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Action of (*E*)-2′-Deoxy-2′-(fluoromethylene)cytidine on DNA Metabolism: Incorporation, Excision, and Cellular Response

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ABSTRACT

(*E*)-2′-deoxy-2′-(fluoromethylene)cytidine (FMdC) is a new analog of deoxycytidine with promising anticancer activity. We investigated the action of FMdC on DNA metabolism by evaluating its incorporation into DNA, its excision from DNA in vitro, and the role of the incorporation of FMdC into DNA in causing cytotoxicity. In vitro DNA primer extension demonstrated that FMdC nucleotides were incorporated with relatively high substrate efficiency into the C sites of the elongating DNA strand. Once incorporated, FMdC became a poor substrate for further chain elongation by DNA polymerases, resulting in a termination of DNA synthesis at the sites of incorporation. Furthermore, the 3′ → 5′ exonuclease activity of DNA polymerase ϵ or wild-type p53 protein was ineffective in removing the incorporated FMdC from DNA in vitro. FMdC also showed potent cytotoxic activity against human leukemia and solid tumor cells. Incubation with a low concentration of FMdC (10 nM) induced cell cycle arrest at S or G_1 phases, but the cells eventually died as the time of incubation increased. Compared with HL-60 cells, human myeloid ML-1 cells with wild-type p53 were more sensitive to FMdC, but the S or G_1 phase arrest did not seem to depend on the presence or absence of p53. Inhibiting the incorporation of FMdC into cellular DNA by aphidicolin suppressed the cytotoxic effect of the compound. We conclude that the incorporated FMdC nucleotide profoundly disrupts DNA synthesis and resists excision by exonucleases, and that incorporation of this analog into DNA is a key molecular event responsible for the drug's cytotoxicity.

(*E*)-2′-Deoxy-2′-(fluoromethylene)cytidine (FMdC) is a novel deoxycytidine analog developed as a specific inhibitor of ribonucleotide reductase (McCarthy et al., 1991). Early studies demonstrated that the diphosphate of this compound affects ribonucleotide reductase through a mechanism-based inhibition (van der Donk et al., 1996) similar to that of gemcitabine. However, unlike gemcitabine and 1-β-D-arabinofuranosylcytosine, FMdC is relatively resistant to deamination by cytidine deaminase (Takahashi et al., 1998). This favorable metabolic property may contribute in part to the analog's potent anticancer activity. In vivo pharmacology study in animals demonstrated that at the dose of 200 and 400 mg/kg, FMdC is eliminated from the plasma and normal tissue with a plasma half-life ($t_{1/2}$) of about 60 min. The kidney is shown to be the major site of initial distribution of FMdC (Adams et al., 1996). After entering the cells, FMdC is first converted to its 5′-monophosphate and is then further phosphorylated to diphosphate and triphosphate. The nucleotides of FMdC are thought to be the active metabolites of this drug. The diphosphate of FMdC has been shown to inhibit ribonucleotide reductase, whereas the triphosphate seems to directly interfere with DNA polymerization (for review, see Seley, 2000). It has been demonstrated that FMdC displays a strong cytotoxic activity against a variety of human solid tumor cell lines in culture and shows a potent antitumor activity against murine tumor models and human tumor xenografts in nude mice (Bitonti et al., 1994, 1995; Snyder, 1994; Piepmeier et al., 1996; Wright et al., 1996; Sun et al., 1997, 1998; Takahashi et al., 1998; Kotchetkov et al., 1999). Clinical trials of FMdC, known as tezacitabine, have recently begun in patients with hematological malignancies and solid tumors (Noriyuki et al., 1999; Rodriguez et al., 1999; Faderl et al., 2000).

Many therapeutic nucleoside analogs share similar metabolic pathways and pharmacological actions. After entering the cells, nucleoside analogs are metabolically converted to nucleotides through a series of sequential phosphorylation steps. The analog nucleotides are believed to be the active forms of the drugs, which in many cases have multiple sites of action in the cells. For example, ribonucleotide reductase and DNA polymerases are inhibited by the diphosphate and triphosphate of gemcitabine and fludarabine (Gandhi et al.,

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ABBREVIATIONS: FMdC, (*E*)-2′-deoxy-2′-(fluoromethylene)cytidine (tezacitabine); pol, DNA polymerase; FMdCMP, (*E*)-2′-deoxy-2′-(fluoromethylene)cytidine-5′-monophosphate; FMdCTP, (*E*)-2′-deoxy-2′-(fluoromethylene)cytidine-5′-triphosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PBS, phosphate-buffered saline; wt, wild-type.

1994; Hui and Reitz, 1997; Storniolo et al., 1997). Fludarabine also inhibits DNA ligation, DNA-dependent RNA primer formation, and RNA synthesis (Huang and Plunkett, 1991; Yang et al., 1992; Catapano et al., 1993). Despite these multiple sites of action, incorporation of the analogs into DNA, which results in the termination of DNA synthesis, seems to be the most prominent event and is closely associated with the cytotoxic action of the analogs. Inhibition of the incorporation of analogs into DNA usually leads to a significant reduction of the analog-induced cell death (Huang et al., 1995).

Like other nucleoside analogs, FMdC seems to have multiple sites of action. The inhibition of ribonucleotide reductase by FMdC diphosphate has been well characterized (van der Donk et al., 1996; Kanazawa et al., 1998). In vitro studies suggest that the triphosphate of FMdC may inhibit human DNA polymerase (pol) α and cause a pause in further DNA synthesis (Yonetani and Mizukami, 1996). However, the incorporation of FMdC triphosphate into DNA and its biological significance have not been systemically evaluated. In this study, we investigated the incorporation of FMdC triphosphate into DNA and its consequences in vitro and in whole cells. We also examined the ability of DNA pol ϵ and p53, two cellular molecules with intrinsic $3' \rightarrow 5'$ exonuclease activity, to remove the incorporated FMdC from DNA in vitro.

Materials and Methods

Chemicals and Reagents. FMdC and its triphosphate (FMdCTP) were provided by Dr. H. S. Gill (Hoechst Marion Roussel, Inc., Cincinnati, OH). Propidium iodide was obtained from Sigma Chemical (St. Louis, MO), RNase and proteinase K from Roche Molecular Biochemicals (Indianapolis, IN), and ethidium bromide from Bio-Rad (Hercules, CA). The 17-base M13 sequencing primer (5′-GTAAAACGACGGCCAGT-3′) and high-performance liquid chromatography-purified dATP, dCTP, dGTP, and dTTP were obtained from Amersham Biosciences, Inc. (Piscataway, NJ). The 25-base template (5′-CACACACGACTGGCCGTCGTTTTAC-3′) was synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX). [γ-^{32}p]ATP (specific activity 4500 Ci/mmol) was purchased from ICN Radiochemicals, Inc. (Irvine, CA). T4 polynucleotide kinase and the large fragment of *Escherichia coli* DNA polymerase I (Klenow fragment) were obtained from United States Biochemical Corp. (Cleveland, OH). DNA polymerases α (pol α) and ϵ (pol ϵ) were purified from human T-lymphoblastoid cells and characterized as described previously (Huang et al., 1990). The wild-type (wt) p53 protein was purified from human leukemia ML-1 cells by precipitation of the protein extracts with 50 mM $(NH_4)_2SO_4$, followed by fractionation with the Pharmacia fast-performance liquid chromatography system using a MonoQ column with a linear gradient of 20 to 700 mM NaCl in 50 mM Tris buffer (1 ml/min/fraction, 40 min). The p53 protein in the fractions was characterized as described previously (Huang, 1998). Fraction 25 was found to contain the highest level of p53 protein with $3' \rightarrow 5'$ exonuclease activity. Immunodepletion of p53 by anti-p53 antibody (Ab-6; Oncogene Science, Cambridge, MA) also removed the $3' \rightarrow 5'$ exonuclease activity from this fraction. Thus, fraction 25 was used for the excision assays in this study.

