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Comparison of the Mechanism of Cytotoxicity of 2-Chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine, 2-Chloro-9-(2-deoxy-2-fluoro-β-D-ribofuranosyl)adenine, and 2-Chloro-9-(2-deoxy-2,2-difluoro-β-D-ribofuranosyl)adenine in CEM Cells

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ABSTRACT

In an effort to understand biochemical features that are important to the selective antitumor activity of 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine [Cl-F(↑)-dAdo], we evaluated the biochemical pharmacology of three structurally similar compounds that have quite different antitumor activities. Cl-F(↑)-dAdo was 50-fold more potent as an inhibitor of CEM cell growth than were either 2-chloro-9-(2-deoxy-2-fluoro-β-D-ribofuranosyl)adenine [Cl-F(↓)-dAdo] or 2-chloro-9-(2-deoxy-2,2-difluoro-β-D-ribofuranosyl)adenine [Cl-diF(↑↓)-dAdo]. The compounds were similar as substrates of deoxycytidine kinase. Similar amounts of their respective triphosphates accumulated in CEM cells, and the rate of disappearance of these metabolites was also similar. Cl-F(↑)-dAdo was 10- to 30-fold more potent in its ability to inhibit the incorporation of cytidine into deoxycytidine nucleotides than either Cl-F(↓)-dAdo or Cl-diF(↑↓)-dAdo, respectively, which indicated that ribonucleotide reductase was differentially inhibited by these three compounds. Thus, the differences in the cytotoxicity of these agents toward CEM cells were not related to quantitative differences in the phosphorylation of these agents to active forms but can mostly be accounted for by differences in the inhibition of ribonucleotide reductase activity. Furthermore, the inhibition of RNA and protein synthesis by Cl-F(↓)-dAdo and Cl-diF(↑↓)-dAdo at concentrations similar to those required for the inhibition of DNA synthesis can help explain the poor antitumor selectivity of these two agents because all cells require RNA and protein synthesis.

2-Chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine [Cl-F(↑)-dAdo] is a promising new antitumor agent that has structural similarities to both 2-chloro-2′-deoxyadenosine (Cl-dAdo; cladribine) and 2-fluoro-9-(β-D-arabinofuranosyl)-adenine (F-araA or fludarabine), both of which are Food and Drug Administration-approved drugs for the treatment of certain types of cancer (Bonnet and Robins, 1993). In the past, Cl-F(↑)-dAdo has been abbreviated as Cl-F-araA and CAFdA. However, for clarity of presentation, we have changed its abbreviation. In addition to its activity against hematologic malignancies (Carson et al., 1992; Waud et al., 1992), Cl-F(↑)-dAdo is also active against solid tumors such as renal and colon carcinomas (W. R. Waud, personal communication). The mechanism of action of Cl-F(↑)-dAdo has been extensively studied (Parker et al., 1991; Carson et al., 1992; Xie and Plunkett, 1995, 1996) and is similar to that of Cl-dAdo and F-araA (Parker et al., 1991; Plunkett and Saunders, 1991). These agents are metabolized via 2′-deoxycytidine (dCyd) kinase to their respective triphosphates, which are potent feedback inhibitors of ribonucleotide reductase and are used as substrates by DNA polymerases.

During the development of Cl-F(↑)-dAdo, 2-chloro-9-(2-deoxy-2-fluoro-β-D-ribofuranosyl)adenine [Cl-F(↓)-dAdo], and 2-chloro-9-(2-deoxy-2,2-difluoro-β-D-ribofuranosyl)adenine [Cl-diF(↑↓)-dAdo] (Fig. 1) were also synthesized and evaluated for antitumor activity. In contrast to Cl-F(↑)-dAdo, these two compounds were not potent inhibitors of cell growth and did not have significant selectivity in animal antitumor models (W. R.

