0026-895X/01/5904-725–731$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 59:725–731, 2001

Vol. 59, No. 4
584/891504
Printed in U.S.A.

2′-*C*-Cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine: A Novel Anticancer Nucleoside Analog that Causes Both DNA Strand Breaks and G_2 Arrest

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Received September 15, 2000; accepted December 15, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The mechanism of 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine (CNDAC) action was investigated in human lymphoblastoid CEM cells and myeloblastic leukemia ML-1 cells. CNDAC was metabolized to its 5′-triphosphate and incorporated into DNA, which was associated with inhibition of DNA synthesis. After incubation of cells with [^{3}H]CNDAC, metabolites were detected in 3′→5′ phosphodiester linkage and at the 3′ terminus of cellular DNA. Specific enzymatic hydrolysis of DNA demonstrated that the parent nucleoside and its 2′-epimer 2′-*C*-cyano-2′-deoxy-2-*ribo*-pentofuranosylcytosine accounted for approximately 65% of the total analogs incorporated into DNA and essentially all of the drug in the 3′→5′ phosphodiester linkage. In contrast, all detectable radioactivity at 3′ termini was associated with 2′-*C*-cyano-2′,3′-didehydro-2′,3′-dideoxycytidine. This de facto DNA chain-terminating nucleotide arises from an electronic characteristic and cleavage of the 3′-phosphodiester bond subsequent to the addition of a nucleotide to the incorporated CNDAC moiety by β-elimination, a process that generates a single strand break in DNA. Investigation of the biological consequences of these actions indicated that, after incubation with cytostatic concentrations of CNDAC, cell cycle progression was delayed during S phase, but that cells arrested predominantly in the G_2 phase. This differed from the S phase-arresting actions of ara-C and gemcitabine, other deoxycytidine analogs that inhibit DNA replication but do not cause strand breaks. Thus, once incorporated into DNA, the CNDAC molecule appears to act by a dual mechanism that 1) delays the progress of further DNA replication, but 2) upon addition of a deoxynucleotide results in the conversion of the incorporated analog to a de facto DNA chain terminator at the 3′ terminus of a single strand break. It is likely that DNA strand breaks trigger cell cycle arrest in G_2.

Nucleoside analogs, the derivatives of the natural purine and pyrimidine nucleosides, are effective in the clinical treatment of human cancer or viral diseases. Arabinosylcytosine (ara-C), gemcitabine, fludarabine, and 2-chlorodeoxyadenosine are examples of this class of drugs that have therapeutic activity against human malignancies (Saven and Piro, 1994; Estey, 1996; Kaye, 1998; Keating et al., 1998). The key biochemical pathway responsible for the cytotoxic action of nucleoside analogs is mediated mainly by the incorporation of the drug into DNA, which subsequently results in a pause in or termination of DNA synthesis (Townsend and Cheng, 1987; Huang et al., 1990, 1991; Chunduru et al., 1993). For example, the triphosphates of ara-C and gemcitabine, two deoxycytidine analogs having potent anticancer activity, are incorporated into the C sites of the DNA strand in primer extension assays and cause a cessation of DNA strand elongation at the drug incorporation sites (Townsend and Cheng, 1987; Huang et al., 1991). However, useful as such assays may be, they do not reflect the complexity of the in vivo situation. For example, each analog is found predominantly in 3′→5′ phosphodiester linkage in DNA extracted from cells incubated with either analog (Major et al., 1982; Huang et al., 1991). Inhibition of the analog incorporation by aphidicolin results in suppression of the cytotoxic activity of the compounds (Huang and Plunkett, 1995). Clearly, the close correlation between the degree of drug-induced cell death and the amount of incorporated analog molecules in cellular DNA strongly suggests that the incorporation of these nucleoside analogs into DNA is a key cytotoxic event (Kufe et al., 1980; Huang et al., 1991).

2′-*C*-Cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine (CNDAC) (Fig. 1) is a newly synthesized analog of deoxycytidine (Matsuda et al., 1990, 1991). Its novel chemical properties

This work was funded in part by Grant CA28596 from the National Cancer Institute, Department of Health and Human Services, Bethesda, MD, and by Sankyo Company Ltd., Tokyo, Japan.

ABBREVIATIONS: ara-C, arabinosylcytosine; CNDAC, 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine; CNDACMP, CNDAC 5′-monophosphate; CNDC, 2′-*C*-cyano-2′-deoxy-1-β-D-*ribo*-pentofuranosylcytosine; CNddC, 2′-*C*-cyano-2′,3′-didehydro-2′,3′-dideoxycytidine; HPLC, high-performance liquid chromatography.

and promising anticancer activity in experimental systems (Matsuda et al., 1991; Tanaka et al., 1992; Azuma et al., 1993) have encouraged investigation of its activities in clinical trials (Donehower et al., 2000). This compound was designed as a mechanism-based DNA self-strand-breaking nucleoside (Matsuda et al., 1991). It was hypothesized that introduction of a cyano group at the 2′-β-position as an electron-withdrawing moiety would increase the acidity of the 2′α-proton. It was predicted that phosphorylation of the 3′-hydroxyl group would alter the electronic structure of the sugar moiety of CNDAC and that this structure would be extremely unstable. It was also envisioned that addition of a subsequent nucleotide by a DNA polymerase to a CNDAC moiety in DNA would initiate such instability, causing a break in the DNA strand. Specifically, Matsuda et al. (1991) postulated that this would lead to dephosphorylation
at the 3′-phosphodiester linkage in a β-elimination process that would result in the conversion of the CNDAC molecule to form 2′-*C*-cyano-2′,3′-didehydro-2′,3′-dideoxycytidine (CNddC), which is unique to this process. Because CNddC lacks a 3′-hydroxyl group, it is a de facto DNA chain terminator; therefore, its formation would probably cause a single-strand DNA break that could not be repaired by ligation.