Cell Culture. Human leukemia cell lines HL-60 and ML-1 were maintained in suspension culture RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% CO_2. During exponential growth, the population doubling time was approximately 22 h. All experiments were carried out using exponentially growing cell cultures. The cell lines were tested periodically to ensure that they were free from *Mycoplasma* species.

Cytotoxicity Assays. Cell growth inhibition was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. HL-60 or ML-1 cells were seeded onto 96-well plates at the initial density of 4000 cells/well. After incubation with various concentrations of FMdC for 72 h, 50 μl of MTT reagent (3 mg/ml) was added to each well, and incubation was continued for another 4 h. The cells were then centrifuged (1500 rpm, 10 min), and the medium was removed. The cell pellets were dissolved in 200 μl of dimethyl sulfoxide. Absorbance was measured at 570 nm within 1 h, using the Dynatech MR 5000 plate reader (Dynatech Labs, Chantilly, VA). The percentage of growth inhibition was calculated by dividing the absorbance of each FMdC-treated well by that of the untreated control. The antiproliferative activity was expressed as the drug concentration that induced 50% growth inhibition (IC_{50}).

To examine the change in cell morphology, the FMdC-treated cells were centrifuged (550 rpm, 5 min) onto glass slides, using a Shandon-Elliot cytospin (London, UK). The slides were fixed with 100% methanol for 45 min, air-dried, and then stained with Wright's Giemsa stain solution (Biochemical Sciences Inc., Swedesboro, NJ). Cells were examined for morphological changes characteristic of apoptosis. Photomicrographs were taken using a 20× objective (Nikon, Tokyo, Japan).

Apoptosis was further confirmed by a DNA fragmentation assay. After treatment with FMdC, approximately 5×10^6 cells were collected, and the cell pellets were digested in 1 ml of buffer containing 10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 10 μl of proteinase K (1 mg/ml, added fresh) at 45°C overnight. The samples were analyzed on 1.8% agarose gel in 1× Tris-borate/EDTA buffer (100 mM Tris-borate, pH 8.3, 2 mM EDTA). After electrophoresis, the gel was incubated overnight in 400 ml of 0.1× Tris-borate/EDTA buffer containing 20 μl of RNase (500 μg/ml) and then photographed. DNA bands were quantitated using the Chemi-Imager 4400 imager system (Alpha Innotech Corporation, San Leandro, CA).

Cell Cycle Analysis by Flow Cytometry. Exponentially growing cells were exposed to 10 nM FMdC for 6, 12, 24, 36, or 48 h, washed with cold phosphate-buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4), and resuspended in PBS at 10^6 cells/ml. The cells were fixed in 70% ethanol at −20°C overnight, washed twice with cold PBS, and stained with 1 ml of PBS containing propidium iodide (15 μg/ml), Tween 20 (0.5%), and 5 μl of RNase (500 μg/ml). The DNA content was measured using a FACSVantage flow cytometer (BD Biosciences, San Jose, CA). Data acquisitions and analyses were performed using Cell-Quest software (BD Biosciences).

Preparation of 18-Base Oligomer with an Incorporated FMdC Nucleotide at 3′ End. The 17-base M13 universal sequencing primer was labeled at the 5′ end with [γ-^{32}P]ATP using the T4 polynucleotide kinase and then annealed to the 25-base template as described previously (Huang et al., 1990). The sequence of ^{32}P-labeled 17-base primer/25-base template hybrid was as follows: (5′) ^{32}P-GTAAAACGACGGCCAGT (3′) CATTTTGCTGCCGGTCAGCACACAC.

This hybrid was incubated with 10 μM FMdCTP and DNA pol I at 37°C for 30 min. The reaction product was separated by electrophoresis through a 10% polyacrylamide sequencing gel, and the band containing the 18-base DNA oligomer with FMdC incorporated at its 3′ end was excised from the gel. The oligomer was eluted from the gel slice by first smashing the gel slice into small pieces, followed by incubating in 0.5 M NH_4Ac and 1 mM EDTA at 45°C for 2 h with occasional stirring. The eluted 18-oligomer was precipitated with 80% ethanol and annealed to the 25-base template. The radioactivity of both the labeled 17-base/template and 18-base/template hybrids was determined by liquid scintillation counting, and the specific radioactivity was calculated based on the oligomer concentrations and the respective radioactivity.

DNA Primer Extension Assay. The 17-base primer/template and 18-base primer/template hybrids were used as the substrates for

DNA primer extension assays by DNA pol α and pol I. The reaction mixtures contained 20 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 mM NaCl, bovine serum albumin (20 μg/ml), and the labeled DNA substrates at various concentrations, dNTPs, FMdCTP, as well as pol I or pol α. The reactions were incubated at 37°C for 30 min and analyzed by electrophoresis through a 10% polyacrylamide sequencing gel. After autoradiography, the radioactivity of each DNA band in the gel was quantitated using a gel analyzer (InstantImager; Packard Instrument Co., Meriden, CT).

DNA Excision Assay. The 17- and 18-base primer/template hybrids were used as the substrates for excision by 3′ → 5′ exonuclease associated with human pol ϵ and wt p53 protein. The reaction mixtures contained 20 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 mM NaCl, bovine serum albumin (20 μg/ml), and various concentrations of the labeled DNA substrates, as well as pol ϵ or p53. The reactions were incubated at 37°C for 30 min and analyzed by electrophoresis through a 10% polyacrylamide sequencing gel. After autoradiography, the radioactivity of the DNA bands in the gel was quantitated by the InstantImager. The K_m and V_{max} values from three separate experiments were calculated based on the Michaelis-Menten equation, using the Winzyme computer program (Biosoft, Ferguson, MO).

Results

Incorporation of FMdC Nucleotide into DNA. Because most nucleoside analogs cause cytotoxicity after being incorporated into cellular DNA, we first tested the ability of human DNA pol α to incorporate FMdCTP into DNA in vitro, using a primer extension assay with the ^{32}P-labeled 17-/25-base hybrid as the primer/template. As illustrated in Fig. 1A, FMdCTP was incorporated into the C site of the primer strand at position 18 by pol α in the absence of dNTPs (lanes 2 and 3). In the presence of each of the 4 dNTPs at a concentration of 0.5 μM, increasing concentrations of FMdCTP competed with dCTP for incorporation, resulting in a concentration-dependent pause of DNA extension at position 18 and a reduction of the full-length products (lanes 4–7). Evaluation of DNA pol I indicated that FMdCTP competed poorly with dCTP, as evidenced by the efficient elongation of the strand to the full-length product in the presence of various concentrations of FMdCTP (Fig. 1B, lanes 4–7). Even at the FMdCTP/dCTP ratio of 20:1 (10:0.5 μM), no significant reduction of the full-length product was seen (lane 7). Thus, FMdCTP seemed to be a better substrate for human DNA pol α than for the *E. coli* enzyme, which strongly preferred normal dNTPs as the substrates. However, in the absence of dNTPs, pol I was still able to incorporate FMdCTP into the DNA (Fig. 1B, lanes 2 and 3).