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ABBREVIATIONS: Cl-dAdo, 2-chloro-2′-deoxyadenosine; Cl-F(↑)-dAdo, 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine; Cl-F(↓)-dAdo, 2-chloro-9-(2-deoxy-2-fluoro-β-D-ribofuranosyl)adenine; Cl-diF(↑↓)-dAdo, 2-chloro-9-(2-deoxy-2,2-difluoro-β-D-ribofuranosyl)adenine; Cl-F(↑)-dATP, Cl-F(↑)-dAdo 5′-triphosphate; Cl-F(↓)-dATP, 2-chloro-9-(2-deoxy-2-fluoro-β-D-ribofuranosyl)adenine 5′-triphosphate; Cl-diF(↑↓)-dATP, 2-chloro-9-(2-deoxy-2,2-difluoro-β-D-ribofuranosyl)adenine 5′-triphosphate; Cyd, cytidine; dCyd, 2′-deoxycytidine; dThd, thymidine; F-araA, 2-fluoro-9-(β-D-arabinofuranosyl)adenine; SAX, strong anion exchange; Urd, uridine.

Waud and L. W. Hertel, personal communications). Cl-diF(↑↓)-dAdo also has structural features similar to 2′,2′-difluoro-dCyd (gemcitabine), another new antitumor nucleoside analog recently approved by the Food and Drug Administration for the treatment of pancreatic cancer (Plunkett et al., 1996). Because of the similarities in structure among Cl-F(↑)-dAdo, Cl-F(↓)-dAdo, and Cl-diF(↑↓)-dAdo, it was important to our understanding of the mechanistic implications of specific structural variations to determine why these changes resulted in very different antitumor activities. Of particular interest was the use of this information for our detailed understanding of the biochemical activities of Cl-F(↑)-dAdo that are important to its highly selective antitumor activity.

Experimental Procedures

Materials. Cl-F(↑)-dAdo and Cl-F(↓)-dAdo were synthesized in our laboratories (Secrist et al., 1988; Thomas et al., 1994), and Cl-diF(↑↓)-dAdo was synthesized at Eli Lilly and Company (Indianapolis, IN) (Grindey and Hertel, 1991, 1995). [*methyl*-^{3}H]Thymidine(dThd) (69 Ci/mmol), [5-^{3}H]uridine (Urd) (20 Ci/mmol), [5-^{3}H]cytidine (Cyd) (25 Ci/mmol), [5-^{3}H]dCyd (26 Ci/mmol), and [4,5-^{3}H]leucine (20 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). CEM cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) containing 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 1 mg/ml sodium bicarbonate, 10 units/ml penicillin, 10 μg/ml streptomycin, and 50 μg/ml gentamycin. Cells were routinely checked for the presence of mycoplasma and were discarded if contaminated. The Aquasil C18 and Partisil-10 strong anion exchange (SAX) HPLC columns were obtained from Keystone Scientific Inc. (Bellefonte, PA). Other compounds were of standard analytical grade.

Measurement of DNA, RNA, and Protein Synthesis in Intact Cells. The effect of compounds on the incorporation of radiolabeled precursors ([5-^{3}H]Urd, [*methyl*-^{3}H]dThd, or [4,5-^{3}H]leucine) into RNA, DNA, or protein was determined as described previously (Hershko et al., 1971; Bennett et al., 1978). The incorporation of Urd into RNA is determined by subtracting the incorporation of radiolabel into the alkali-stable/acid-precipitable fraction (DNA) from the total acid-precipitable fraction (DNA plus RNA). [5-^{3}H]Urd is primarily incorporated into RNA but also can be incorporated into DNA as dCyd. The incorporation of dThd and leucine into acid-precipitable material is a measure of their incorporation into DNA and protein, respectively.

