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Cellular pharmacokinetics and pharmacodynamics of the deoxycytidine analog 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine (CNDAC)

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Abstract

The pharmacokinetics and pharmacodynamics of the novel clinical candidate 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine (CNDAC) were investigated in human lymphoblastoid CCRF-CEM cells and human myeloblastic leukemia ML-1 cells. Formation of CNDAC 5′-mono-, di-, and triphosphate (CNDACTP) was concentration-dependent; nucleotide accumulation was greater in the lymphoid cells than in the myeloid cells. The nucleotides were eliminated with linear kinetics from both lines, but were retained more effectively by the ML-1 cells. DNA synthesis was selectively inhibited by a 4-hr treatment with CNDAC in CCRF-CEM and ML-1 cells; the IC_{50} values were 1 and 0.8 μM, respectively. Evaluation of the polymerization reaction of a primer on an M13mp19(+) template by human DNA polymerase α indicated that CNDACTP was incorporated effectively (K_m = 0.22 μM) opposite a complementary dGMP in the template strand. CNDACTP competed with the normal substrate, dCTP, for incorporation, and the two nucleotides showed similar substrate efficiencies (V_{max}/K_m: dCTP = 0.91; CNDACTP = 0.77). Primer extension was potently inhibited by CNDAC triphosphate (K_i = 23 nM); once the analog had been incorporated, further extension was not observed *in vitro*, suggesting that primers containing a 3′-terminal nucleotide analog were high K_m substrates for polymerase α. Thus, the ability of human leukemia cells to effectively accumulate and retain CNDACTP, coupled with the favorable kinetics of competition for incorporation into DNA, and the relatively strong ability of the analog to terminate further extension, are likely to contribute to the cytotoxic action of CNDAC.

Keywords: Nucleoside analog; CNDAC; DNA synthesis; DNA polymerase

1. Introduction

Nucleoside antimetabolites have become a major class of chemotherapeutic agents for the treatment of cancer and viral diseases. ara-C (Fig. 1) is a standard component in remission induction [1,2] and maintenance therapy [3] for acute myelogenous leukemia, although it is less useful in other hematologic malignancies, and is inactive against solid tumors. In contrast, gemcitabine, another carbohydrate-modified analog of deoxycytidine [4], is effective against a variety of solid tumors [5,6], and preliminary studies suggest that it may have therapeutic value in hematological malignancies [7–9].

With respect to their mechanisms of action, it appears that incorporation of each analog into DNA is the dominant cause of cytotoxicity, and for this the formation of each triphosphate is requisite. Indeed, inhibition of DNA synthesis is the most prominent activity for ara-C [10,11] and gemcitabine [12,13]. This has been correlated with the intracellular concentration of each nucleoside triphosphate [12], and analog incorporation into DNA has been causally related to cytotoxicity [13,14]. Models of DNA synthesis using purified DNA polymerases that extend primers over either activated DNA, single-strand viral DNA, or synthetic oligodeoxynucleotide templates of defined sequences have

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Abbreviations: ara-C, ara-CMP, and ara-CTP, 1-β-D-arabinofuranosylcytosine and its 5′-mono- and triphosphate, respectively; CNDAC, CNDACMP, CNDACDP, and CNDACTP, 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine and its 5′-mono-, di-, and triphosphate, respectively; dNTP, deoxynucleoside triphosphate; CEM, CCRF-CEM human lymphoblastoid cells; and pol, DNA polymerase.

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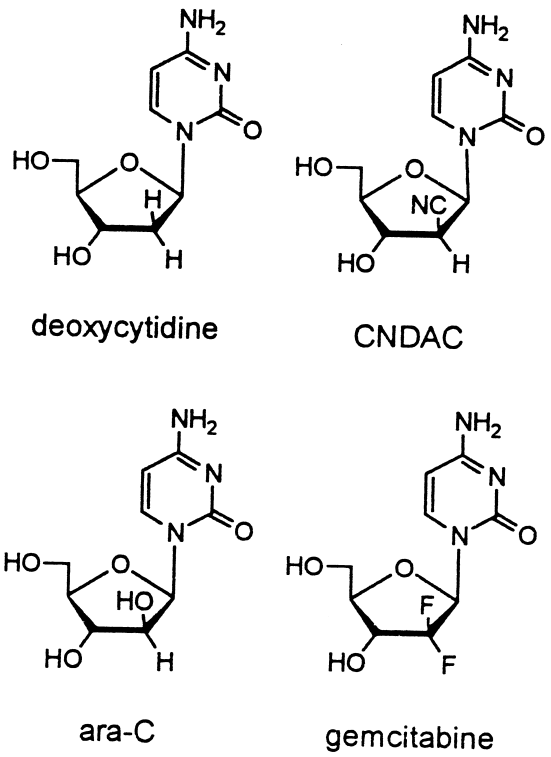

Fig. 1. Structures of deoxycytidine, ara-C, gemcitabine, and CNDAC.

been used to investigate the effects of the triphosphate of each nucleotide [10,13,15–17]. Each analog triphosphate competes with dCTP for incorporation by polymerases, a process in which the relative concentrations of these metabolites in cells and the affinity of the polymerases for them are likely to be the major determinants. Once incorporated, ara-C nucleotide acts as a high K_m substrate for the addition of a subsequent nucleotide [18], whereas DNA polymerases are capable of extending a 3′-terminal gemcitabine nucleotide, albeit at a reduced rate [13,17]. In a separate action, it is thought that ara-C nucleotides do not affect cellular dNTP levels directly [19]. In contrast, ribonucleotide reductase is inhibited by gemcitabine diphosphate [20,21], an action that decreases the cellular concentration of dCTP and other deoxynucleotides [22,23], likely favoring the incorporation of gemcitabine triphosphate into DNA. Thus, these structurally similar cytosine nucleoside analogs differ markedly and unpredictably with respect to both their mechanisms of action and the spectrum of their clinical activity.