Because FMdCTP seemed to be a relatively good substrate for pol α, we further compared its incorporation kinetics with that of dCTP catalyzed by pol α. Various concentrations of FMdCTP or dCTP were incubated with pol α and the ^{32}P-labeled primer/template, and the reaction products were analyzed. As shown in Fig. 1C, we observed a concentration-dependent incorporation of FMdCMP or dCMP into position 18. When the radioactivity of each band was quantitated (Fig. 1D), dCTP seemed to be a somewhat better substrate for pol α than was FMdCTP.

DNA Chain Termination by FMdC Nucleotide. To further characterize the chain termination properties of FMdC, we prepared an 18-base primer containing a FMdCMP at the 3′ end of the oligomer and annealed it to the 25-base template as described under *Materials and Methods*. This analog-primer/template hybrid was used as the substrate for extension by pol α and pol I, in comparison with the normal 17-base primer/template. As shown in Fig. 2A, both pol α and pol I extended the normal DNA substrate efficiently in the presence of four normal dNTPs. In contrast, the presence of FMdCMP at the 3′ end of the primer substantially inhibited strand elongation by each enzyme. Small amounts of extension products were detected in samples incubated with pol I for prolonged periods (30–40 min); no significant extension was observed in samples incubated with pol α. The time courses of primer extension of both normal and analog-terminated primers by pol α and pol I are shown in Fig. 2B.

To further characterize the extension in samples with the normal (17-base) primer and the analog-containing (18-base) primer, we incubated various concentrations of the DNA substrates with pol α. We observed a concentration-dependent increase of extension products in samples with the normal DNA substrate (Fig. 2C, lanes 2–8), whereas the extension of the analog primer was hardly detectable (lanes 10–16). The quantitative data are shown in Fig. 2D. Kinetic analysis revealed an apparent K_m value of 76.9 nM for the normal 17-base primer as the substrate for pol α. Because of inadequate primer extension, we were unable to determine the K_m value for the analog-containing primer.

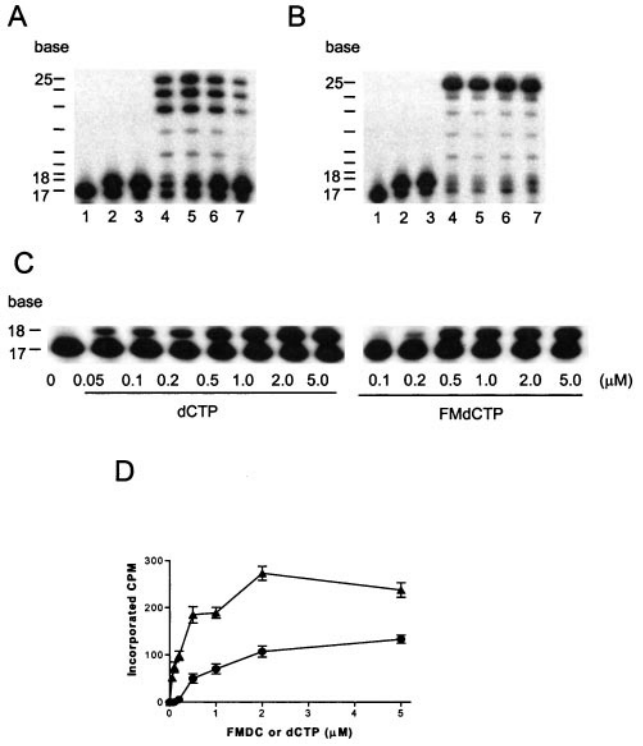

Fig. 1. Incorporation of FMdCTP and dCTP into DNA. A 17-base primer labeled with ^{32}P at the 5′ end and annealed to its complementary site on a 25-base DNA template was used as the substrate for extension. The in vitro DNA primer extension reaction assays were carried out as described under *Materials and Methods*. The assays show incorporation of and competition between FMdCTP and dNTPs by pol α (A) and pol I (B). Lane 1, the 17-base primer/template alone; lanes 2 and 3, reactions with 0.3 and 10 μM FMdCTP, respectively; lanes 4–7, reactions with 4 dNTPs at 0.5 μM plus 0.3, 1, 3, and 10 μM FMdCTP, respectively. C, kinetic incorporation of FMdCTP and dCTP into DNA by pol α. Various concentrations of FMdCTP and dCTP are indicated. D, quantitation of radioactivity in the 18-base band shown in C. The extension activity was expressed as the incorporated radioactivity (cpm). ●, FMdCTP; ▲, dCTP. Data are presented as the mean ± S.D. from three separate experiments.

Excision of FMdC from 3′ End of DNA by pol ϵ and p53. Because FMdC showed potent DNA chain termination activity in vitro (Fig. 2), we sought to determine whether the incorporated analog could be removed from DNA by enzymes. Two proteins, DNA pol ϵ and the wt p53, each with intrinsic 3′ → 5′ exonuclease activity (Mummenbrauer et al., 1996; Burgers, 1998), were tested for their abilities to remove FMdCMP from the 3′ terminus of the 18-base primer when annealed to the 25-mer template. The 3′-FMdCMP primer was prepared as described above; the normal 17-base primer was processed through the same procedures and used as a control. As shown in Fig. 3A, pol ϵ effectively removed the normal nucleotides from the 17-base primer; most of the 17-mers were degraded to shorter oligonucleotides within 15 min. The p53 protein exhibited moderate activity in excising nucleotides from the 17-base primer; about 50% of the 17-mers still remained intact after a 45-min incubation. When the 18-base primer containing 3′-terminal FMdCMP was used as the substrate, pol ϵ and p53 each showed a substantially reduced ability to remove the analog from the 18-base primer. Figure 3B shows a quantitative comparison of the time courses of excision of the normal 17-base primer and the FMdCMP-18-mer by pol ϵ and p53. Clearly, the FMdCMP-containing DNA was a poor substrate for excision by either enzyme.

The substrate efficiency of the normal 17-base primer/template and the 3′-FMdCMP-containing 18-base primer/template for excision by pol ϵ and p53 was further characterized using a kinetic assay. Various concentrations of each primer/template were incubated with each enzyme, and the excision products were quantitated (Fig. 3, C and D). The generation of excision products by each enzyme was concentration-dependent with both substrates, although the 18-base primer with FMdCMP incorporated at its 3′ end was much more resistant to excision by both enzymes than was the normal 17-base primer. The apparent kinetic parameters were calculated based on the primer/template substrate concentrations and the respective radioactivity associated with the excision products. As shown in Table 1, in the case of pol ϵ, the apparent K_m value for the normal 17-base primer (4.7 nM) was substantially greater than that for the FMdCMP-primer (0.4 nM), whereas the V_{max} for excision of the FMdCMP was only 3% of that for excision of dCMP. Similarly, for p53, there was a much greater affinity for the analog-containing oligonucleotide, as measured by the apparent K_m values of the normal 17-base primer (26.1 nM) and the 18-base primer (2.0 nM). As with pol ϵ, the V_{max} for removal of FMdCMP was less than 6% of that for the removal of dCMP. Thus, although the affinity of the repair activities was substantially greater for the analog-terminated DNA than for normal primers, the rates at which pol ϵ and p53 were able to excise the fraudulent nucleotide was greatly diminished relative to the deoxynucleotide.