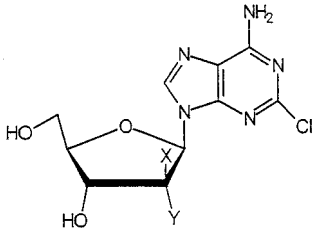
The effect of these compounds on the incorporation of [5-^{3}H]Cyd into DNA was determined as a measure of their effect on ribonucleotide reductase activity. In these experiments, CEM cells were incubated with 10 nM [^{3}H]Cyd plus varying concentrations of the nucleoside analogs. After 10 min, the incorporation of [^{3}H]Cyd into the alkali-stable/acid-insoluble fraction (DNA) was determined as described previously (Hershko et al., 1971; Bennett et al., 1978). Hydroxyurea, at 1 mM, completely inhibited the incorporation of [5-^{3}H]Cyd into the alkali-stable/acid-insoluble fraction, which indicated that this fraction was not contaminated with RNA. Preliminary studies had determined that the rate of incorporation of [^{3}H]Cyd into DNA was linear for at least 1 h.

To determine the effect of the compounds on the conversion of [5-^{3}H]Cyd to acid-soluble dCyd nucleotides, cells were treated with varying concentrations of the compounds and [5-^{3}H]Cyd for 10 min, and the acid-soluble extract was obtained as described below. The extracts were incubated at 37°C with 20 units of alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) for 24 h. The enzyme reaction was stopped by adding perchloric acid to 0.5 M, and the samples were neutralized with KOH and potassium phosphate buffer, pH 7.4. dCyd was separated from Cyd using reversed phase HPLC (Aquasil C18 column (150 × 4.6 mm; isocratic elution with 10 mM ammonium dihydrogen phosphate buffer in 1% acetonitrile at a flow rate of 1 ml/min). Radioactivity in Cyd and dCyd was determined by counting 1-min fractions that eluted from the column.

Extraction and Analysis of Acid-Soluble Nucleotide Pool. Cells were collected by centrifugation and resuspended in ice-cold 0.5 M perchloric acid. The samples were centrifuged at 12,000*g* for 20 min, and the supernatant fluid was removed and neutralized with 1 M potassium phosphate, pH 7.4, and 4 M KOH. $KClO_4$ was removed by centrifugation, and a portion of the supernatant fluid was injected onto a Partisil-10 SAX column. Elution of the nucleotides was accomplished with a 50-min linear gradient from 5 mM $NH_4H_2PO_4$ (pH 2.8) to 750 mM $NH_4H_2PO_4$ (pH 3.7) buffer with a flow rate of 2 ml/min. The nucleotides were detected by measurement of the UV absorbance at 260 nm.

Measurement of dCyd Kinase Activity. dCyd kinase was purified 24,000-fold from MOLT-4 cells to greater than 95% purity. The procedures for the isolation and for assay of nonlabeled nucleosides as substrates have been described elsewhere (Shewach et al., 1992). The Michaelis-Menten parameters were determined from linear double reciprocal plots of 1/velocity versus 1/concentration of the substrate. The best line was determined using linear regression from at least five data points, and the K_m and V_{max} values were determined from the *x*- and *y*-intercepts.

dCyd kinase activity was also measured in crude CEM cell extracts. CEM cells were homogenized with a Dounce homogenizer using pestle A. The extract was centrifuged at 100,000*g* and was dialyzed against 50 mM Tris, pH 7.5, 0.5 mM EDTA, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Glycerol was added after dialysis to a final concentration of 30%. dCyd kinase activity was measured at 37°C in 50-μl volumes containing 50 mM Tris, pH 8.0, 2 mM ATP, 7.5 mM $MgCl_2$, 1 μM [^{3}H]dCyd (2 Ci/mol), 20 mM NaF, and extract. After 30 min, the reactions were stopped by spotting 50 μl of the reaction mix onto DE-81 filters. The disks were washed three times with 95% ethanol/5% H_2O, and the radioactivity on each disk was determined. Preliminary experiments had determined that the reaction rate was linear for 30 min.



a) X = F; Y = H; Cl-F(↑)-dAdo
b) X = H; Y = F; Cl-F(↓)-dAdo
c) X = Y = F; Cl-diF(↑↓)-dAdo
d) X = Y = H; Cl-dAdo

Fig. 1. Structures of Cl-dAdo, Cl-F(↑)-dAdo, Cl-F(↓)-dAdo, and Cl-diF(↑↓)-dAdo.