We have designed and synthesized a new deoxycytidine analog, CNDAC [24,25], which is structurally similar to ara-C and gemcitabine in that the 2′-hydrogen of the sugar moiety has been replaced with a cyano group positioned in the arabino configuration (Fig. 1). In the conceptualization of this molecule, it was hypothesized that once the nucleotide of this analog was incorporated into DNA, the addition of a subsequent nucleotide at the 3-hydroxyl would initiate β-elimination, resulting in a single-strand break in the elongating DNA strand containing CNDAC nucleotide. Conversion of the analog during this process would produce 2′-*C*-cyano-2′,3′-didehydro-2′,3′-dideoxycytidine, a *de facto* chain-terminating nucleotide that is diagnostic of the β-elimination process. Subsequent studies in a cell-free model demonstrated that single-strand DNA breaks occurred after CNDACTP was incorporated into the elongating DNA strand by a bacterial DNA polymerase [26]. Recent investigations demonstrated that 2′-*C*-cyano-2′,3′-didehydro-2′,3′-dideoxycytidine was a minor component of radioactively labeled CNDAC metabolites isolated from the DNA of whole cells [27]. Experimental therapy studies indicated that CNDAC is relatively resistant to deamination by cytidine deaminase [28], and that cells lacking deoxycytidine kinase were resistant to CNDAC. Importantly, CNDAC has been shown to have effective growth inhibitory activity against human tumor cell lines *in vitro* and *in vivo* [24–29]. Compared with ara-C, CNDAC is more potent, and activity has been observed in a wider variety of cell lines and model murine and human tumors [28]. This information supported the recent initiation of clinical trials of the N^4-palmitoyl derivative of CNDAC (know as CS-682) in the United States [30].

Although the cytotoxicity of CNDAC and its efficacy against murine tumor models have been well established, the pharmacokinetics of the CNDAC nucleotides in cells and their actions on cellular processes have not been reported. The present study was conducted to investigate the intracellular metabolism of CNDAC and the incorporation of its nucleotide into cellular nucleic acids in human leukemia cells. The consequences of CNDACTP incorporation into DNA were also examined, using an *in vitro* system with purified human DNA polymerase α and a defined sequence DNA primer/template. The results suggest that CNDACTP is effectively accumulated and retained in cells, that it is a relatively good substrate for incorporation into DNA, and that this action strongly inhibits further DNA chain extension.

2. Materials and methods

2.1. Chemicals

CNDACTP was chemically synthesized using procedures described previously [31]. CNDAC and [5-^{3}H] CNDAC (specific activity 15 Ci/mmol) were supplied by the Sankyo Co., Ltd. [*methyl*-^{3}H]Thymidine and [5-^{3}H]uridine were from ICN Radiochemicals, Inc. The 17-base M13 universal sequencing primer and HPLC-purified dATP, dGTP, dCTP, and dTTP were obtained from Pharmacia LKB Biotechnology, Inc. M13mp19(+) DNA was from Life Technologies, Inc. 3(3,4-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from the Sigma Chemical Co. Pol α was purified as described previously [32].

2.2. Cell culture

The T-lymphoblastic cell line CEM was obtained from the American Type Culture Collection. The ML-1 myeloblastic leukemia cell line was a gift from Dr. M.B. Kastan. These two cell lines were maintained in exponential growth phase in RPMI 1640 suspension culture medium, supplemented with 5% fetal bovine serum (FBS) for CEM cells and 10% FBS for ML-1 cells. The cells were incubated at 37° in a humidified atmosphere containing 5% CO_2.

2.3. Effect of CNDAC on cell growth in vitro

The tetrazolium-based semiautomated colorimetric assay (MTT assay) was used to determine cell growth [33]. Cells (0.5×10^5 cells/mL) were incubated with 1–3000 nM CNDAC. After incubation for 4, 24, and 48 hr with CNDAC, the cells were washed twice with drug-free warm medium, and then incubated further in drug-free medium for a total of 96 hr. At the end of the incubation, 80 μL of the cell samples was seeded in 96-well round-bottom tissue culture plates (Falcon 3077, Becton Dickinson) containing 120 μL of fresh medium. MTT (25 μL, 3 mg/mL sterile in phosphate-buffered saline: NaCl, 8.1 g; KCl, 0.22 g; Na_2HPO_4, 1.14 g; KH_2PO_4, 0.27 g; per liter of H_2O, pH 7.4) was added to each well, and the samples were incubated for another 4 hr at 37°. The microplate was centrifuged at 450 g for 10 min at 4°, and then the medium was removed. Cell pellets were dissolved in 200 μL of dimethyl sulfoxide. After thorough mixing, the absorbance of the samples was read at 570 nm with a microplate reader MR 5000 (Dynatech Laboratories).

2.4. Measurement of intracellular nucleotides by HPLC

Cellular nucleotides were extracted with 60% MeOH overnight, and following centrifugation (12,000 *g* for 30 min at 4°), the supernatant was dried under vacuum and stored at −20° until analysis by high-pressure liquid chromatography. The dried extracts were reconstituted in phosphate-buffered saline, applied to a Partisil 10-SAX anion exchange column (250 × 4 mm, Waters Corp.), and eluted with a linear concentration gradient from 100% buffer A (0.005 M $NH_4H_2PO_4$, pH 2.8) to 100% buffer B (0.5 M $NH_4H_2PO_4$, 0.25 M KCl, pH 2.8) at a flow rate of 1.5 mL/min. The eluate was monitored by ultraviolet absorption at 256 nm, and the nucleoside mono-, di-, and triphosphates were quantitated by electronic integration with reference to external standards. The radioactivity associated with the respective nucleotides was measured with an on-line radioactive flow detector (model A 250; Packard Instrument Co.). The eluate was mixed automatically with scintillation fluid (Flo-Scint IV; Packard Instrument Co.) at a ratio of 1:3. The amount of each CNDAC nucleotide in an extract was calculated after reference to the specific activity of [^{3}H]CNDAC. The intracellular concentrations of nucleotides contained in the extract were calculated from a knowledge of the number of cell equivalents in the analyzed extract and the mean cell volume of the population. This calculation assumes that these nucleotides are uniformly distributed in total cell water.

2.5. Determination of DNA and RNA syntheses in whole cells

CEM cells and ML-1 cells in exponential growth phase were incubated with various concentrations of CNDAC for 4 hr in a 96-well filtration plate (Multiscreen-GV 96-well filtration plate, Millipore), and then either [^{3}H]thymidine (0.1 μCi/well) or [^{3}H]uridine (0.1 μCi/well) was added. Cells were incubated for another 30 min, and then diluted with cold phosphate-buffered saline and collected on filters using a vacuum manifold (Millipore). Each filter was then washed four times with cold 8% trichloroacetic acid, water, and 70% ethanol. The filters were dried, punched out, and the radioactivity retained on the filter was determined by liquid scintillation counting (1900CA; Packard Instrument Co.).

