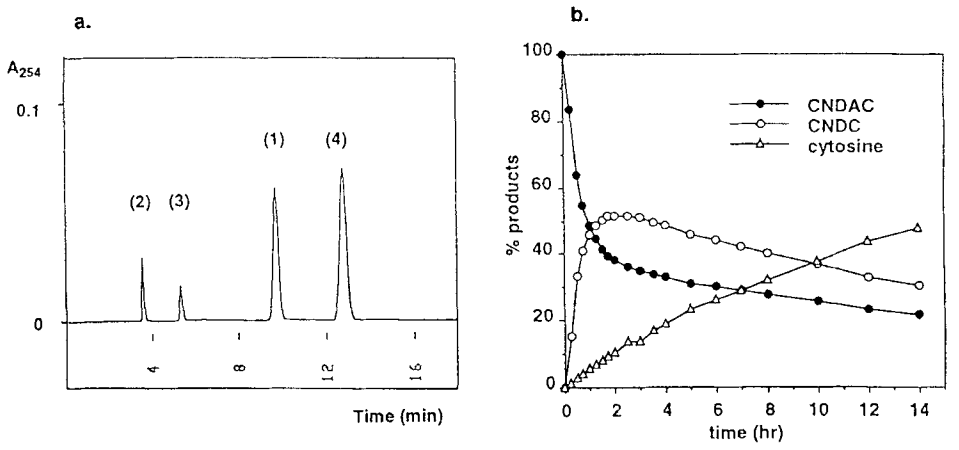

Figure 2. a) HPLC Chromatogram of the Epimerization-Degradation Reaction of CNDAC. b) Course of the Epimerization-Degradation Reaction of CNDAC.

thermodynamically stable than CNDAC. Increasing the pH of the buffer accelerated the reaction. However, we could not measure the time required to reach the equilibrium over pH 11.3 because the degradation of CNDAC to cytosine and the glycal proceeded too fast. Although we could not measure the pKa of the 2'α proton of CNDAC, we found that the 2'α proton would be acidic enough to be eliminated by weak bases. From these experiments, therefore, if CNDAC is incorporated into DNA, the phosphate oxygen could pick up the 2'α proton to result in strand scission.

It is noteworthy that CNDAC underwent epimerization in cell culture medium such as RPMI 1640-10% FCS under the conditions used to measure its tumor cell growth inhibitory activity. Although the glycal did not show any tumor cell growth inhibitory effects up to 100 μg/mL, CNDC had growth inhibitory activity against L1210 cells with an IC_{50} of 0.8 μg/mL, while for CNDAC the IC_{50} was 0.36 μg/mL.

Inhibitory Mode of CNDACTP Toward DNA Polymerase

Chemically synthesized CNDACTP from the corresponding CNDAC was treated with calf thymus DNA polymerase α using an activated DNA (Figure 4a). CNDACTP was a potent inhibitor of the polymerase with a Ki value of 0.16 μM, competitive to dCTP, the Km of which was 1.15 μM (Figure 4b).

Next, we examined in detail the inhibitory mode of action of CNDACTP toward DNA polymerase α using a chain extension method. As a primer and template for the