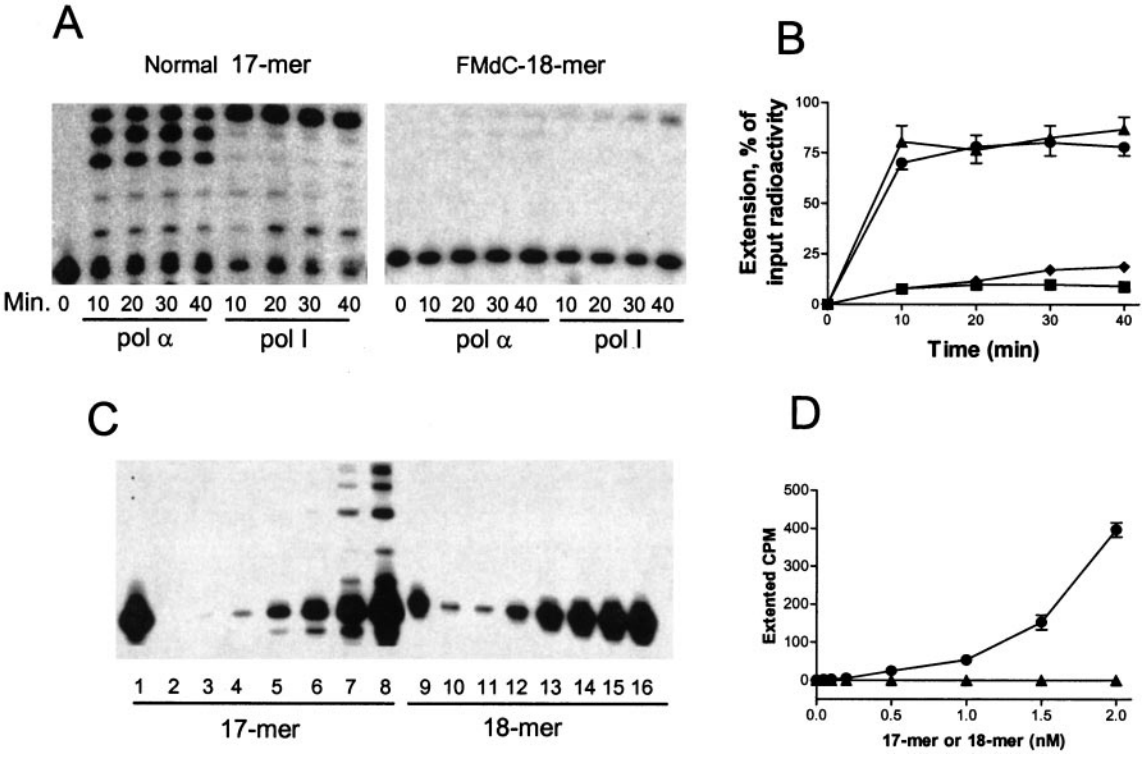

Fig. 2. Extension of 3′-FMdCMP-terminated DNA. The oligomer (18-base primer) with FMdCMP incorporated at the 3′ end was constructed as described under *Materials and Methods*. The normal 17-base primer/template was used as the control. The primer extension reactions were carried out as described under *Materials and Methods*. A, normal 17-base and FMdCMP-terminated 18-base primer extension reactions by pol α and pol I were measured at 10, 20, 30, and 40 min. B, extension activity was measured by quantitating the radioactivity associated with the excision products and expressed as the percentage of the total input radioactivity. ●, 17-mer, pol α; ▲, 17-mer, pol I; ■, 18-mer, pol α; ♦, 18-mer, pol I. Data are presented as the mean ± S.D. from three separate experiments. C, kinetic assay was performed for 17-base or 18-base primer extension by pol α. Lane 1, the normal 17-base primer/template alone; lanes 2 to 8, reactions with 0.05, 0.1, 0.2, 0.5, 1.0, 1.5, and 2.0 nM 17-base primer, respectively; lane 9, the 18-base primer/template alone; lanes 10 to 16, reactions with 0.05, 0.1, 0.2, 0.5, 1.0, 1.5, and 2.0 nM 18-base primer, respectively. D, quantitation of the radioactivity associated with primer extension products. The extension activity was expressed as the extended radioactivity in cpm. ●, 17-mer; ▲, 18-mer. Data are presented as the mean ± S.D. from three separate experiments.

Cellular Responses to FMdC Treatment. Incubation of human leukemia HL-60 and ML-1 cells in various concentrations of FMdC in vitro caused a potent growth inhibition in both cell lines, although it seemed that the ML-1 cells were more sensitive to FMdC than HL-60 cells (Fig. 4A). The drug concentrations required to achieve IC_{50} during a continuous 72-h incubation were 10.4 nM for HL-60 cells and 3.5 nM for ML-1 cells. A colony formation assay showed that FMdC also exhibited potent activity in human pancreatic cancer cells. Incubation of Panc-1 cells with FMdC substantially inhibited the ability of the cells to form colonies, with IC_{50} values of 148.4 nM after a 24-h incubation and 11.8 nM after continuous (10-d) exposure (data not shown).

Because FMdC showed potent DNA chain termination activity in vitro, we used a flow cytometric analysis to determine whether incubation of cells in FMdC caused changes in cell-cycle distribution (Fig. 4B). After exposing HL-60 cells to 10 nM FMdC, we observed a time-dependent accumulation of cells in the S phase up to 24 h. Thereafter, cell death occurred, as evidenced by the appearance of a cell population with sub-G_1 DNA content. By 48 h, approximately 50% of the cells had shifted to the sub-G_1 region with a concomitant decrease of the S phase cell population. Incubation of ML-1 cells with 10 nM FMdC caused a moderate accumulation of G_1 cells (i.e., from 45 to 55%) during the first 12 h, followed by the appearance of a population of cells in the sub-G_1 region (Fig. 4C). Consistent with the results of the MTT assay (Fig. 4A), ML-1 cells were more sensitive to FMdC, and evidence of cell death became apparent after 12 h of incuba-