Several lines of experimental evidence support this hypothesis. As a model reaction of the phosphorylation, chemical ligation of the 3′-hydroxyl group of CNDAC with *N*″,*N*′-carbonyldiimidazole resulted in the production of CNddC (Azuma et al., 1993). A chemically synthesized dinucleoside monophosphate of CNDAC-*p*-halogenated thymidine was also shown to be very unstable under basic conditions (Hayakawa et al., 1998). Recent studies have shown that CNDAC triphosphate is a good substrate for insertion by human DNA polymerase-α in a primer extension assay, but once the analog is incorporated into the extending DNA primer, it is a poor substrate for the addition of a subsequent nucleotide by the enzyme (Azuma et al., 2001). Extension of the CNDAC nucleotide in DNA using a primer extension assay employing a bacterial DNA polymerase resulted in the formation of CNddC at the 3′ terminus of the primer (Matsuda and Azuma, 1995). Finally, Hanaoka et al. (1999) demonstrated the presence of CNddC in hydrolysates of DNA isolated from KB cells after CNDAC treatment, indicating that β-elimination occurs in intact cells. However, the prevalence of this reaction and the fate of the analog molecules in DNA remain to be determined.

This type of DNA damage is clearly different from that caused by other nucleoside analogs such as ara-C and gemcitabine, which terminate or pause DNA synthesis at the site of incorporation (Townsend and Cheng, 1987; Huang et al., 1991) and presumably stall the progress of the replication fork in intact cells. Because of this major difference in their modes of action, we hypothesize that the cellular responses to CNDAC are qualitatively different from those to ara-C and gemcitabine. Thus, the present study was conducted to test the hypothesis by investigating the extent to which CNDAC is incorporated into DNA, the identity and location of its metabolites in DNA, and the incidence of β-elimination after CNDAC incorporation into cellular DNA in intact human leukemia cells. Furthermore, the cellular responses to CNDAC incorporation were compared with those to ara-C and gemcitabine with respect to changes in cell cycle distribution and the associated molecular events.

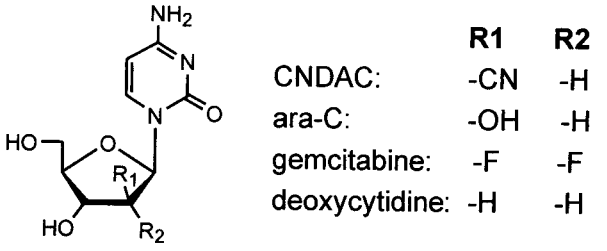

Fig. 1. Structure of CNDAC, ara-C, gemcitabine, and deoxycytidine.

Materials and Methods

Chemicals and Antibodies. CNDAC and [^{3}H]CNDAC (specific activity, 15 Ci/mmol) were supplied by Sankyo Co., Ltd. (Tokyo, Japan). CNddC was synthesized as described previously (Azuma et al., 1993). Arabinosylcytosine, nocodazole, and aphidicolin were obtained from Sigma Chemical Co. (St. Louis, MO). Gemcitabine was provided by Lilly Research Laboratories (Indianapolis, IN).

cdc2 Kinase polyclonal antibody (C-terminal) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Phospho-specific cdc2 (Tyr15) antibody was obtained from New England BioLabs, Inc. (Beverly, MA). Cyclin B1 polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Amersham Pharmacia Biotech (Piscataway, NJ) supplied β-actin monoclonal antibody.

Cell Culture. The T-lymphoblastic cell line CCRF-CEM was obtained from the American Type Culture Collection (Manassas, VA). The ML-1 myeloblastic leukemia cell line was a gift from Dr. M. J. Kastan (Johns Hopkins University, Baltimore, MD). The cells were maintained at 37°C in RPMI 1640 suspension culture medium, supplemented with 5% (CEM) or 10% (ML-1) fetal bovine serum in a humidified atmosphere containing 5% CO_2.