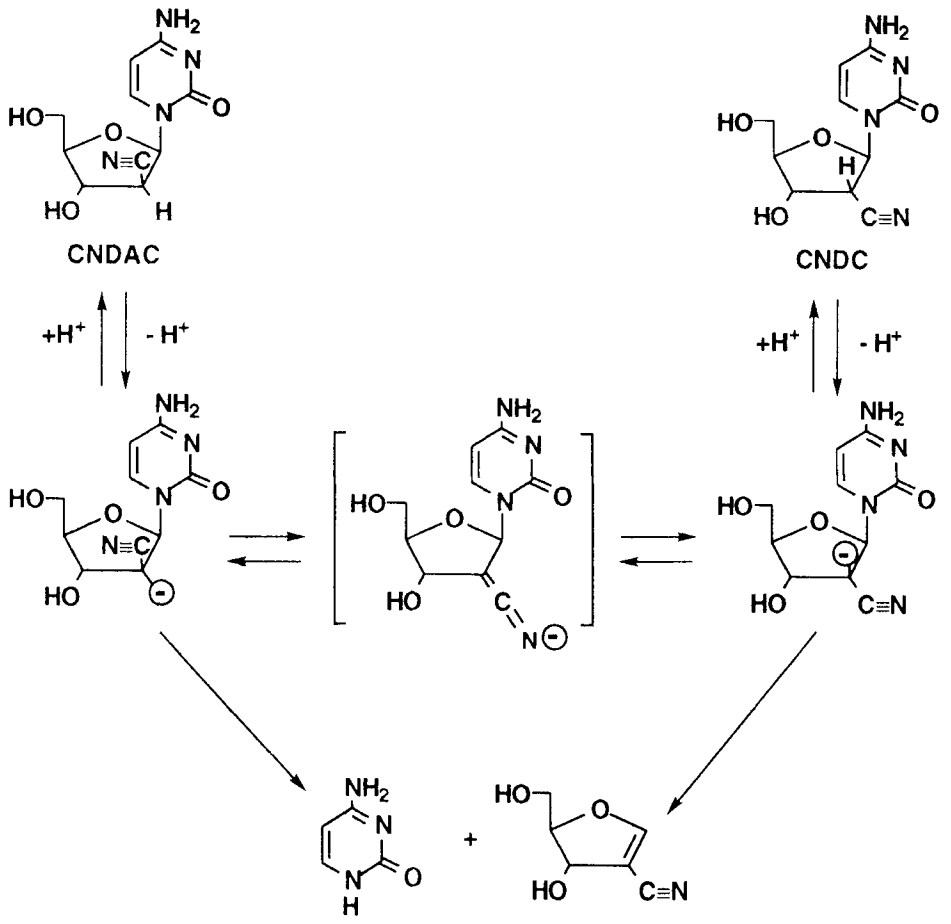

Figure 3. Epimerization and Degradation of CNDAC

enzyme reaction, ^{32}P-labeled 5'-GGACTTTCGC-3' and 3'-CCTGAAAGCGCAGTCG-ACT-C-5', respectively, were used. As shown in Figure 5, when each natural dNTP was added to the reaction mixture, chain-extension reactions proceeded smoothly (lanes 1-5). When CNDACTP, instead of dCTP, was added, it was incorporated into the primer opposite to G in the template (lane 6). Further addition of dATP to the reaction mixture, however, did not result in chain elongation (lane 7). Increasing the reaction time did not afford further extension of the primer. On the other hand, in the control experiment, addition of *ara*-CTP along with dGTP, TTP, and dATP gave a further extended band as shown in lane 8. This result is consistent with previous observations.[14] Thus, CNDACTP appeared to be a potent chain terminator.