2.6. DNA primer extension assay

The 17-base M13 sequencing primer was labeled with ^{32}P at its 5′-end by T4 polynucleotide kinase and then annealed to its complementary site on the M13mp19(+) DNA template as described previously [13].

5′-^{32}P-GTAAAACGACGGCCAGT

3′-. . . .CATTTTGCTGCCGGTCACTTAA

GCTCGAGCCA. . . .

The reaction mixture (10 μL) contained the ^{32}P-labeled primer/template (0.3 μg/μL), various concentrations of dNTP and CNDACTP, and 0.15 unit of pol α as indicated in the figure legends. The DNA primer extension assay and sequencing gel analysis were carried out as described previously [13]. The reactions were incubated at 37° and analyzed on a 10% polyacrylamide sequencing gel. The radioactivity of the DNA bands of interest in the sequencing gel was quantitated with a Betascope 603 blot analyzer (Betagen Corp.). To determine the kinetic parameters of CNDACTP incorporation, the ^{32}P-labeled 17-mer primer/M13mp19(+) DNA was used as the template for DNA synthesis in the presence of 30 μM each of dGTP, dATP, and dTTP and various concentrations of CNDACTP. The relative velocity of incorporation was determined by dividing the radioactivity in the target site (site 23) by the radioactivity of the band one nucleotide smaller (site 22) [34,35]. The K_m and V_{max} values were calculated based on the Michaelis–Menten equation, using a computer-assisted program [36].

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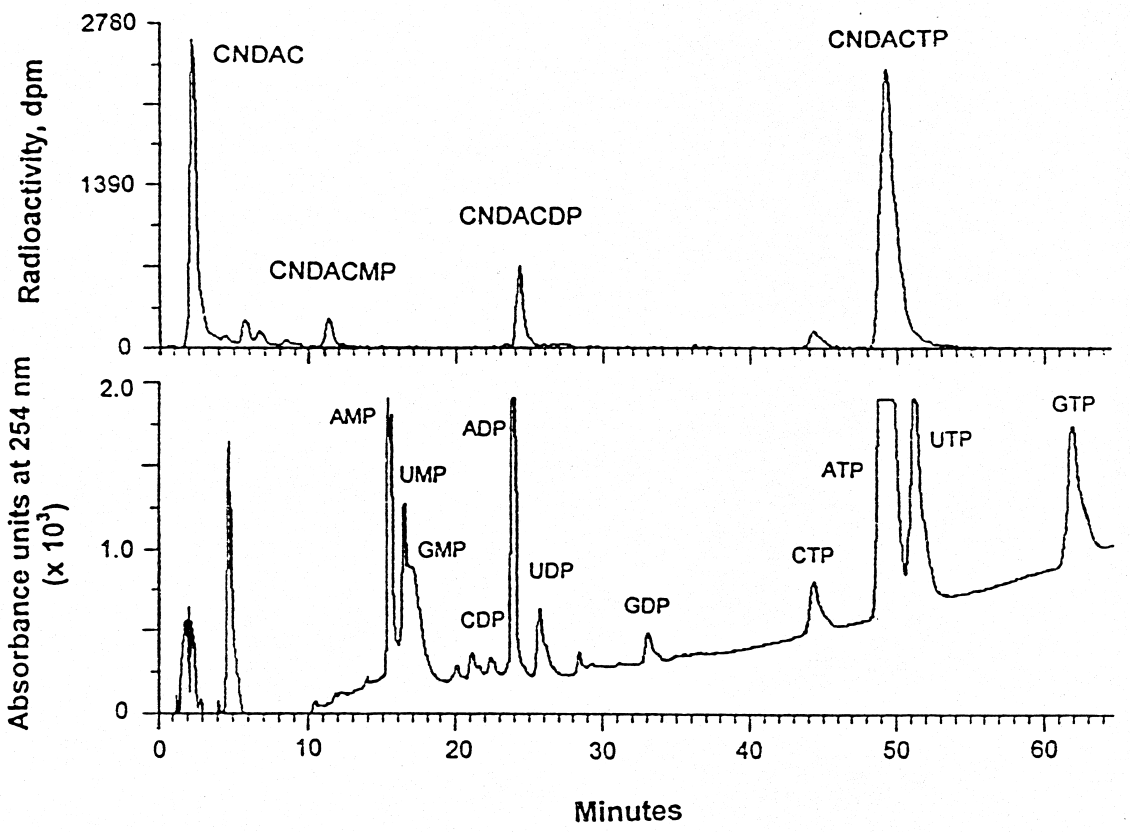

Fig. 2. HPLC analysis of [^{3}H]CNDAC metabolites in CEM cells. CEM cells were incubated with 30 μM [^{3}H]CNDAC for 4 hr, and then nucleotides were extracted with 60% methanol. Components of the extract from the equivalent of 5×10^5 cells were separated by HPLC. Radioactivity (upper) and absorbance units at 254 nm (lower) were quantitated as described in "Materials and methods."

CNDACMP by DNA primer extension *in vitro*, the 17-base M13 sequencing primer was annealed to a synthetic 41-base template for DNA synthesis.

5′-GTAAAACGACGGCCAGT

3′-CATTTTGCTGCCGGTCACACACA**G**CCA

CACACACACACACA

The reaction mixture (10 μL) contained the primer/41-mer template (0.6 μg/μL), 10 μM dTTP, 10 μM [α-^{32}P]dGTP (1 mCi/mL), 10 μM dCTP or 1 μM CNDACTP, and pol α as indicated in the figure legends. The reaction mixture was incubated at 37° for 30 min, and the reaction products were analyzed on a 10% polyacrylamide sequencing gel.

3. Results

3.1. Growth inhibition effects of CNDAC in CEM cells and ML-1 cells

The cell growth inhibitory effects of CNDAC were concentration- and time-dependent (not shown). After incubation of cells with CNDAC for 4, 24, and 48 hr, the cells were washed free of drug and incubated further in the drug-free medium for a total of 96 hr. The IC_{50} value for a 4-hr incubation was 126 ± 18 nM in CEM cells, but this decreased to 20 nM after a 24-hr incubation, the approximate population doubling time of this line. There was no further decrease in IC_{50} with longer exposures. In the case of ML-1 cells, the IC_{50} value for a 4-hr incubation was 158 ± 7 nM, but maximal growth inhibitory potency was observed after 48 hr of incubation, at an IC_{50} of 60 nM.