Results

Inhibition of CEM Cell Growth. Cl-F(↑)-dAdo was approximately 50-fold more potent as an inhibitor of CEM cell growth than either Cl-F(↓)-dAdo or Cl-diF(↑↓)-dAdo (Fig. 2). The IC_{50} values of Cl-F(↑)-dAdo, Cl-F(↓)-dAdo, and Cl-diF(↑↓)-dAdo were 6 ± 1, 313 ± 47, and 317 ± 78 nM, respectively (mean ± S.D. from three experiments). The addition of dCyd completely protected against the cytotoxicity of these three compounds (data not shown), which suggested that these compounds were phosphorylated to active metabolites by dCyd kinase.

Phosphorylation in CEM Cells. Differential activation of these compounds could explain their differences in cytotoxicity to CEM cells; therefore, the level of conversion of each compound to its respective triphosphate analog was determined (Fig. 3). A 2-h incubation with 25 μM Cl-F(↑)-dAdo, Cl-F(↓)-dAdo, or Cl-diF(↑↓)-dAdo resulted in 75 ± 13, 67 ± 14, or 76 ± 9 pmol of triphosphate analog formed/10^6 cells (mean ± S.D. from three experiments). For each of the compounds, the accumulation of triphosphate increased during an 8-h incubation period and the amount of triphosphate formed were directly related to the concentration of compound in the culture medium (1–50 μM; data not shown). Because of the much smaller peaks formed at lower concentrations, GTP and the other ribonucleotides were removed from the extracts by boronate column chromatography (Shewach, 1992) before analysis by SAX HPLC. The conversion of these nucleoside analogs to their respective nucleoside triphosphates was inhibited by incubation with dCyd (data not shown), which again indicated that dCyd kinase was the enzyme primarily responsible for the metabolism of these agents in CEM cells.

The triphosphates of these nucleoside analogs also had a similar initial $T_{1/2}$ for disappearance (Fig. 4). After a 2-h incubation with 25 μM concentration of compound, the $T_{1/2}$ values for the disappearance of Cl-F(↑)-dAdo 5′-triphosphate [Cl-F(↑)-dATP], 2-chloro-9-(2-deoxy-2-fluoro-β-D-ribofuranosyl)adenine 5′-triphosphate [Cl-F(↓)-dATP], and 2-chloro-9-(2-deoxy-2,2-difluoro-β-D-ribofuranosyl)adenine 5′-triphosphate [Cl-diF(↑↓)-dATP] were 3.2 ± 2.1, 1.6 ± 0.7, and 1.6 ± 0.7 h, respectively (mean ± S.D. from at least three experiments). Xie and Plunkett (1995) have shown that Cl-F(↑)-dATP was eliminated in a nonlinear manner with an initial $T_{1/2}$ of 1.2 h, which is in good agreement with our results. The terminal $T_{1/2}$ for Cl-F(↑)-dATP was in the order of 29 h. Due to the lack of radiolabeled compounds, we were not able to determine a terminal $T_{1/2}$ for these three compounds. Our results indicated that differences in the rate of accumulation of triphosphate analog or the rate of their disappearance were not sufficient to explain the large differences seen in cytotoxicity of these agents in CEM cells.

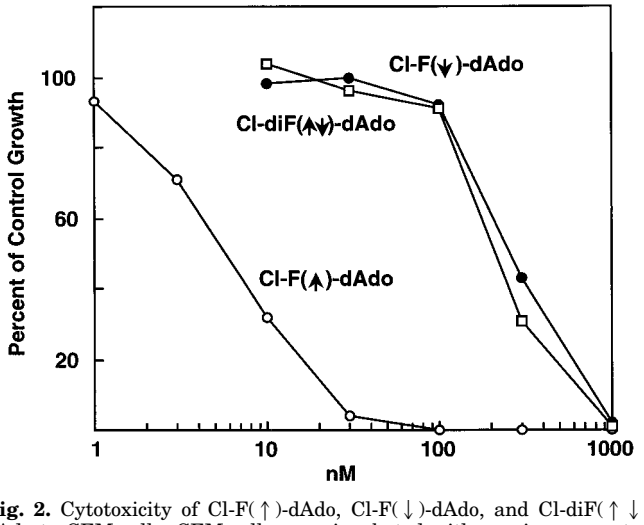


Fig. 2. Cytotoxicity of Cl-F(↑)-dAdo, Cl-F(↓)-dAdo, and Cl-diF(↑↓)-dAdo to CEM cells. CEM cells were incubated with varying concentrations of compounds for 72 h, and the cell numbers were determined using a Coulter Counter. The results shown are from a representative experiment from a total of three that were done.