Incorporation of CNDAC 5′-Monophosphate (CNDACMP) into DNA and RNA in Whole Cells. Exponentially growing cells (10^7 cells) were incubated with [^{3}H]CNDAC for 24 h, washed twice with cold PBS (0.123 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4), and then digested in a solution containing 10 mM Tris-HCl, pH 8, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and proteinase K (200 μg) at 50°C for 12 h. Subsequently, cellular nucleic acids were obtained by successive extractions with phenol/chloroform/isoamyl alcohol (25:24:1) (Iwasaki et al., 1997). The nucleic acid was recovered by ethanol precipitation, dissolved in 5 mM EDTA, and supplemented with formamide to 50% (v/v) in a final volume of 1 ml. The solution was heated to 80°C for 5 min, brought to 4.5 ml with 5 mM EDTA, and then mixed with 4.5 ml of saturated Cs_2SO_4. The solution was centrifuged in a Ti75 rotor, first at 50,000 rpm for 16 h and then at 40,000 rpm for 1 h in a Beckman ultracentrifuge (model L5–75; Beckman Instruments, Palo Alto, CA). The resulting gradients were aspirated from the top with a Densi-Flow IIC apparatus (Buchler, Fort Lee, NJ), and 0.6-ml fractions were collected. Each fraction was diluted with H_2O to 1 ml, and the UV absorbance at 254 nm was determined by spectrophotometry (Response UV-VIS; Gilford, Oberlin, OH). The radioactivity associated with each fraction was determined by liquid scintillation counting. In separate cultures, the fractions containing [^{3}H]thymidine-labeled DNA and [^{3}H]uridine-labeled RNA were determined in parallel experiments and used as the controls for identifying the DNA and RNA peak fractions in samples labeled with [^{3}H]CNDAC.

Degradation of Cellular DNA to 3′-Monophosphates and Nucleosides. Exponentially growing cells (6×10^6 cells) were treated with 1 μM [^{3}H]CNDAC for 24 h, and the nucleic acids were extracted as described above. Total nucleic acids were dissolved in H_2O, digested with RNase (DNase free, 10 μg/ml; Roche Molecular Biochemicals, Indianapolis, IN) at 37°C for 2 h, and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1)

(Roche Molecular Biochemicals). The resulting cellular DNA was precipitated with 3 volumes of ethanol and then dissolved in H_2O. To hydrolyze the DNA directly to nucleosides, cellular DNA was digested at 37°C in a 1-ml solution of 0.5 M Tris-HCl, pH 7.0, 4 mM $MgCl_2$, and 20 units of DNase I. After 1 h, 25 μmol of $CaCl_2$, 50 μg of venom phosphodiesterase (Roche Molecular Biochemicals), and 20 μg of alkaline phosphatase (Sigma Chemical Co.) were added, and the solution was then incubated at 37°C for another 12 h. To limit the digestion of DNA to internal nucleoside 3′-monophosphates and 3′-terminal nucleosides, DNA was incubated in a solution (360 μl) of 3 mM Tris-acetate, pH 9.0, 5 mM $CaCl_2$, and 30 units of micrococcal nuclease (Roche Molecular Biochemicals) at 37°C for 3 h. Fifty micrograms of spleen phosphodiesterase (Roche Molecular Biochemicals) followed by 2 N HCl (1.3 μl) was then added and the solution incubated at 37°C for an additional 12 h. To further convert the digested products to nucleosides, 20 μg of alkaline phosphatase was added to an aliquot of the reaction mixture and incubated at 37°C for 12 h.

The digested products were subjected to HPLC on a YMC-ODS-AM reversed-phase column (250 × 4.6 mm; YMC Inc., Wilmington, NC) and eluted at a flow rate of 1.0 ml/min on a 45-min gradient of 100% solution A (5% MeOH) for the first 15 min and 20% solution B (80% MeOH) through 45 min. The column eluate was monitored by ultraviolet absorption at 270 nm, and the peaks representing different nucleosides were quantitated by electronic integration with reference to standard curves generated with external standards. The radioactivity associated with the respective nucleosides was measured with a radioactive flow detector as described above. Under these analytical conditions, CNDAC and its two nucleoside metabolites are separated, but monophosphates are retained on the stationary phase.

Cell Cycle Analysis. Exponentially growing cells (10^6 cells) were incubated with each drug for the times indicated in the legends to Figs. 4 and 5. After being washed twice with cold PBS, cells were fixed with 70% EtOH and kept at 4°C overnight. Cells were then washed with PBS and suspended in 1 ml of PBS containing 15 μg of propidium iodide, 2.5 μg of RNase (DNase free), and 0.5% Tween 20. Cell cycle distribution was assessed using an Epics Profile flow cytometer (Coulter, Hialeah, FL). The populations of cells were estimated using the Multicycle program (Phoenix Flow Systems, San Diego, CA).

Cell Synchronization. ML-1 cells were treated with 100 μM nocodazole for 24 h, which resulted in an accumulation of cells in M phase. To enrich the G_2/M phase population, cells were treated with 1 μM aphidicolin for 24 h to block cells in S phase. After washing into fresh medium, cells were incubated for 8 h to generate a population that was maximally enriched in cells with a G_2/M DNA content.