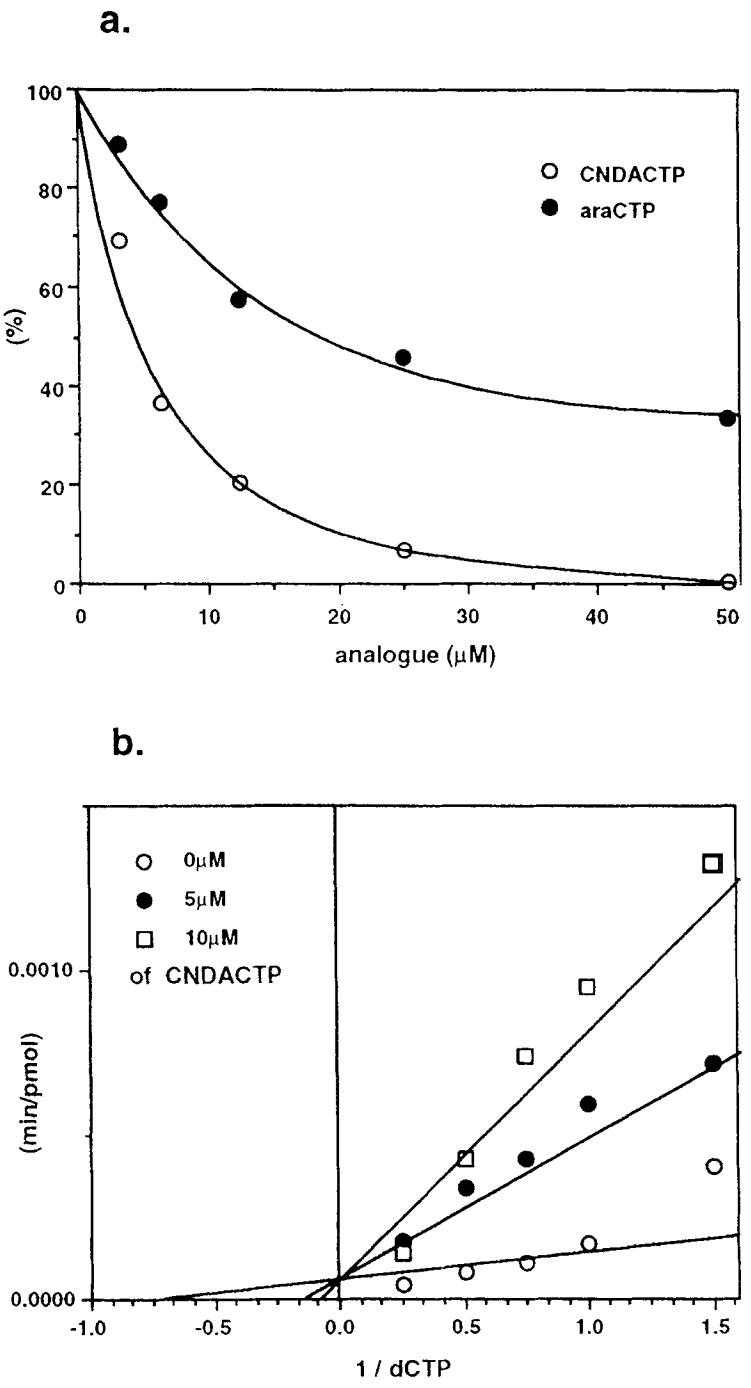

Figure 4. a) Inhibition of Calf-Thymus DNA Polymerase α by CNDACTP and *ara*-CTP. b) A Lineweaver-Burk Plot of the Enzyme Inhibition by CNDACTP.

As shown in Figure 1, during chain elongations of the newly synthesized DNA, the phosphate group of the nucleotide unit at the 3'-end position, which is next to CNDAC incorporated in the DNA, could possibly pick up the 2'α proton of CNDAC to cause a strand break by β-elimination. This possibility was next examined by analysis of nucleoside compositions of the corresponding bands in lane 6 and 7 in Figure 5. Without addition of dATP, the structure of the 3'-terminal nucleoside would be CNDAC or isomerized CNDC. However, after addition of dATP, if 2'-*C*-cyano-2',3'-didehydro-2',3'-dideoxycytidine (ddCNC) were detected, it is a proof of our DNA-self-strand breaking hypothesis.

This experiment was done using Vent DNA polymerase (exo$^-$) and the same primer (without ^{32}P-labeling)/template system in 0.02 M Tris-HCl buffer (pH 8.8) at 75 °C. The corresponding 13-mer band was extracted by H_2O from the polyacrylamide gel and the resulting oligomer was completely hydrolyzed to the corresponding nucleosides by a mixture of snake venom phosphodiesterase and alkaline phosphatase in Tris-HCl buffer (pH 7). The nucleoside mixture was analyzed on HPLC with a reverse phase column. Results are shown in Figure 6. Without addition of dATP, new peaks corresponding to CNDAC and CNDC were observed and these peaks was confirmed by authentic samples with co-migration experiments (Figure 6a). The composition of the nucleosides calculated from areas of the peaks [dA : dG : T : dC : C* (CNDAC+CNDC)] was 1.0 : 4.4 : 3.9 : 2.8 : 0.93 (3.4 : 6), the ratio of which is close to the theoretical value (1 : 4 : 4 : 3 : 1, respectively). In contrast to this experiment, when dATP was added, another new peak due to ddCNC was detected between the peaks corresponding to CNDAC and CNDC (Figure 6b). The ratio of dA : dG : T : dC : C* (CNDAC+ddCNC+CNDC) was 1.1 : 4.3 : 3.9 : 3.0 : 1.0 (3 : 5.3 : 1.7), this ratio is again close to the theoretical ratio. The ratio of ddCNC in C* was increased further when the incubation period was extended. Thus, it is clear that the detection of the peak corresponding to ddCNC provided evidence of the self-strand-breaking due to the β-elimination from the acidic 2'α proton of CNDAC.