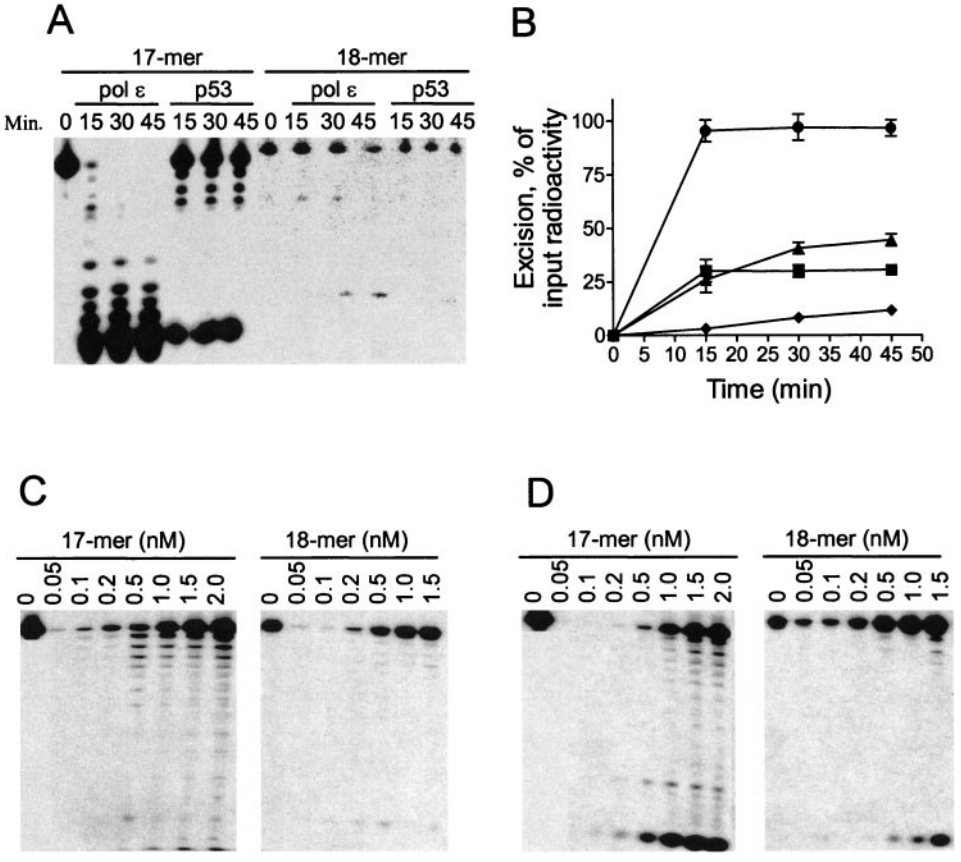

Fig. 3. Excision of incorporated FMdC from DNA. The oligomer (18-base primer) with FMdCMP incorporated at the 3′ end was constructed as described under *Materials and Methods*. The normal 17-base primer was used as the control. The primer excision reactions were carried out as described under *Materials and Methods*. A, 17- and 18-base primer excision reactions by pol ϵ and p53 were measured at 15, 30, and 45 min. B, graph shows the quantitation of radioactivity. The excision activity was expressed as the percentage of total input radioactivity. ●, 17-mer, pol ϵ; ▲, 17-mer, p53; ■, 18-mer, pol ϵ; ■, 18-mer, p53. Data are presented as the mean ± S.D. from three separate experiments. Kinetic assay for 17- or 18-base primer excision by pol ϵ (C) and p53 (D). The concentrations of 17- and 18-base primers are indicated.

TABLE 1

Apparent kinetic parameters of nucleotide excision by DNA polymerase ε and p53

Various concentrations of the normal 17-base primer/template and 3′-FMdCMP-18-base primer/template were incubated with purified either pol ε or p53 in excision reactions for 30 min as described under *Materials and Methods*, and the excision products were quantitated. Values were calculated using the Michaelis-Menten equation. Each data point represents the mean ± S.D. of three separate experiments.

Enzyme	Normal 17-mer			3′-FMdCMP 18-mer		
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m
		nM			*nM*	
pol ε	169.1 ± 13.4	4.7 ± 0.3	36.1 ± 5.0	5.0 ± 0.9	0.4 ± 0.1	14.7 ± 5.6
p 53	115.0 ± 11.6	26.1 ± 3.3	4.4 ± 0.7	6.6 ± 0.8	2.0 ± 0.8	3.7 ± 1.5

tion with FMdC. By 24 h, more than 30% of the cells had lost their DNA content and shifted to the sub-G_1 region.

The appearance of cells with a sub-G_1 DNA content suggested that FMdC probably induced apoptosis in the leukemia cell lines. Morphological evaluation and a DNA fragmentation assay were used to explore this possibility. As shown in Fig. 5A, morphological changes typical of apoptosis, including cell shrinkage, condensation, and fragmentation of nuclei, and cell membrane blebbing, were observed in HL-60 cells incubated with FMdC. Apoptosis became apparent after 8 h of incubation with 0.1 μM FMdC or 4 h of incubation with 1 μM compound. These time courses were consistent with those of nucleosomal DNA fragmentation (Fig. 5B). At a lower concentration (10 nM), no apparent apoptotic morphology was observed up to 24 h (data not shown), although this concentration of FMdC did cause a moderate arrest of the cells at S phase (Fig. 4).

To determine whether incorporation of FMdC into DNA was a key event in triggering apoptosis, we incubated HL-60 cells with FMdC in the presence of aphidicolin, a potent inhibitor of DNA replication. Such treatment would be expected to limit the incorporation of a nucleoside analog into DNA. HL-60 cells were incubated with 1.0 FMdC for 4 h after exposure to various concentrations (0.03–1.0 μM) of aphidicolin for 2 h. Under these experimental conditions, aphidicolin inhibited FMdC-induced DNA fragmentation in a concentration-dependent manner. In contrast, aphidicolin alone (0.1 and 1.0 μM) did not induce DNA fragmentation (Fig. 5C).

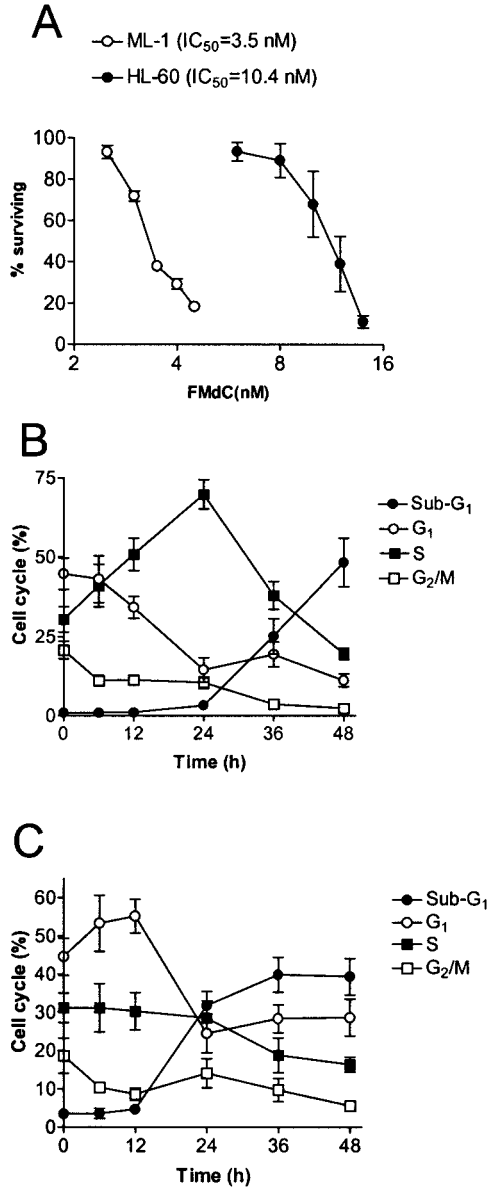

Fig. 4. Effects of FMdC on cell growth and cell cycle distribution in human leukemia cells. A, graph shows the FMdC-induced growth inhibition curves in ML-1 (○) and HL-60 (●) cells. MTT assay was carried out as described under *Materials and Methods*. The IC_{50} values are indicated. Exponentially growing HL-60 (B) or ML-1 (C) cells were exposed to 10 nM FMdC for 6, 12, 24, 36, and 48 h. Changes in the cell cycle distribution were determined as described under *Materials and Methods*. Data are presented as the mean ± S.D. from three separate experiments.