Immunoblotting. Cells were treated with 1 μM CNDAC for the times indicated in the legend to Fig. 6, and the protein extracts were prepared as described previously (Poon et al., 1996). Cell lysates (100 μg of protein) were resolved by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto an Immobilon P nitrocellulose membrane (Millipore, Bedford, MA). After blocking with 4% nonfat milk or 4% bovine serum albumin in Tris-buffered saline with 0.05% Tween 20 for 2 h, the membranes were probed with anti-cdc2 (Upstate Biotechnology Inc.), anti-phospho-specific cdc2 (New England BioLabs, Inc.), anti-cyclin B1 (Santa Cruz Biotechnology, Inc.), or anti-β-actin antibody (Amersham Pharmacia Biotech) for 16 h at 4°C. The membranes were washed in Tris-buffered saline with 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody for 1 h. The resulting protein bands were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The relative expressions of each protein were quantitated using a densitometer and normalized to the value obtained for actin within the same samples.

Results

Incorporation of CNDAC into Nucleic Acids. Both ML-1 and CEM cells accumulate CNDAC triphosphate effectively in a time- and concentration-dependent fashion, and in vitro studies demonstrated CNDAC has a substrate efficiency for incorporation into DNA that is similar to that of the natural substrate dCTP (Azuma et al., 2001). To evaluate the ability of these cells to utilize the analog in intact cells, DNA and RNA were isolated from CEM and ML-1 cells incubated with [^{3}H]CNDAC (30 nM for CEM; 100 nM for ML-1 cells) for 24 h, and the drug incorporation into the nucleic acids was determined as described under *Materials and Methods*. As shown in Fig. 2, the majority of radioactivity was detected in the DNA-containing fractions. Quantitative analysis revealed that there was 4.9 ± 0.2 fmol of incorporated CNDACMP per μg of DNA in CEM cells and 2.6 ± 0.01 fmol/μg in ML-1 cells. A small amount of radioactivity was also detected in the RNA-containing fractions (0.09 ± 0.02 fmol/μg for CEM cells; 0.38 ± 0.09 fmol/μg for ML-1 cells). In parallel experiments, cells labeled with [^{3}H]thymidine or [^{3}H]uridine confirmed that the radioactive DNA band was located in fractions 12 to 15, whereas the radioactivity associated with RNA was in fractions 27 and 28 (data not shown).

***β*-Elimination-Mediated DNA Strand Breaks in Whole Cells.** The β-elimination hypothesis (Matsuda et al., 1991) indicates that DNA strand breakage mediated by this mechanism would produce CNddC at the 3′ terminus of the DNA strand, whereas CNDAC would remain unchanged if it were incorporated internally without β-elimination or if cells failed to extend the incorporated CNDAC at the 3 terminus (Fig. 3A). CNDAC may spontaneously epimerize to 2′-*C*-cyano-2′-deoxy-1-β-D-*ribo*-pentofuranosylcytosine (CNDC) when the pH is greater than 7.5, in which case the two molecules will eventually reach an equilibrium (Azuma et al., 1995; Matsuda and Azuma, 1995). Thus, analysis of CNddC, CNDAC, and CNDC in cellular DNA is a critical step in evaluating the occurrence of β-elimination in whole cells. To investigate this possibility, cellular DNA isolated from cells

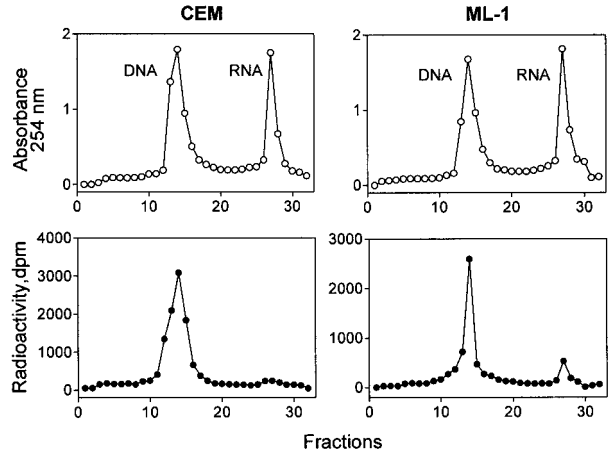

Fig. 2. Incorporation of CNDAC into nucleic acids in CEM and ML-1 cells. CEM and ML-1 cells were labeled with [^{3}H]CNDAC (30 and 100 nM, respectively) for 24 h and then extracted with phenol/chloroform/isoamyl alcohol (25:24:1). Cellular DNA was separated from RNA by banding on a Cs_2SO_4 gradient. The UV absorbances at 254 nm (top) and radioactivity (bottom) associated with each fraction (0.6 ml) were determined as described under *Materials and Methods*.