Metabolism of CNDAC

Before studying the metabolism of CNDAC, we examined the effects of CNDAC, CNDC, and *ara*-C on the synthesis of DNA, RNA, and protein using [^{3}H]-thymidine, [^{3}H]-uridine, and [^{3}H]-leucine, respectively, in colo 320DM cells. After 2 h of incubation of the cells with 1 μg/ml of each nucleoside, the DNA synthesis was mainly inhibited by *ara*-C (98%), while 81% and 32% inhibition was observed by CNDAC and CNDC. However, although *ara*-C did not inhibit RNA synthesis (3%), CNDAC and CNDC inhibited it by 32% and 35%, respectively. At the same time, inhibition of protein synthesis was negligible with each nucleoside. After 6 h, all the nucleosides had inhibited DNA synthesis more than 93%. Again, CNDAC and CNDC inhibited the RNA synthesis

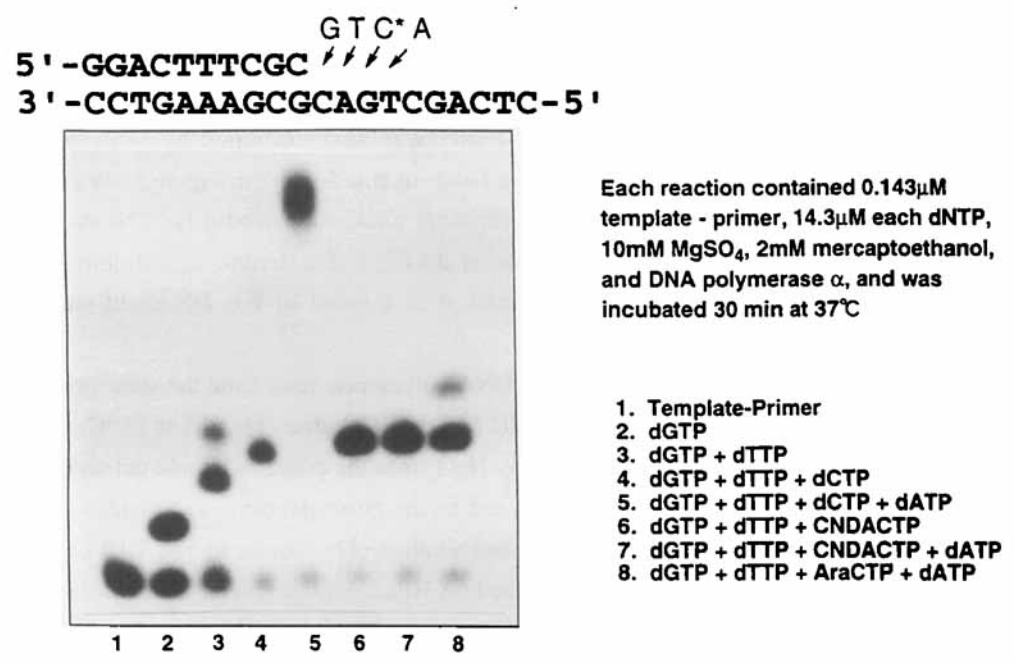

Figure 5. Effects of CNDACTP and *ara*-CTP on DNA Strand Elongation with DNA Polymerase α and a Synthetic Template/Primer Complex.

by 33% and 50%, respectively, as compared to 17% for *ara*-C. Therefore, the action of CNDAC and CNDC would be somewhat different from that of *ara*-C, and they inhibited both DNA and RNA synthesis.