Substrate Activity with Purified dCyd Kinase. The Michaelis-Menten constants for these three compounds were determined with pure dCyd kinase isolated from Molt-4 cells (another cell line derived from human T cells) (Table 1). Even though the K_m values for the dAdo analogs were much higher than the K_m value for dCyd, their maximum velocities were also much higher, so the relative efficiencies were closer to that for dCyd. Of the three dAdo analogs, Cl-F(↑)-dAdo was the best substrate, but it was only 2-fold better than Cl-diF(↑↓)-dAdo and 7-fold better than Cl-F(↓)-dAdo. The K_m value for Cl-F(↓)-dAdo was 10-fold greater than the K_m value for Cl-F(↑)-dAdo or Cl-diF(↑↓)-dAdo. These differences in the substrate parameters between Cl-F(↑)-dAdo and Cl-diF(↑↓)-dAdo were not

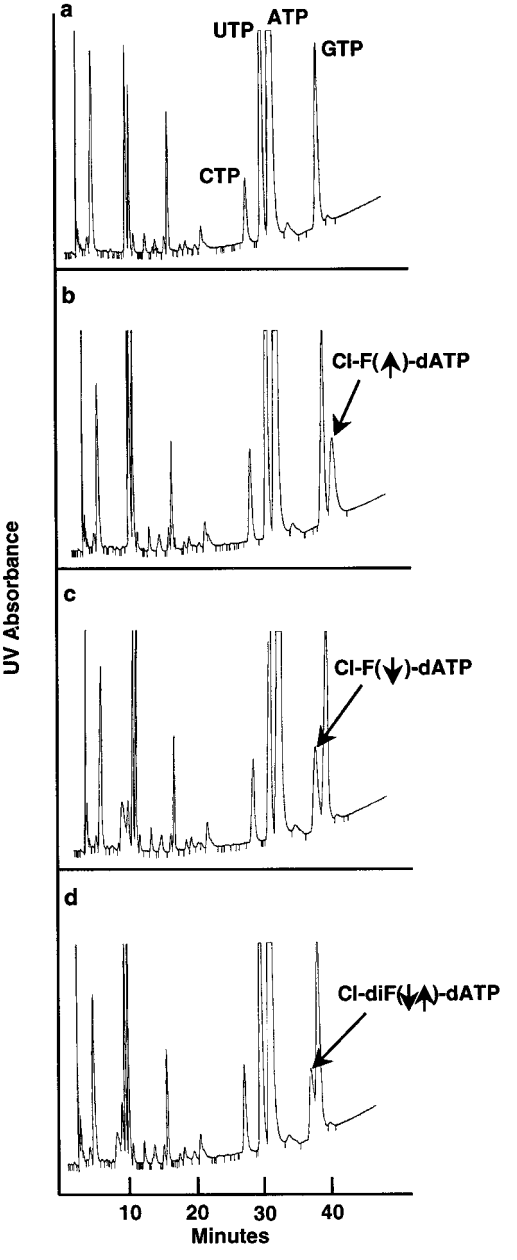


Fig. 3. Phosphorylation of Cl-F(↑)-dAdo, Cl-F(↓)-dAdo, and Cl-diF(↑↓)-dAdo in CEM cells. CEM cells were incubated with no drugs (a) or 25 μM Cl-F(↑)-dAdo (b), Cl-F(↓)-dAdo (c), or Cl-diF(↑↓)-dAdo (d) for 2 h. The acid-soluble fraction was collected and analyzed by SAX HPLC as described in *Materials and Methods*. This is a representative experiment from a total of three that were done.

sufficient to account for the differences in the cytotoxicities of these two agents. However, the 7-fold difference in the preference of dCyd kinase for Cl-F(↑)-dAdo versus Cl-F(↓)-dAdo could help explain some of the differences in potency of these two compounds.