3.2. Metabolism of CNDAC in CEM cells and ML-1 cells

Exponentially growing cells were incubated with [^{3}H]CNDAC for 4 hr, and then the cellular nucleotides were extracted and separated by HPLC. The radioactivity of the respective CNDAC mono-, di-, and triphosphates was eluted at the times expected from prior analysis of authentic compounds (Fig. 2). Under these conditions, the analog triphosphate was the major cellular metabolite. After accounting for the specific activity of [^{3}H]CNDAC and the mean cell volume, the peak of radioactivity associated with the [^{3}H]CNDACTP indicated a cellular concentration of about 75 μM. The presence of substantial CNDAC in the cells and the relatively low amounts of the mono- and

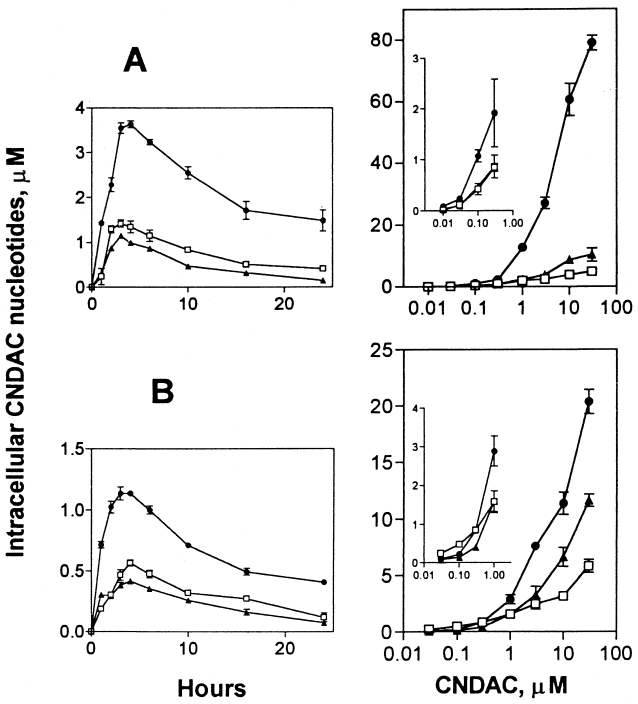

Fig. 3. Accumulation of CNDAC mono-, di-, and triphosphates in CEM (A) and ML-1 (B) cells. (Left) CEM and ML-1 cells were incubated with [^{3}H]CNDAC (30 and 100 nM, respectively) for the indicated times. Intracellular nucleoside mono-, di-, and triphosphates were extracted by methanol and analyzed by HPLC as described in "Materials and methods." Values are means ± SD. Key: (□) CNDACMP; (▲) CNDACDP; and (●) CNDACTP. (Right) Cells were incubated with different concentrations of [^{3}H]CNDAC for 4 hr, and then nucleotides were extracted and analyzed by HPLC as above. Key: (□) CNDACMP; (▲) CNDACDP; and (●) CNDACTP. Values are the means ± SD of single determinations in three identical experiments.

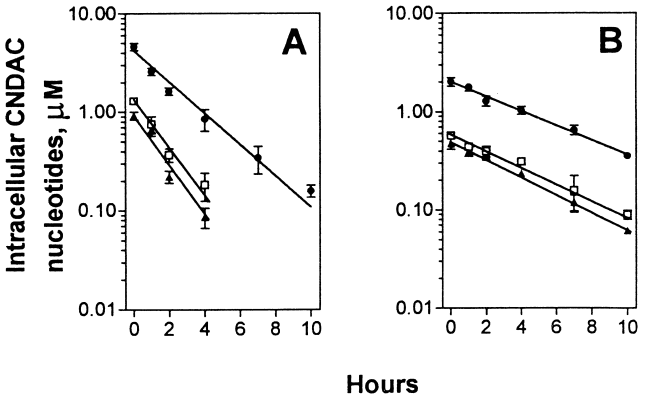

Fig. 4. Cellular elimination of CNDAC mono-, di-, and triphosphates. CEM (A) and ML-1 (B) cells were incubated with [^{3}H]CNDAC (30 and 100 nM, respectively) for 4 hr, washed twice with warm, drug-free medium, and then incubated in drug-free medium. At the indicated time points, intracellular nucleoside mono-, di-, and triphosphates were extracted with methanol and analyzed by HPLC as described in "Materials and methods." Key: (□) CNDACMP; (▲) CNDACDP; and (●) CNDACTP. Values are the means ± SD of single determinations in three identical experiments.

diphosphates are consistent with the conclusion that phosphorylation of CNDAC to the monophosphate, most likely by deoxycytidine kinase, is the rate-limiting step in triphosphate formation. Under these chromatographic conditions, CNDAC and its nucleotides eluted coincident with cellular metabolites that absorbed at 254 nm; therefore, all quantitation of nucleotides was conducted with reference to the specific activities of [^{3}H]CNDAC used in each experiment.

The accumulation of CNDAC mono-, di-, and triphosphates in CEM and ML-1 cells with respect to time and the CNDAC concentration was investigated next. Incubation of cells with [^{3}H]CNDAC (30 nM for CEM cells and 100 nM for ML-1 cells) for up to 24 hr resulted in an increasing accumulation of CNDACMP, CNDACDP, and CNDACTP in the first 4 hr, after which the analog nucleotide pools gradually decreased (Fig. 3, A and B, left). CNDAC nucleotides accumulated to somewhat greater concentrations in CEM cells than in ML-1 cells. The accumulation of CNDAC nucleotides was concentration-dependent when cells were incubated with up to 30 μM [^{3}H]CNDAC (Fig. 3, right panels). CNDACTP was the major metabolite of CNDAC in both CEM (Fig. 3A, right) and ML-1 cells (Fig. 3B, right). This was also true when cells were incubated with as little as 0.01 to 1 μM CNDAC, as shown in the insets to these figures. Although the temporal patterns of CNDAC nucleotide accumulation were similar in both cell lines, the concentrations of CNDACTP accumulated by CEM cells were 4-fold greater than those in ML-1 cells treated with the same CNDAC concentrations. In contrast, the accumulation of CNDACMP and CNDACDP was not significantly different between CEM and ML-1 cells. This could be explained if the nucleoside diphosphate kinase activity in CEM cells [20] was substantially greater than that in ML-1 cells.