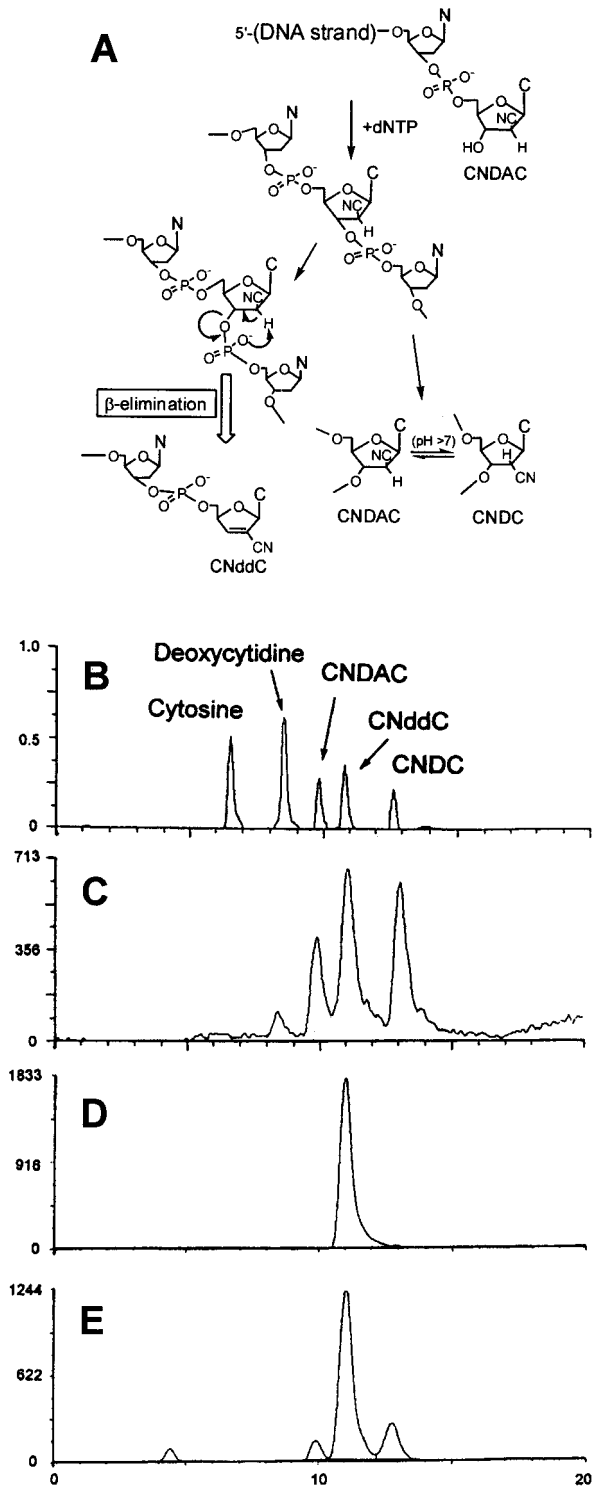

Fig. 3. Identification of CNDAC and its metabolites in DNA. ML-1 cells were incubated with 1 μM [^{3}H]CNDAC for 24 h before DNA was extracted and enzymatically hydrolyzed as described under *Materials and Methods*. Components of DNA hydrolysates were separated by reversed-phase HPLC, and radioactivity was quantitated. A, reaction pathway of DNA strand breaks after incorporation of CNDAC into DNA. B, an authentic standard of each nucleoside was separated by reversed-phase HPLC; ordinate is UV absorption at 270 nm. HPLC analysis of hydrolysis products of degraded with DNase I, phosphodiesterase, and alkaline phosphatase (C), with micrococcal nuclease and spleen phosphodiesterase (D), or with micrococcal nuclease, spleen phosphodiesterase, and alkaline phosphatase (E). Ordinate units for C, D, and E are radioactivity in the eluate, expressed as counts per minute sampled at 0.1-min intervals.

incubated with [^{3}H]CNDAC (0.5 μM for CEM cells; 1 μM for ML-1 cells) for 24 h was degraded to nucleosides by DNase I, phosphodiesterase, and alkaline phosphatase. Analysis of the digestion products by reversed-phase HPLC revealed radioactivity peaks at 10, 11, and 13 min (Fig. 3C). The retention times of those three peaks matched those of authentic CNDAC, CNddC, and CNDC standards (Fig. 3B). Because the presence of CNddC is diagnostic for the β-elimination-mediated DNA strand break, it is clear that this process did occur in whole cells. Quantitation of the radioactivity associated with each peak showed that CNddC constituted about 36% of the total radioactivity in DNA from ML-1 cells and 31% in DNA from CEM cells.

Because β-elimination could occur only after the incorporated CNDAC was extended by further addition of deoxynucleotides during DNA polymerization, the presence of approximately 60 to 70% of the radioactivity in the CNDAC and CNDC peaks suggests the following two possibilities: 1) a major portion of CNDAC remained at the 3′ terminus without further extension; and 2) the incorporated CNDAC molecules had been internalized into the DNA chain, but only 30 to 40% of the incorporated analogs caused β-elimination-mediated strand breaks. To differentiate these possibilities, DNA isolated from cells labeled with [^{3}H]CNDAC was degraded to internal 3′-monophosphates and 3′-terminal nucleosides by sequential digestion with micrococcal nuclease and spleen phosphodiesterase as described previously (Manor et al., 1971). HPLC separation revealed only one radioactive nucleoside peak in the hydrolysates of DNA from ML-1 cells; the retention time of this peak corresponded to that of CNddC (Fig. 3D). The lack of CNDAC and CNDC at the 3′ terminus indicates that most of the analog was incorporated internally by 3′→5′ phosphodiester linkage and that the CNddC at the 3′ terminus was the product of β-elimination. When alkaline phosphatase was added to the reactions to dephosphorylate 3′-monophosphates, the internal analog nucleotides were further degraded to nucleosides, resulting in the appearance of two additional peaks; these corresponded to CNDAC and CNDC (Fig. 3E). Somewhat less radioactivity was associated with these two peaks than with the primary hydrolysate DNA (Fig. 3C), possibly because of incomplete degradation of the internal nucleotides under the sequential digestion conditions.