The kinetic parameters for Cl-dAdo, F-araA, and 2′,2′-diF-dCyd are included in Table 1 for comparison purposes. The relative efficiencies of the 2′-halo-2-Cl nucleosides with dCyd kinase were similar to that observed with Cl-dAdo, although Cl-dAdo had lower K_m and V_{max} values than any of the 2′-halo analogs. All of the 2-Cl analogs were much better substrates for dCyd kinase than were either dAdo or F-araA. Tisdale et al. (1993) have shown that the kinetic parameters of 2′-F(↑)-dAdo with calf thymus dCyd kinase were similar to dAdo. However, with human dCyd kinase, the V_{max} value for Cl-F(↑)-dAdo was 10 times that of Cl-dAdo, whereas the V_{max} for 2′-F(↑)-dAdo with calf thymus dCyd kinase was only 50% of that seen with dAdo.

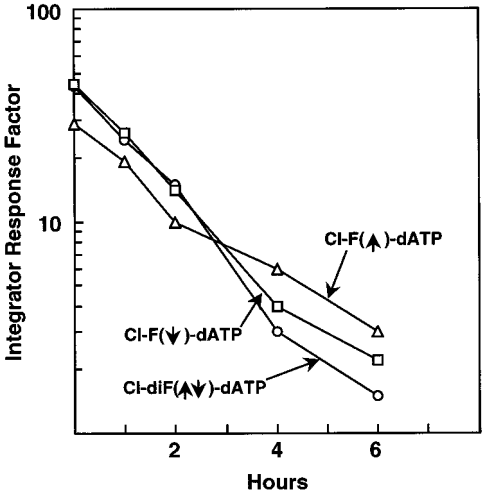


Fig. 4. Half-life for disappearance of Cl-F(↑)-dATP, Cl-F(↓)-dATP, and Cl-diF(↑↓)-dATP. CEM cells were treated with 25 μM Cl-F(↑)-dAdo, Cl-F(↓)-dAdo, or Cl-diF(↑↓)-dAdo. After 2 h, the cells were collected by centrifugation and resuspended in fresh media that contained none of the above compounds. At 0, 1, 2, 4, and 6 h after resuspension, a sample of the cell extract was collected, and the amount of analog triphosphate was determined as described in *Materials and Methods*. The results shown are from a representative experiment from a total of three that were done.

TABLE 1
Substrate characteristics of Cl-F(↑)-dAdo, Cl-F(↓)-dAdo, and Cl-diF(↑↓)-dAdo with dCyd kinase isolated from MOLT-4 cells

Compound	K_m	Relative V_{max}	V_{max}/K_m	Relative Efficiency
	μM			
dCyd	1.3	1	0.77	100
2′,2′-diF-dCyd[a]	9.3	44	4.7	610
dAdo	501	1	0.002	0.26
Cl-dAdo[b]	5	0.9	0.18	23
Cl-F(↑)-dAdo	14	9.9	0.71	87
Cl-F(↓)-dAdo	141	14.5	0.10	13
Cl-diF(↑↓)-dAdo	14	4.3	0.31	40
F-araA[a]	1600	42	0.026	3.4

Each value is average of at least two assays. Each assay was done in duplicate, and kinetic constants were determined with a computer program (Wilkinson, 1961). Standard errors for each assay value were less than 10%. K_m and V_{max} values varied between assays by 1.1- to 2.3-fold.
[a] Data taken from Shewach et al. (1992).
[b] Data taken from Eriksson et al. (1991).