To evaluate the ability of CEM and ML-1 cells to retain CNDAC nucleotides, cells were incubated with [^{3}H] CNDAC (30 and 100 nM, respectively) for 4 hr, and cellular [^{3}H]CNDAC nucleotides were measured at various times after washing cells in drug-free medium. The elimination kinetics of CNDAC nucleotides were similarly monophasic for each line, but the elimination rates of the analog nucleotides were more rapid from CEM cells than from ML-1 cells (Fig. 4). The elimination half-lives of CNDACMP, CNDACDP, and CNDACTP were 1.8, 1.8, and 2.4 hr, respectively, in CEM cells, whereas values of 4.9, 5.1, and 5.0 hr were observed in ML-1 cells.

3.3. Effect of CNDAC on deoxynucleotide pools

The diphosphate of gemcitabine acts as a mechanism-based irreversible inhibitor of ribonucleoside diphosphate reductase [21,37], an action that is associated with a decrease in dNTP pools [22,23]. Because of the competition between the deoxynucleotides and analog nucleotide

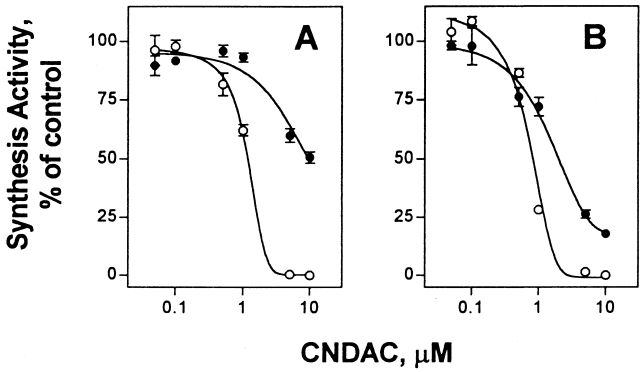

Fig. 5. Effect of CNDAC on DNA and RNA syntheses in CEM (A) and ML-1 (B) cells. Cells were incubated with the indicated concentrations of CNDAC for 4 hr and then labeled with [^{3}H]thymidine or [^{3}H]uridine for 30 min. The radioactivity incorporated into the acid-insoluble material was determined as described in "Materials and methods." Key: (○) percentage of DNA synthesis; and (●) percentage of RNA synthesis. Values are the means ± SD of triplicate determinations in three identical experiments. Values from each experiment were normalized to controls of that study. Absolute control values (mean ± SD) of radioactivity (dpm) incorporated for three experiments were: CEM cells: [^{3}H]Urd, 23,405 ± 4,129; [^{3}H]dThd, 13,476 ± 1,228; ML-1 cells; [^{3}H]Urd, 26,612 ± 5,728; and [^{3}H]dThd, 18,247 ± 3,257.

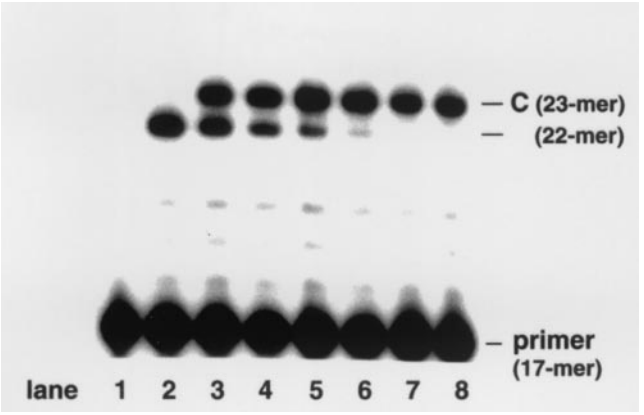

Fig. 6. Action of CNDACTP on DNA primer extension. The ability of human DNA polymerase α (0.15 unit/10 μL of reaction mixture) to extend the 5′-^{32}P-labeled primer/M13mp19(+) DNA (0.3 μg/μL) was evaluated in the presence of dATP, dGTP, dTTP, and various concentrations of CNDACTP. Lane 1, primer; lane 2, reaction with pol α and 30 μM each of dATP, dGTP, and dTTP; lanes 3–8, reactions identical to lane 2 but with 0.03, 0.1, 0.3, 1, 3, and 10 μM CNDACTP, respectively.

triphosphates for incorporation into DNA, the altered ratios of the cellular concentrations of the analog triphosphates and dCTP would favor an increased incorporation of the analogs into DNA. However, treatment with 0.03 to 10 μM CNDAC did not show a significant effect on dNTP pools in either cell line (not shown), although at 10 μM CNDAC, the intracellular CNDACDP concentration reached 5–10 μM (Fig. 3). Thus, it is unlikely that CNDACDP is an effective inhibitor of ribonucleoside diphosphate reductase at these concentrations. Similarly, CNDAC treatment did not appear to perturb the cellular levels of ribonucleoside triphosphates (not shown).

3.4. Action of CNDAC on DNA and RNA syntheses

The action of CNDAC on the syntheses of DNA and RNA was evaluated by measuring the incorporation of [^{3}H]thymidine and [^{3}H]uridine into cellular DNA and RNA, respectively. When cells were incubated with various concentrations of CNDAC for 4 hr, DNA synthesis was inhibited in a concentration-dependent manner in CEM (Fig. 5A) and ML-1 (Fig. 5B) cells. The IC_{50} values were 1.06 ± 0.04 and 0.83 ± 0.04 μM, respectively.

RNA synthesis in the CEM cells was not inhibited significantly by CNDAC at a concentration of 3 μM during a 4-hr incubation. When CEM cells were incubated with 10 μM CNDAC, RNA synthesis was reduced to about 50% of the control (Fig. 5A). In contrast, RNA synthesis in ML-1 cells was inhibited by a lower concentration of CNDAC. The RNA synthesis IC_{50} value in ML-1 cells was 2.13 ± 0.15 μM (Fig. 5B).