Effects of CNDAC on Cell Cycle Progression in CEM and ML-1 Cells. The foregoing results demonstrate that CNDAC acts, in part, by a different mechanism than is recognized for other nucleoside analogs. To evaluate the biological significance of this action, exponentially growing CEM cells and ML-1 cells incubated for 24 h with 0.5 μM or 1 μM CNDAC, respectively, were analyzed by flow cytometry. The results indicated that this treatment increased the accumulation of cells with a G_2/M DNA content (Fig. 4, C and D). Quantitation of the cell populations revealed an increase in the percentage of G_2/M phase cells from 10 to 33% in CEM cells and from 9 to 55% in ML-1 cells. In contrast, exponentially growing ML-1 cells treated with 50 nM ara-C (Fig. 4E) or 10 nM gemcitabine (Fig. 4F) for 24 h exhibited a prominent S phase arrest. Thus, the effect of CNDAC on cell cycle progression was qualitatively different from that of other deoxycytidine analogs. Detailed analysis of cell cycle distribution in ML-1 cells treated with CNDAC for various times

demonstrated that cells were somewhat delayed in their transit through S phase but were able to progress more slowly to G_2/M phases, where cell cycle progression was blocked (Fig. 5). Further experiments with nocodazole indicated that CNDAC arrested cells in the G_2 phase because the mitotic index in the CNDAC-treated cells was similar to that of exponentially growing cells (<5%).

Expression of G_2 Checkpoint-Associated Proteins. Because CNDAC arrested cells in the G_2 phase, we further evaluated the effect of CNDAC on the expression of proteins that regulate the $G_2 \rightarrow M$ phase progression by measuring the levels of cdc2, its phosphorylation status, and the levels of cyclin B1. As shown in Fig. 6, the total cdc2 protein levels increased as the cell slowly progressed through the S phase and eventually arrested in the G_2 phase. Immunoblotting analysis using an antibody specific for cdc2 phosphorylated at Tyr-15 showed that the phosphorylation of Tyr-15 in cdc2 was substantially increased in the CNDAC-treated cells at 16 to 32 h (lanes 3–5). This timing coincides with the accumulation of cells in the G_2 phase (Fig. 5). Cells synchronized to enrich the G_2/M population (49%) to a proportion almost equal to the proportion of CNDAC-induced G_2/M cells (57% at 32 h) showed substantially less Tyr-15 phosphorylation (Fig. 6, lane 6). In contrast, cells arrested in M phase (61%) by nocodazole showed little phosphorylation at Tyr-15 (Fig. 6, lane 7), although the hypophosphorylated cdc2 protein was present as a more rapidly migrating band. The protein level of cyclin B1 did not change significantly in CNDAC-treated cells or in cells arrested in G_2/M or M phase. The protein levels of cdc25C phosphatase and wee1 kinase were not significantly changed by CNDAC treatment (data not shown).

Discussion

Inhibition of DNA synthesis is the most prominent activity of ara-C and gemcitabine. The mechanism of such inhibition after the incorporation of the monophosphates of ara-C (ara-CMP) or gemcitabine (dFdCMP) into DNA strands by DNA