Tumor cell growth inhibition of CNDAC toward KB cells was reversed by addition of 2'-deoxycytidine. This suggests that CNDAC would be phosphorylated to CNDACMP by the action of deoxycytidine kinase in the tumor cells. Further phosphorylation of CNDACMP was demonstrated using [^{14}C]-CNDAC to show the presence of CNDACDP and CNDACTP in KB cells. Therefore, CNDAC was metabolized by certain kinases to CNDACTP.

We next examined the action of cytidine deaminase (from guinea pig kidney), which efficiently deaminates *ara*–C to chemotherapeutically inactive *ara*-U. The rate of the deamination of cytidine (Km = 0.22 mM) was compared with other derivatives. *Ara*-C (Km = 0.35 mM), CNDC (Km = 0.52 mM), and CNDAC (Km = 1.0 mM) were substrates of the enzyme, and deamination rates (relative Vmax for cytidine) were 0.4, 0.16, and 0.08, respectively. Thus, CNDAC and CNDC are substrates of the cytidine deaminase with deamination rates that are 1/20 or 1/6 of that of cytidine, respectively.

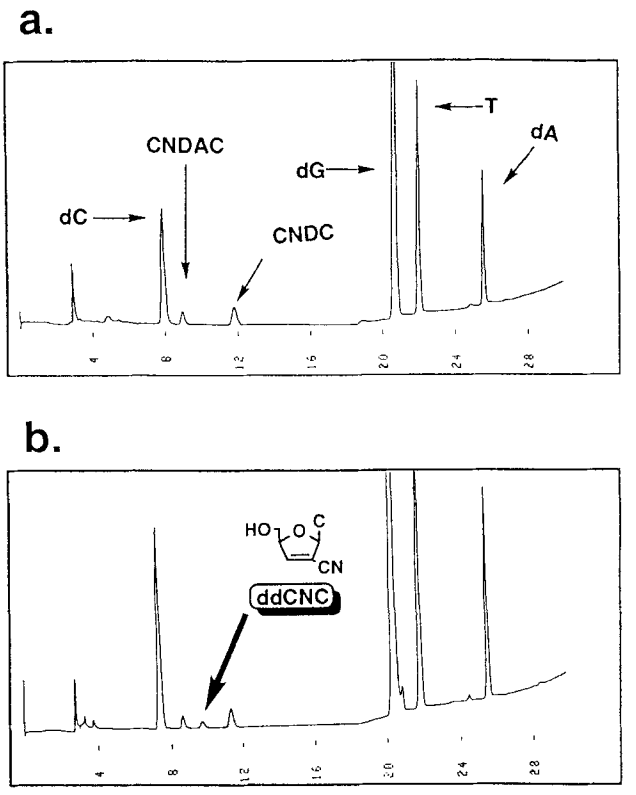

Figure 6. HPLC Analysis of a Mixture of Nucleosides Obtained by Complete Hydrolysis of the Chain-Elongated Primer. a) Chain-elongation without addition of dATP. b) Chain-elongation with addition of dATP.

Conclusion

Although CNDC might be a substrate of cytidine/uridine kinase, it is readily deaminated to CNDU by the deaminase and decomposed rapidly to uracil and the glycal. Chemically synthesized CNDACDP was not a potent inhibitor of *E. coli* ribonucleoside diphosphate reductase.[15] Since 5'-phosphates of CNDAC would be in equilibrium with 5'-phosphates of CNDC, the antitumor activity of CNDAC and CNDC would mainly be caused by its metabolites, CNDACTP and CNDCTP. CNDACTP inhibits DNA polymerase α by its incorporation into DNA and subsequent cleavage of the DNA strand by the β-elimination as we hypothesized in Figure 1. CNDCTP would inhibit RNA polymerase by a mechanism that is still unknown. In cell-cycle kinetics of CNDAC using

P388 mouse leukemic cells, it blocked cell distribution in the G2/M phase. This was different from the effect of *ara*-C, which caused cell growth arrest in the S phase.

Acknowledgments

This investigation was supported in part by Grants-in-Aid for Cancer Research and Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture of Japan.

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