3.5. Action of CNDACTP on DNA strand extension in vitro

A DNA primer extension assay was used to further investigate the mechanism of action of CNDAC on DNA synthesis. Initial studies were conducted in the presence of dATP, dGTP, and dTTP but without dCTP to evaluate the incorporation of CNDACTP into an elongating DNA strand *in vitro* (Fig. 6). The bottom band indicates the position of the unextended 17-mer primer (lane 1). In the presence of dATP, dGTP, and dTTP, DNA polymerase α extended the primer to the pre-C site (site 22) and paused there due to the absence of dCTP required for incorporation into site 23 (lane 2). When various concentrations of CNDACTP (0.03, 0.1, 0.3, 1, 3, and 10 μM) were included in the reaction mixture (lanes 3–8), the analog was incorporated into the C site (site 23) by pol α. The density of the band at the C site (site 23) increased in a CNDACTP concentration-dependent manner with a concurrent decrease of the band density at the pre-C site (site 22). This indicated that CNDACTP was recognized by pol α as a substrate and incorporated at the C site.

The radioactivity associated with the bands at sites 22 and 23 was quantitated in a series of identical experiments to determine the incorporation kinetics of the analog. The apparent K_m value of CNDACTP incorporation into the C site by pol α was 0.22 ± 0.03 μM, and the apparent maximum velocity of the incorporation value (V_{max}) was 0.17 ± 0.02 fmol/min/unit (Table 1). Although the K_m for the incorporation of dCTP was approximately 10-fold greater than that of CNDACTP, the V_{max} for dCTP incorporation was more favorable than for CNDACTP, leading to similar values for their substrate efficiencies (V_{max}/K_m).

Table 1
Apparent kinetic parameters of CNDACTP and dCTP

Nucleotide	K_m (μM)	V_{max} (fmol/min/unit)	V_{max}/K_m	K_i (nM)
CNDACTP	0.22 ± 0.03	0.17 ± 0.02	0.77 ± 0.01	23 ± 1.0
dCTP	2.2 ± 0.23	2.0 ± 0.76	0.91 ± 0.02	

Values are means ± SD for determinations from three identical experiments.

3.6. Competition between CNDACTP and dCTP for incorporation into DNA

The action of CNDACTP on DNA primer extension by pol α was then examined in the presence of dCTP. In control reactions, the primer extension by pol α paused at the pre-C site because of the absence of dCTP in the reaction mixture (Fig. 7, lane 2). In contrast, full-length extension occurred when all four dNTPs were present in the reaction (lane 8). However, when 0.2 μM CNDACTP was added in a reaction mixture lacking dCTP (lane 3), primer extension paused at the first C site, and further elongation did not occur. Addition of 0.2 μM CNDACTP to the reaction mixture containing 0.3 to 10 μM dCTP resulted in DNA chain termination patterns consistent with competition between CNDACTP and dCTP (lanes 4–7). Furthermore, the intensity of pause bands caused by the incorporation of CNDACTP at the C sites diminished, and the amount of larger products (>23 nucleotides) increased in a dCTP concentration-dependent manner. This suggests that the ratio of intracellular CNDACTP and dCTP would be an important determinant of the quantity of the analog incorporated into DNA.

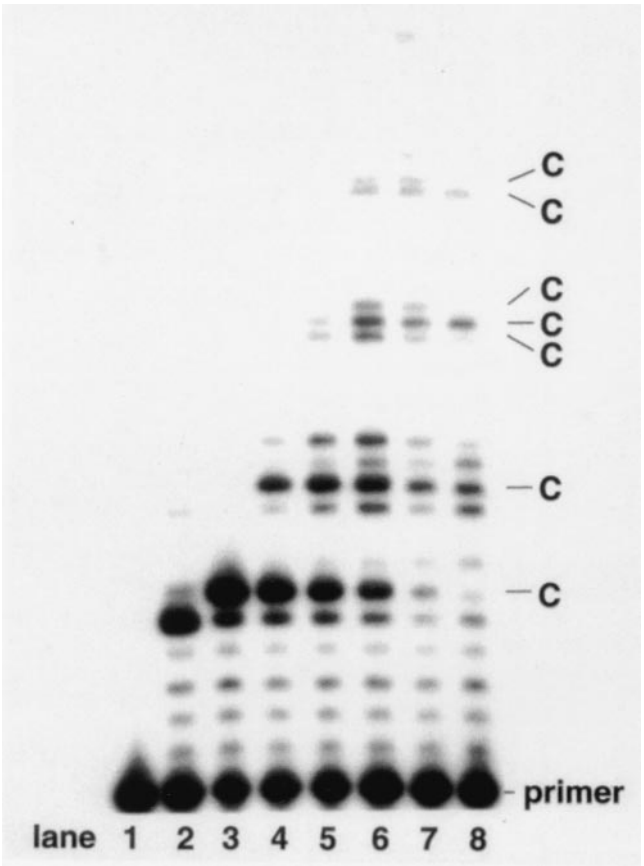

Fig. 7. Competition between dCTP and CNDACTP for incorporation into DNA by pol α. A primer extension assay was carried out in the presence of a 5′-^{32}P-labeled primer annealed to M13mp19(+) DNA (0.3 μg/μL), 0.15 unit of enzyme, 30 μM each of dATP, dGTP, and dTTP, and various concentrations of dCTP and CNDACTP. Lane 1, primer; lane 2, reaction with pol α and 30 μM dATP, dGTP, and dTTP, without dCTP and CNDACTP; lanes 3–7, reactions identical to lane 2 but with 0.2 μM CNDACTP and 0, 0.3, 1, 3, and 10 μM dCTP, respectively; lane 8, reactions identical to lane 2 but without CNDACTP and with 10 μM dCTP.

The competition between CNDACTP and dCTP for incorporation into DNA by pol α was characterized further by kinetic studies using a filter disk assay. The unlabeled 17-mer primer/M13mp19(+) DNA was used as the primer/template, and the incorporation of various concentrations of [^{3}H]dCTP into the primer by pol α was quantitated in the absence and presence of CNDACTP. When 30 μM dATP, dGTP, and dTTP, and 0.3 μg/μL of primer/template were in the reaction mixture, the incorporation of dCTP followed Michaelis–Menten kinetics, with an apparent K_m value of 2.2 ± 0.2 μM and an apparent V_{max} value of 2.0 ± 0.8 fmol/min/unit (Table 1). This reaction was inhibited by CNDACTP (30 and 100 nM) in a competitive manner. The apparent K_i value of 23 ± 1.0 nM was determined for pol α, and a K_i/K_m value of 0.01 was calculated for CNDACTP under these conditions.