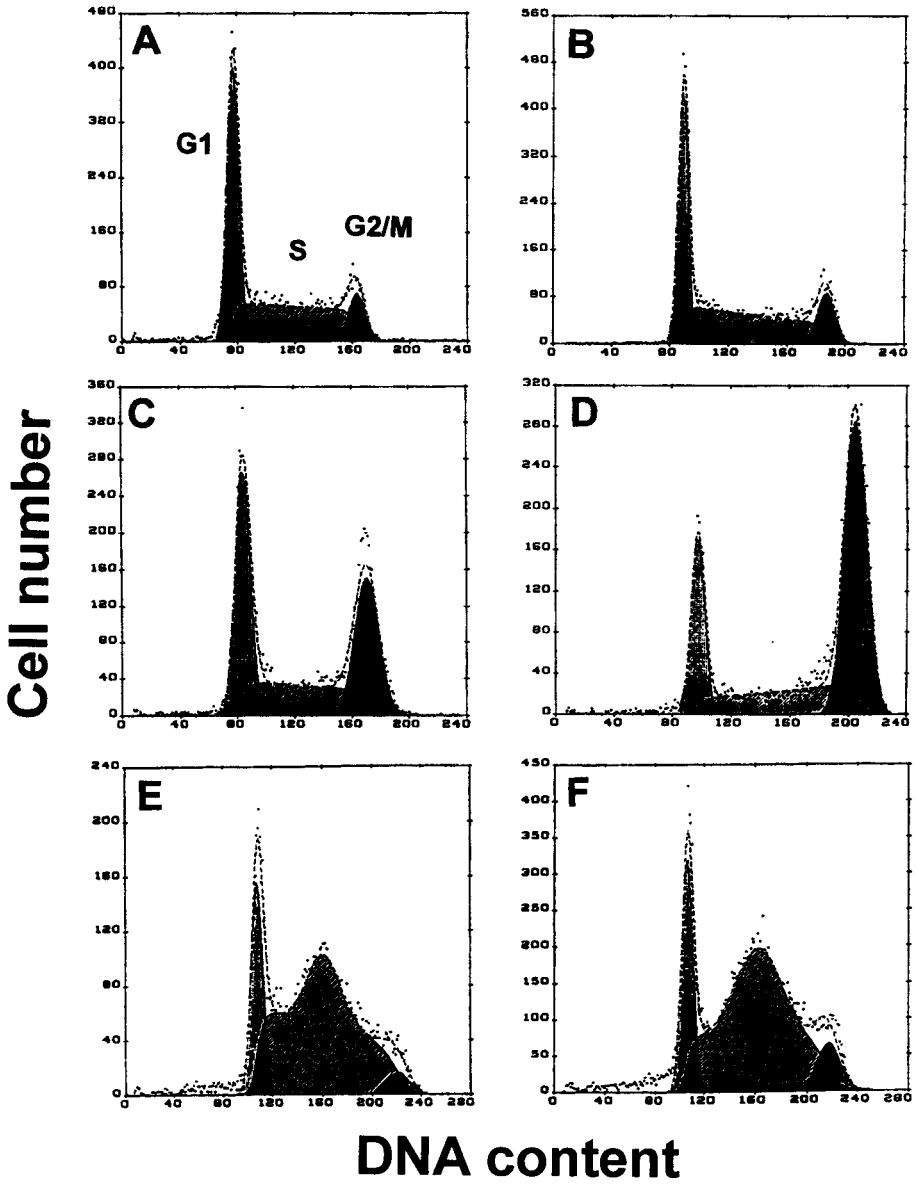

Fig. 4. The effect of CNDAC, ara-C, and gemcitabine on cell cycle distribution. The flow cytometry plots of exponentially growing CEM (A) and ML-1 (B) cells. CEM (C) and ML-1 (D) cells were incubated with CNDAC (0.5 and 1 μM, respectively) for 24 h. ML-1 cells were incubated with 50 nM ara-C (E) or 10 nM gemcitabine (F) for 24 h. The cells were harvested for analysis by flow cytometry as described under *Materials and Methods*. The G_1, S, and G_2/M cell populations have been highlighted in the plots.

polymerase has been demonstrated in vitro and in vivo (Townsend and Cheng, 1987; Huang et al., 1991). The present study demonstrated that CNDAC, like ara-C and gemcitabine, is mainly incorporated into cellular DNA. However, there are major differences in the biochemical consequences of the analog's incorporation and the cellular response to the drug's action. For example, although CNDACMP was incorporated into the C sites of elongating DNA strands by bacterial DNA polymerase (Matsuda and Azuma, 1995) and purified DNA polymerase-α (Azuma et al., 2001) and this action blocked further elongation in vitro, CNDAC nucleotides were predominantly incorporated in whole cells at the internal positions of cellular DNA (Fig. 3). These data suggest that DNA can be elongated further from the 3′-hydroxyl group of CNDAC in whole cells. The observation that cells were able to progress through the S phase in the presence of CNDAC (Fig. 5) is consistent with this conclusion. In contrast, ara-C and gemcitabine seem to inhibit DNA strand elongation more effectively in intact cells, thus blocking the cells in the S phase (Fig. 4, E and F).

The ability of CNDAC to induce DNA strand breaks by a β-elimination-mediated mechanism after its internal incorporation into the DNA strand is novel among nucleoside analogs. This unique strand-breaking action seems to be the basis of its ability to induce cell cycle arrest at the G_2 phase, as distinct from the S phase block seen in the cells treated with ara-C and gemcitabine (Fig. 4). However, it should be noted that only a portion (30–40%) of incorporated CNDAC led to β-elimination-mediated strand breaks and produced CNddC in whole cells by 24 h. Nevertheless, the scope of DNA strand breaks under the experimental conditions we used seems to be sufficient to trigger the cellular checkpoint mechanism responsible for G_2 arrest. The fact that 60 to 70% of the incorporated CNDAC was detected at internal positions, either as CNDAC or its epimer CNDC, contrasts with the chemical behavior of the compound. A model reaction of the 3′-hydroxyl group of CNDAC by *N*″,*N*′-carbonyldiimidazole (Azuma et al., 1993) and nucleotide addition at this site (Hayakawa et al., 1998) both caused quantitative conversion to the β-elimination product CNddC. Thus, the tertiary structure of DNA and the multitude of associated proteins probably influence the rate at which this process occurs in whole cells. It is then reasonable to assume that the β-elimination process may be affected by normal metabolic events that alter the structure of DNA such as transcription and DNA replication. If so, it may be possible to modulate this process with other agents.