Discussion

Incorporation into DNA is the most prominent action of nucleoside analogs and is closely correlated with cytotoxicity (Kufe et al., 1984; Huang et al., 1990, 1991, Xie and Plunkett, 1995). In many cases, such incorporation causes a disturbance of DNA synthesis that is characterized by in vitro assays as a kinetic delay of DNA chain elongation at or near the sites of the incorporated analogs. The relative potency of the chain termination effect varies among different analogs,

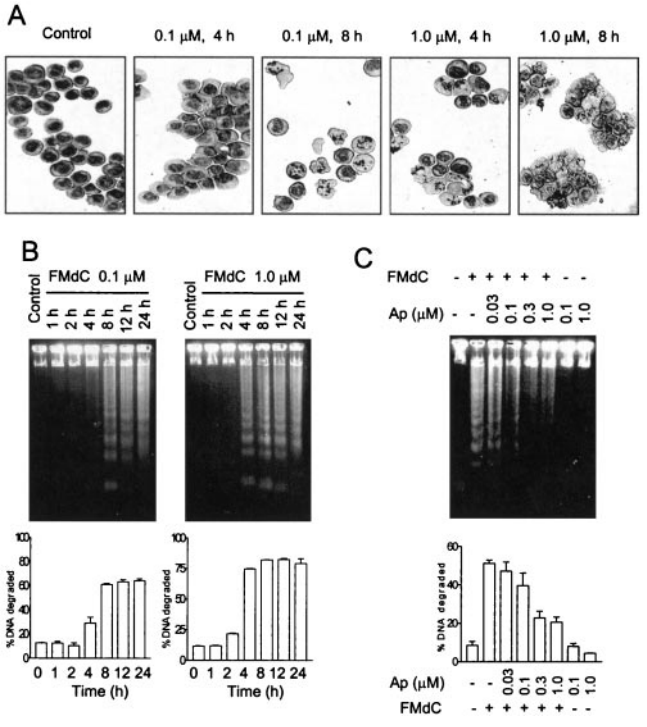

Fig. 5. FMdC-induced apoptosis in HL-60 cells. A, microscopic images show the FMdC-induced morphological changes characteristic of apoptosis. After cells were incubated with FMdC under the conditions indicated, the samples were stained and photographed as described under *Materials and Methods*. B, assay shows FMdC-induced DNA fragmentation after treatment of exponentially growing cells with 0.1 or 1.0 μM FMdC for 1, 2, 4, 8, 12, and 24 h. DNA fragmentation was determined as the percentage of DNA released from the well and quantitated as described under *Materials and Methods*. Data are presented as the mean ± S.D. from three separate experiments. C, effect of aphidicolin on FMdC-induced DNA fragmentation. Exponentially growing cells were incubated with 1.0 μM FMdC for 4 h after 2 h of exposure to aphidicolin (0.03–1.0 μM). Data are presented as the mean ± S.D. from three separate experiments.

and there may be an apparent discrepancy in chain termination observed in vitro and in whole cells. For instance, the deoxyadenosine analog fludarabine shows potent DNA chain termination activity both in vitro and in whole cells (Huang et al., 1990), whereas the deoxycytidine analog 1-β-D-arabinofuranosylcytosine was effective in terminating DNA synthesis in vitro but was incorporated mainly internally into cellular DNA in cell culture (Kufe et al., 1984; Grant, 1998). Another deoxycytidine analog, gemcitabine, is incorporated into the C sites of the elongating DNA strand and can be extended by a normal deoxynucleotide in vitro before DNA synthesis pauses (Huang et al., 1991). Consistent with this molecular pharmacological behavior, gemcitabine nucleotide is also found internally in DNA extracted from cells after incubation with this nucleoside analog.

The present study demonstrated that FMdCTP was incorporated into the C sites by DNA pol α (Fig. 1). Once FMdCTP was incorporated into the DNA, it potently terminated further DNA strand elongation (Fig. 2). These findings are in contrast to the action of gemcitabine nucleotide, which can be extended by pol α and pol ϵ, and which is found predominantly (>95%) in phosphodiester linkage in DNA extracted from whole cells (Huang et al., 1991). In our study, the competing normal nucleotide dCTP seemed to be preferred to FMdCTP by pol α (Fig. 1A) and was selected over FMdCTP almost exclusively by pol I (Fig. 1B). Therefore, it is likely that the ratio of dCTP to FMdCTP in cells can be used to predict the amount of analog incorporation into DNA. The extent to which FMdCDP can inhibit ribonucleotide reductase and thereby decrease the dCTP levels in whole cells may significantly alter the cellular FMdCTP/dCTP ratio to enhance the incorporation of FMdCTP. In designing a therapeutic strategy, a biochemical modulation approach that decreases dCTP levels, such as those using either fludarabine (Gandhi et al., 1993) or chlorodeoxyadenosine (Gandhi et al., 1996) may be usefully applied to FMdC. It should be pointed out that although FMdC nucleotide seemed to be a potent DNA chain terminator in vitro when DNA pol α is used as the catalyzing enzyme, it is possible that other DNA polymerases in the cells might be able to extend the DNA substrate containing FMdCMP at 3′ end. This possibility can be tested both in vitro by using various types of purified DNA polymerases, and in whole cells by using radioactive labeled FMdC to determine the location of the incorporated analog in DNA (terminal or internal incorporation).

Because incorporation of nucleotide analogs into DNA is a critical event in causing cytotoxicity, their excision from cellular DNA by a 3′ → 5′ exonuclease activity is presumably an important mechanism in cell resistance to nucleoside analogs. Several cellular molecules, including DNA pol δ, pol ϵ, and wt p53, have intrinsic 3′ → 5′ exonuclease activity (Mummenbrauer et al., 1996; Burgers, 1998; Huang, 1998). The present study demonstrated that neither pol ϵ nor p53 effectively removed the incorporated FMdCMP from DNA in vitro (Fig. 3). The kinetic analysis revealed an interesting interaction between the drug-containing DNA and pol ϵ or p53. As shown in Table 1, p53 and pol ϵ were each able to bind with a much higher affinity to the FMdCMP-containing oligomer than to the normal oligodeoxynucleotide. However, the rate of analog excision was slow, as evidenced by the V_{max} values, which were lower for the FMdCMP oligodeoxynucleotide than the normal oligodeoxynucleotide. These kinetic data suggest that once incorporated into the DNA, FMdCMP might have caused a change in the DNA configuration at the 3′ terminus so that the analog-DNA was preferentially recognized by the proofreading exonuclease of pol ϵ or p53. Importantly, this preferential binding did not result in an effective excision; this failure also may have been due to an analog-induced structural change at the 3′ terminus that was unfavorable for the excision process. This interesting phenomenon has also been observed with the purine nucleoside analog fludarabine (Kamiya et al., 1996). The findings that 3′-terminal FMdCMP is a poor substrate for extension, and that it is resistant to excision indicate that the analog is likely to be a strong DNA chain-terminating nucleotide in whole cells. This attribute is likely to have implications for mechanism-based combination strategies aimed at increasing nucleotide analog incorporation into the DNA of cells undergoing excision repair processes (Sandoval et al., 1996; Yang et al., 2000).