To evaluate whether CNDAC incorporation could lead to DNA strand breaks upon addition of a subsequent nucleotide, an unlabeled 17-base primer was annealed to a 41-base template, and the primer/template was used for extension by pol α in the presence of [α-^{32}P]dGTP, dTTP, and either dCTP or CNDACTP (Fig. 8). In this primer/template construct, three of the six nucleotides incorporated by pol α prior to reaching the C site (site 24) would be [^{32}P]dGMP, thus labeling the extended primer. Successful extension beyond the C site would also label the portion of the primer beyond the analog, providing a probe to detect a fragment of primer cleaved by β-elimination subsequent to CNDACMP incorporation and extension. The formation of a [^{32}P]oligomer of 17 nucleotides or less would be consistent with a strand break via a β-elimination process. A 5′-^{32}P-labeled primer was included on the gel to designate its position (lane 2). In the absence of dCTP, pol α extended the primer to the pre-C site (site 23), but was unable to proceed farther (lane 3). There was a substantial increase in site 23 product when 5-fold pol α was added (lane 4); this also produced a small amount of full-length product, which was most likely due to misincorporation of dGTP or dTTP in the C site (site 24) and subsequent extension. In contrast, full-length extension occurred in the presence of 10 μM dCTP (5x K_m, lanes 7 and 8). When 1 μM CNDACTP (5x K_m) was added to the reactions in the absence of dCTP, it was clear that the

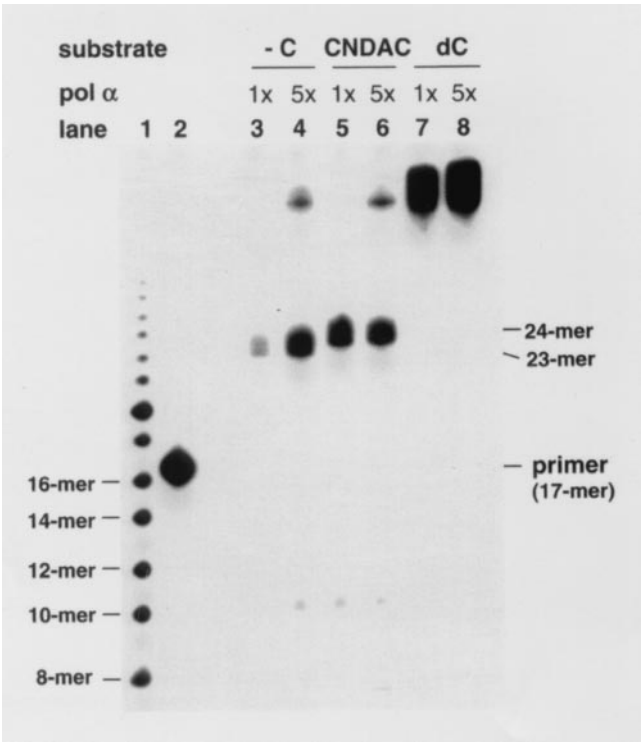

Fig. 8. Evaluation of primer extension after CNDAC nucleotide incorporation. A primer extension assay was carried out in reaction mixtures containing: a 17-mer primer annealed to a 41-mer template (0.6 μg/μL), 0.15 or 0.75 unit of pol α, 10 μM dTTP, 10 μM [α-^{32}P]dGTP (1 mCi/mL), and 10 μM dCTP or 1 μM CNDACTP. Lane 1, DNA size marker; lane 2, primer; lanes 3 and 4, reaction without CNDACTP or dCTP and 0.15 and 0.75 unit of pol α, respectively; lanes 5 and 6, reaction with CNDACTP and 0.15 and 0.75 unit of pol α, respectively; lanes 7 and 8, reaction with dCTP and 0.15 and 0.75 unit of pol α, respectively.

polymerase proceeded to site 24, demonstrating the incorporation of the analog (lane 5) into the extending primer. In this model, increasing the amount of polymerase (lane 6) failed to raise the amount of full-length product above the level of that due to misincorporation (compare with lane 4). If extension after CNDACMP incorporation occurred, and if DNA was cleaved at the 3′ position of CNDACMP after addition of deoxynucleotide(s), ^{32}P-labeled short-length DNA (17 nucleotides or less) should have appeared on the gel using this method. However, no detectable bands appeared in the region between 8 and 17 nucleotides, even when the reaction contained 5x polymerase (lane 6). Unfortunately, the area of the gel that would contain DNA fragments shorter than 8 nucleotides exhibited background in all the reactions which precluded evaluation of fewer than 7 nucleotides (not shown). The use of 10x dTTP and dGTP in attempts to force the polymerase to extend the primer beyond the CNDACMP incorporation site were not successful (not shown). This is consistent with either the rapid formation of a 3′-deoxy product subsequent to β-elimination or a 3′-terminal CNDAC nucleotide substrate with an extremely high K_m for deoxynucleotide addition by pol α.

4. Discussion

The most prominent mechanism of cytotoxicity by ara-C and gemcitabine is the inhibition of DNA synthesis. The loss of cell viability caused by these drugs has been strongly correlated with intracellular accumulation of the analog triphosphates, and subsequently their incorporation into DNA [12–14]. The present study evaluated the intracellular metabolism of the new cytosine nucleoside analog, CNDAC, and the actions of its triphosphate on DNA synthesis.

Accumulation of CNDAC nucleotides in both cell lines increased in a concentration-dependent manner over a 1000-fold range of CNDAC concentrations during 4-hr exposures (Fig. 3). The accumulation of CNDACTP, the major cellular metabolite, was greater in CEM cells than in ML-1 cells, and was retained more effectively by the myeloblastic leukemia cells (Fig. 4). Nevertheless, the concentration of CNDACTP that accumulated during a 4-hr incubation was smaller than that of either ara-C triphosphate or gemcitabine triphosphate in CEM cells [20]. This may be attributed to different substrate characteristics for membrane transport, metabolic clearance by deaminases, phosphorylation by kinases, and the activities of nucleotidases and phosphatases that govern the cellular pharmacokinetics of normal nucleosides and their analogs. These parameters clearly differ among cell lines and are sensitive to structure.