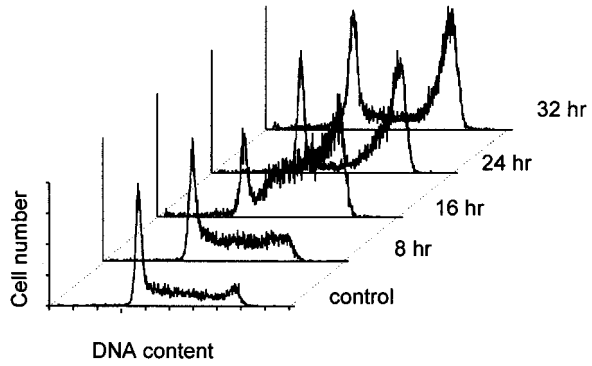

Fig. 5. The effect of CNDAC on cell cycle distribution of ML-1 cells over time. ML-1 cells were incubated with CNDAC (1 μM) for 0 to 32 h and then harvested at the indicated times for analysis by flow cytometry as described under *Materials and Methods*.

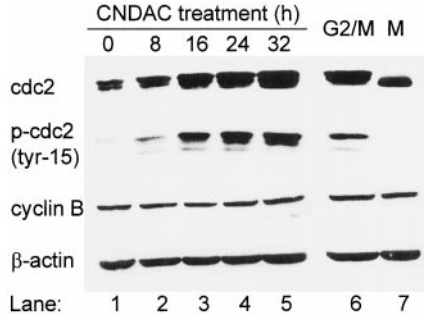

Fig. 6. The protein levels of cdc2, cdc2 phosphorylated at Tyr-15 (*p*-cdc2), cyclin B1, and β-actin in ML-1 cells treated with 1 μM CNDAC. After treatment for indicated times, cells were lysed and cellular proteins were analyzed by immunoblotting with antibodies to the indicated proteins as described under *Materials and Methods*. As controls, cells were treated with either nocodazole to induce accumulation in M phase or with aphidicolin to block cells in S phase and enrich the G_2/M phase population as described under *Materials and Methods*.

In association with cyclin B1, cdc2 kinase plays an important role in cell cycle progression from the G_2 phase to mitosis (Booher et al., 1989; Meijer et al., 1989; Nurse, 1990). The activity of the cdc2 kinase is controlled by phosphorylation at Thr-14, Tyr-15, and Thr-161, which are localized in the ATP-binding site of the kinase (De Bondt et al., 1993; Jeffrey et al., 1995). After formation of a complex with cyclin B1, cdc2 kinase is phosphorylated on these sites in a temporally specific fashion that affects the kinase activity. The phosphorylation of Thr-161 activates the complex, whereas phosphorylation of Thr-14 and Tyr-15 in late S phase and early G_2 phase inhibits progression to M phase. At the G_2 to M phase transition, both Thr-14 and Tyr-15 are dephosphorylated by cdc25C phosphatase, and thus the complex becomes activated (Gautier and Maller, 1991; Kumagai and Dunphy, 1991). The accumulation of phosphorylated cdc2 (Tyr-15) in ML-1 cells treated with CNDAC (Fig. 6) suggests that the cells had down-regulated the kinase function of cdc2 in response to incubation with the drug. The precise mechanism by which CNDAC activated this G_2 cell cycle checkpoint is not clear at present. It is known, however, that certain DNA-damaging agents such as ionizing radiation and cisplatin block the cell cycle at the G_2 phase (Rowley and Leeper, 1985; Sorenson et al., 1990). It is logical to speculate that the β-elimination-mediated DNA strand breaks caused by CNDAC might activate signaling pathways similar to those triggered by radiation and/or cisplatin. In this regard, CNDAC functions as a DNA strand-breaking agent to activate G_2 arrest rather than as a typical nucleoside analog acting on DNA replication processes.

It should also be noted that cells treated with CNDAC progressed through the S phase of the cell cycle at a reduced rate. There was a clear S phase delay at 16 h after CNDAC incubation (Fig. 5). This delay suggests that CNDAC may be able to decrease the rate of DNA synthesis in whole cells. In fact, the inhibitory effect of CNDAC triphosphate on DNA strand elongation has been observed in vitro (Matsuda and Azuma, 1995); its ability to reduce the rate of [^{3}H]thymidine incorporation was also seen in both ML-1 and CEM cells, as characterized by an IC_{50} value of approximately 1 μM (data not shown). This aspect of CNDAC function is similar to that of other nucleoside analogs such as ara-C and gemcitabine.

Thus, once incorporated into DNA, the CNDAC nucleotide appears to have a dual mode of action. It is a poor substrate for addition of subsequent nucleotides and thereby slows cellular DNA synthesis. This is seen as a delay, but not a total inhibition, in the transit through the S phase of the cell cycle. Once in 3′→5′ phosphodiester linkage upon the addition of a subsequent nucleotide, a portion of the analog molecules causes DNA strand breaks by a β-elimination-mediated mechanism. Under conditions of cytostatic incubation with CNDAC, the biological consequences of this action are distinguished from those of other analogs that inhibit DNA replication but do not cause strand breaks or arrest cell cycle transit in the G_2 phase.

Acknowledgment

We are grateful to Jude Richard for critical editorial review of this manuscript.

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