The wt p53 is known to signal apoptosis in response to DNA damage. It is possible that the interaction of p53 with the analog-containing DNA serves as a signal to initiate apoptosis, thus making the cells more sensitive to nucleoside analogs. The high binding affinity of p53 to 3′ FMdCMP-oligodeoxynucleotide and low excision rate make this possibility more likely. Compared with the p53-null HL-60 cells, the wt p53 cells (ML-1) seemed to be more sensitive to FMdC (Fig. 4A). In fact, previous studies demonstrated that transfection of p53-null (H1299) cells with wt p53 increased the sensitivity of the cells to the deoxycytidine analog gemcitabine (Feng et al., 2000). In a separate experiment, we showed that the levels of p53 protein in ML-1 cells increased as early as 4 h after FMdC incubation (10 nM), and the p53 protein accumulation increased in a time-dependent manner (up to 24 h after drug treatment; data not shown). Thus, it is possible that the binding of p53 to the FMdCMP-containing DNA may serve as a signal to accelerate apoptosis. However, the greater sensitivity observed in ML-1 cells should not be solely attributed to their wild-type p53 status, because there may be other differences between ML-1 and HL-60 cells. Factors including drug metabolism, pharmacodynamic factors, and DNA repair capacity may potentially affect the cellular response to FMdC treatment.

Interestingly, in the HL-60 cell line, incubation with FMdC caused a moderate accumulation of cells in the S-phase before the cells succumbed to apoptosis (Fig. 4B). This pause of cells in the S phase may reflect the early cellular response to the drug-induced inhibition or chain termination of DNA synthesis. The concomitant appearance of apoptotic cells (i.e., sub-G_1 population) and the decrease of S or G_1 phase cells suggests that the apoptotic cells were derived from the S-phase cell population. The moderate accumulation of cells in the G_1 phase observed in ML-1 cells (wt p53) might reflect the activation of p53 in response to the drug treatment. As described above, incubation of ML-1 cells with 10 nM FMdC led to a time-dependent accumulation of p53 protein, suggesting this molecule is likely to be involved in the cellular response to FMdC. Interestingly, under the same drug incubation conditions (10 nM FMdC for up to 24 h), no significant change in p21 protein was observed (data not shown). Further studies are needed to assess the molecular mechanisms responsible for the different cell-cycle disturbances caused by FMdC in various cell lines.

The important role of FMdC incorporation into DNA in causing cytotoxicity was further demonstrated in our experiments with aphidicolin, a potent inhibitor of DNA replication. Although inhibition of DNA synthesis by aphidicolin alone did not affect cell viability, incubation of cells with aphidicolin before the addition of FMdC to prevent incorporation of the analog substantially reduced FMdC-induced apoptosis (Fig. 5C). This result suggests that incorporation of FMdC into DNA is a critical event in triggering apoptosis. This observation was consistent with the reported actions of other nucleoside analogs, such as fludarabine and gemcitabine, whose incorporation into cellular DNA is also essential in causing cell death (Huang et al., 1990, 1991, 1995).

In summary, our study indicates that incorporation of FMdC into DNA is a key biochemical event responsible for the cytotoxic action of this analog against tumor cells. FMdC was incorporated into the C site of the DNA strain. Once FMdC nucleotide was incorporated into DNA, further extension of the 3′-FMdCMP-DNA strand by DNA polymerases was difficult. The incorporated analog seemed to be resistant to excision by the 3′ → 5′ exonuclease activity of pol ϵ and p53. The chain-termination activity of FMdC is likely to cause cells to pause in the S phase of the cell cycle and ultimately lead the initiation of processes that promote cell death. It is also possible that if the wild-type p53 protein is present in the cells, the terminally incorporated FMdC residues in DNA may serve as a mechanism to activate p53, trigger cell cycle arrest in G_1/S boundary, and ultimately lead to cell death due to failure in removing the incorporated analog from DNA.

References

Adams ER, Leffert JJ, Craig DJ, Burtness BA, Offord SJ, and Pizzomo G (1996) NMR spectroscopic study of metabolism and distribution of a new anticancer agent (E)-2′-deoxy-2′-(fluoromethylene)cytidine (MDL 101,731) in a colon murine tumor model (Abstract). *Proc Am Assoc Cancer Res* **37:**2498.

Bitonti AJ, Bush TL, Lewis MT, and Sunkara PS (1995) Response of human colon and prostate tumor xenografts to (E)-2′-deoxy-2′-(fluoromethylene)cytidine, an inhibitor of ribonucleoside reductase. *Anticancer Res* **15:**1179–1182.

Bitonti AJ, Dumont JA, Bush TL, Cashman EA, Cross-Doersen DE, Wright PS, Matthews DP, McCarthy JR and Kaplan DA (1994) Regression of human breast tumor xenografts in response to (E)-2′-deoxy-2′-(fluoromethylene)cytidine, an inhibitor of ribonucleoside diphosphate reductase. *Cancer Res* **54:**1485–1490.

Burgers PM (1998) Eukaryotic DNA polymerases in DNA replication and DNA repair. *Chromosoma* **107:**218–227.

Catapano CV, Perrino FW, and Fernandes DJ (1993) Primer RNA chain termination induced by 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-triphosphate: a mechanism of DNA synthesis inhibition. *J Biol Chem* **268:**7179–7185.

Faderl S, Thomas DA, Cortes J, Giles F, Garcia-Manero G, O'Brien S, Koller C, Keating MJ, Cabanillas F, Sarris A, et al. (2000) Phase I study of tezacitabine, an antimetabolite deoxycytidine analog, in patients with relapsed and refractory hematologic malignancies (Abstract). *Blood* **96:**724a.

Feng L, Achanta G, Pelicano H, Zhang W, Plunkett W, and Huang P (2000) Role of p53 in cellular response to anticancer nucleoside analog-induced DNA damage. *Int J Mol Med* **5:**597–604.

Gandhi V, Estey E, Keating MJ, Chucrallah A, and Plunkett W (1996) Chlorodeoxyadenosine and arabinosylcytosine in patients with acute myelogenous leukemia: pharmacokinetic, pharmacodynamic, and molecular interactions. *Blood* **87:**256–264.

Gandhi V, Estey E, Keating MJ, and Plunkett W (1993) Fludarabine potentiates metabolism of arabinosylcytosine in patients with acute myelogenous leukemia during therapy. *J Clin Oncol* **11:**116–124.

Gandhi V, Huang P, and Plunkett W (1994) Fludarabine inhibits DNA replication: a rationale for its use in the treatment of acute leukemias. *Leuk Lymphoma* **2:**3–9.

Grant S (1998) Ara-C: cellular and molecular pharmacology. *Adv Cancer Res* **72:**197–233

Huang P (1998) Excision of mismatched nucleotides from DNA: a potential mechanism for enhancing DNA replication fidelity by the wild-type protein. *Oncogene* **17:**261–270.

Huang P, Chubb S, Hertel LW, Grindey GB, and Plunkett W (1991) Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. *Cancer Res* **51:**6110–6117.