DNA synthesis in whole cells was inhibited by CNDAC treatment in CEM and ML-1 cells (Fig. 5). As CNDAC did not affect dNTP pools, it may be assumed that the mechanism of DNA synthesis inhibition was related to the incorporation of the analog into DNA. Consistent with this, the *in vitro* DNA primer extension experiments demonstrated that CNDACMP was incorporated into the C site of the elongating DNA strand (Fig. 6), and appeared to cause termination of primer DNA elongation at the site of the analog incorporation. DNA chain termination activity was also observed when ara-CTP and other arabinosyl nucleotides were used in studies of similar design [13,15,32]. Interestingly, the chain termination effect elicited by ara-C or gemcitabine nucleotide incorporation was not absolute for DNA elongation continued, albeit at a slower rate, following the incorporation of ara-CMP or gemcitabine monophosphate in cell-free systems [13,15]. In contrast, after CNDACMP was incorporated into the 3′-terminus of the elongating DNA primer strand, further elongation of the DNA strand was not observed in the *in vitro* reaction in the absence of dCTP (Fig. 6). Reactions containing increasing concentrations of dCTP did permit the elongation of primers in the presence of CNDACTP (Fig. 7), illustrating the competitive relationship between these nucleotides for incorporation by the polymerase. CNDACTP was an effective substrate for pol α, which exhibited a substrate efficiency that was similar to that for dCTP incorporation (relative V_{max}/K_m = 84%, Table 1). This is in contrast with the poor substrate properties of gemcitabine triphosphate for this enzyme

(V_{max}/K_m = 2.5%, [13]). These results suggest that CNDACTP is a relatively good substrate for incorporation and the resultant 3′-terminal CNDAC nucleotide is a potent chain terminator. However, the mechanism by which the latter event occurs is not clear.

One explanation for the inability of purified human DNA polymerase α to extend the primer chain once a CNDAC nucleotide has been incorporated (Fig. 7) is that the analog is a relatively poor substrate for addition of a subsequent nucleotide by the enzyme. While the primer extension assay provides useful comparisons among nucleotide analogs and different polymerases, it is of low efficiency relative to more complex models of replication or of the actual processes in intact cells. Thus, the answers given by this system may not quantitatively mimic cellular processes. For instance, it is clear that in whole cells most of the incorporated ara-C nucleotides are associated with internucleotide linkages [38, 39]. In contrast, ara-CMP at the 3′-terminus of a primer appears to be a high K_m substrate for deoxynucleotide addition by several DNA polymerases in primer extension assays [12]. In the present study, the inability of greatly increased concentrations of dTTP and dGTP substrates or elevated enzyme levels to force the polymerase to extend the primer beyond the CNDACMP incorporation site (Fig. 8) could be due to the poor substrate quality of the analog. Alternatively, these findings are also consistent with the formation of a 3′-deoxy product, a *de facto* chain terminator, that would be generated subsequent to a β-elimination process.

To evaluate the β-elimination hypothesis in the current experimental context, we designed an assay with the goal of revealing the formation of short fragments of newly synthesized DNA that might be liberated subsequent to the extension of CNDACMP (Fig. 8). Although this clearly confirmed the primer termination properties of the incorporated analog, no newly synthesized DNA fragments were observed between 8 and 17 nucleotides in length, a size consistent with those expected to be liberated by a strand break after β-elimination. Unfortunately, high background in the region of the gel containing DNA fragments of less than 7 nucleotides was inherent in the assay, despite numerous attempts to eliminate this limitation. This prevented analysis of fragments of these sizes that might have been liberated by β-elimination. In earlier experiments when we used *Vent* DNA polymerase (exo$^-$) to catalyze the primer extension, reverse-phase HPLC analysis of the products demonstrated β-elimination-based DNA strand-breakage [26]. This result suggests that either DNA elongation efficacy by pol α is lower than *Vent* polymerase *in vitro* or that the *in vitro* reaction conditions in the present study may not have been suitable for pol α to extend DNA from the 3′-OH of CNDAC at the 3′-terminus. Although DNA replication in intact cells is much more complex than the simple *in vitro* model we have employed, analysis of the products by this technique may reflect the true nature of the reactions taking place *in vivo*. Recently, Hanaoka et al. [27] used high pressure liquid chromatography and mass spectrometry to determine that a minor portion of the CNDAC nucleotide incorporated into the DNA of KB cells was 2′-*C*-cyano-2′,3′-didehydro-2′,3′-dideoxycytidine, which would be formed following β-elimination. Clearly, further studies must be conducted to provide definitive evidence regarding the ability of CNDAC nucleotides in DNA to initiate a β-elimination process that would result in DNA strand-breakage. Although CNDAC inhibited RNA synthesis measured in whole cells (Fig. 5) and was detected from RNA fractions (data not shown), the mechanism of this action is not known. It may be that if CNDACTP is a substrate for RNA polymerase(s), incorporation into transcripts could affect the information flow from the genome, as is the case for both the purine nucleoside analog fludarabine [40] and the pyrimidine nucleoside 3′-ethynylcytidine [41]. The relative amount of CNDAC incorporated into DNA and RNA was 7:1 in ML-1; a lesser proportion of the analog was detected in the RNA of CEM cells in which the DNA:RNA ratio was 50:1. This comparatively high efficiency of CNDAC incorporation into RNA in ML-1 cells is consistent with the level of RNA synthesis inhibition by CNDAC in these cells relative to that in CEM cells (Fig. 5). This effect on RNA synthesis by CNDAC may contribute to its cytotoxicity, particularly in more indolent cell populations in which RNA synthesis would take on more importance, as appears to be the case for fludarabine [42].

In conclusion, CNDAC is readily anabolized in human leukemia cell lines and accumulates as the triphosphate. CNDACTP is a relatively good substrate for incorporation into DNA by DNA polymerase α, but primers bearing CNDACMP at the 3′-terminus are poor substrates for extension. Accordingly, DNA synthesis is strongly inhibited in cells treated with CNDAC. Promising evidence that extension of this in DNA may lead to strand breaks should be confirmed and pursued, as this action is likely to have implications for novel approaches to combination therapy strategies.

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