Huang P, Chubb S, and Plunkett W (1990) Termination of DNA synthesis by 9-β-D-arabinofuranosyl-2-fluoroadenine, a mechanism for cytotoxicity. *J Biol Chem* **265:**16617–16625.

Huang P and Plunkett W (1991) Action of 9-β-D-Arabinofuranosyl-2-fluoroadenine on RNA metabolism. *Mol Pharmacol* **39:**449–455.

Huang P and Plunkett W (1995) Fludarabine and gemcitabine-induced apoptosis: incorporation of analogs into DNA is a critical event. *Cancer Chemother Pharmacol* **36:**181–188.

Huang P, Robertson LE, Wright S, and Plunkett W (1995) High molecular weight DNA fragmentation: a critical event in nucleoside analog-induced apoptosis in leukemia cells. *Clin Cancer Res* **1:**1005–1013.

Hui YF and Reitz J (1997) Gemcitabine: a cytidine analogue active against solid tumors. *Am J Health Syst Pharm* **54:**162–170.

Kamiya K, Huang P, and Plunkett W (1995) Inhibition of the 3′ → 5′ exonuclease of human DNA polymerase epsilon by fludarabine-terminated DNA. *J Biol Chem* **271:**19428–19435.

Kanazawa J, Takahashi T, Akinaga S, Tamaoki T, and Okabe M (1998) The relationship between the antitumor activity and the ribonucleotide reductase inhibitory activity of (E)-2′-deoxy-2′-(fluoromethylene) cytidine, MDL 101,731. *Anticancer Drugs* **9:**653–657.

Kotchetkov R, Krivtchik AA, Cinatl J, Kornhuber B, and Cinatl J Jr (1999) Selective cytotoxic activity of a novel ribonucleoside diphosphate reductase inhibitor MDL-101,731 against thyroid carcinoma in vitro. *Folia Biol (Praha)* **45:**185–191

Kufe DW, Munroe D, Herrick D, Egan E, and Spriggs D (1984) Effects of 1-β-D-arabinofuranosylcytosine incorporation on eukaryotic DNA template function. *Mol Pharmacol* **26:**128–134.

McCarthy JR, Matthews DP, Stemerick DM, Huber EW, Bey P, Lippert BJ, Snyder RD, and Sunkara PS (1991) Stereospecific method to E and Z terminal fluoroolefins and its application to the synthesis of 2′-deoxy-2′-fluoromethylene nucleosides as potential inhibitors of ribonucleotide diphosphate reductase. *J Am Chem Soc* **113:**7439–7440.

Mummenbrauer T, Janus F, Muller B, Wiesmuller L, Deppert W, and Grosse F (1996) p53 protein exhibits 3′-to-5′ exonuclease activity. *Cell* **85:**1089–1099.

Noriyuki M, Shunichi N, Kouji T, Nobuhide T, Tomonori H, Takashi Y, Norialki K, Takashi K, Satoshi K, Shinzoh K, et al. (1999) Phase I and pharmacologic study of oral (E)-2′-deoxy-2′-(fluoromethylene) cytidine: on a daily × 5-day schedule. *Invest New Drugs* **16:**245–254.

Piepmeier JM, Rabidou N, Schold SC Jr, Bitonti AJ, Prakash NJ and Bush TL (1996) *In vitro* and *in vivo* inhibition of glioblastoma and neuroblastoma with MDL 101,731, a novel ribonucleoside diphosphate reductase inhibitor. *Cancer Res* **56:**359–361.

Rodriguez GL, Brooks DJ and Burtness BA (1999) Phase I clinical trials of intravenous 2′-deoxy-2′-(fluoromethylene) cytidine (FMdC) in patients with advanced solid tumors. *Proc Am Soc Clin Oncol* **18:**15–18.

Sandoval A, Consoli U, and Plunkett W (1996) Fludarabine-mediated inhibition of nucleotide excision repair induces apoptosis in quiescent human lymphocytes. *Clin Cancer Res* **2:**1731–1742.

Seley KL (2000) Tezacitabine Hoechst Marion Roussel. *Curr Opin Invest Drugs* **1:**135–140.

Snyder RD (1994) Effect of 2′-deoxy-2′-(fluoromethylene) cytidine on the ultraviolet and x-ray sensitivity of HeLa cells. *Oncol Res* **6:**177–182.

Storniolo AM, Allerheiligen SR, and Pearce HL (1997) Preclinical, pharmacologic, and phase I studies of gemcitabine. *Semin Oncol* **24 (2 Suppl 7):**2–7.

Sun LQ, Li YX, Guillou L, and Coucke PA (1998) (E)-2′-deoxy-2′-(fluoromethylene) cytidine potentiates radioresponse of two human solid tumor xenografts. *Cancer Res* **58:**5411–5417.

Sun LQ, Li YX, Guillou L, Mirimanoff RO, and Coucke PA (1997) Antitumor and radiosensitizing effects of (E)-2′-deoxy-2′-(fluoromethylene) cytidine, a novel inhibitor of ribonucleoside diphosphate reductase, on human colon carcinoma xenografts in nude mice. *Cancer Res* **57:**4023–4028.

Takahashi T, Nakashima A, Kanazawa J, Yamaguchi K, Akinaga S, Tamaoki T, and Okabe M (1998) Metabolism and ribonucleotide reductase inhibition of (E)-2′-deoxy-2′-(fluoromethylene)cytidine, MDL 101,731, in human cervical carcinoma HeLa S_3 cells. *Cancer Chemother Pharmacol* **41:**268–274.

van der Donk WA, Yu GX, Silva DJ, and Stubbe J (1996) Inactivation of ribonucleotide reductase by (E)-2′-fluoromethylene-2′-deoxycytidine 5′-diphosphate: a paradigm for nucleotide mechanism-based inhibitors. *Biochemistry* **35:**8381–8391.

Wright PS, Cross-Doerson D, Th'ng JPH, Guo XW, Crissman HA, Bradbury EM, Montgomery LR, Thompson FY, Loudy DE, Johnston JO, et al. (1996) A ribonucleotide reductase inhibitor, MDL 101,731, induces apoptosis and elevates TRPM-2 mRNA levels in human prostate tumor xenografts. *Exp Cell Res* **222:**54–60.

Xie C and Plunkett W (1995) Metabolism and actions of 2-chloro-9-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)-adenine in human lymphoblastoid cells. *Cancer Res* **55:**2847–2852.

Yang SW, Huang P, Plunkett W, Becker FF, and Chan JYH (1992) Dual mode of inhibition of purified DNA ligase I from human cells by 9-β-D-arabinofuranosyl-2-fluoroadenine triphosphate. *J Biol Chem* **267:**2345–2349.

Yang LY, Li L, Jiang H, Shen Y, and Plunkett W (2000) Expression of *ERCC1* antisense RNA abrogates gemcitabine-mediated cytotoxic synergism with cisplatin in human colon tumor cells defective in mismatch repair but proficient in nucleotide excision repair. *Clin Cancer Res* **60:**773–781.

Yonetani Y and Mizukami T (1996) Kinetic analysis of inhibition of human ribonucleotide reductase and DNA polymerase α by a new anticancer agent, (E)-2′-deoxy-2′-(fluoromethylene) cytidine. *Proc Am Assoc Cancer Res* **37:**405.

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