Inhibition of dCyd Kinase Activity in Crude CEM Cell Extracts. The concentrations of Cl-F(↑)-dAdo, Cl-F(↓)-dAdo, and Cl-diF(↑↓)-dAdo required to inhibit the phosphorylation in crude CEM extracts of 1 μM dCyd by 50% were 110 ± 23, 225 ± 72, and 105 ± 22 μM, respectively (mean ± S.D. from three experiments, data not shown). This result indicated that the affinity of these compounds for dCyd kinase in CEM cells were similar and supported our conclusion that the differences in the activation of the compounds were not responsible for the differences in cytotoxicity.

Inhibition of Macromolecular Synthesis. Cl-F(↑)-dAdo was 10- to 20-fold more potent in its ability to inhibit the incorporation of dThd into DNA than either Cl-F(↓)-dAdo or Cl-diF(↑↓)-dAdo (Fig. 5, IC_{50} = 0.05 versus approximately 1 μM). Furthermore, both Cl-F(↓)-dAdo and Cl-diF(↑↓)-dAdo inhibited the incorporation of Urd into RNA and leucine into protein at concentrations that were similar to those required to inhibit dThd incorporation into DNA. At 1, 2, and 4 μM, Cl-F(↑)-dAdo, RNA, and protein syntheses were inhibited to a similar degree as that seen with Cl-F(↓)-dAdo and Cl-diF(↑↓)-dAdo (data not shown).

Inhibition of [^{3}H]Cyd Incorporation into dCyd Nucleotides. Because the inhibition of DNA synthesis by Cl-F(↑)-dAdo results from the inhibition of both ribonucleotide reductase and DNA polymerases (Parker et al., 1991), the effect of these compounds on the incorporation of [^{3}H]Cyd into DNA was determined in an attempt to evaluate the inhibition of ribonucleotide reductase by these agents (Fig. 6). The concentrations of Cl-F(↑)-dAdo, Cl-F(↓)-dAdo, and Cl-diF(↑↓)-dAdo that were required to inhibit the incorporation of Cyd into DNA by 50% were 0.26 ± 0.1, 3.2 ± 1.8, and 9.2 ± 2.5 μM, respectively (mean ± S.D. from three experiments). Cl-F(↑)-dAdo was approximately 10-fold more potent than Cl-F(↓)-dAdo and 30-fold more potent than Cl-diF(↑↓)-dAdo in its ability to inhibit ribonucleotide reductase.

Because the inhibition of DNA polymerase activity by these compounds would also result in the inhibition of the incorporation of label into DNA, the effect of these compounds on the conversion of [^{3}H]Cyd to acid-soluble deoxycytidine nucleotides was determined (data not shown). In this experiment, CEM cells were incubated with varying concentrations of the dAdo analogs and [^{3}H]Cyd for 10 min. The nucleotides in the acid-soluble extract were degraded to their respective nucleosides using alkaline phosphatase, and [^{3}H]Cyd was separated from [^{3}H]dCyd using reversed phase HPLC. Incubation of CEM cells with these agents caused a dose-dependent decrease in the conversion of [^{3}H]Cyd to acid-soluble dCyd deoxynucleotides, with IC_{50} values similar to those determined for the inhibition of label into DNA. In addition, at a concentration of 10 μM none of the compounds inhibited the uptake of 10 nM [^{3}H]Cyd, nor did they inhibit the conversion of Cyd to CTP. Therefore, the incorporation of [^{3}H]Cyd into DNA is a good measure of the ribonucleotide reductase activity.

Discussion

The inhibition of ribonucleotide reductase and DNA polymerase activities is believed to be responsible for the antitumor activity of Cl-F(↑)-dAdo (Parker et al., 1991; Carson et al., 1992; Xie and Plunkett, 1995, 1996). Cl-F(↑)-dAdo is

phosphorylated in human cells to Cl-F(↑)-dATP, which potently inhibits ribonucleotide reductase as an allosteric regulator. Cl-F(↑)-dATP is also used as a substrate by DNA polymerase α, and its incorporation into the 3′ end of the growing DNA chain causes disruption of further DNA synthesis. Because Cl-F(↑)-dATP competes with dATP for incorporation into DNA, the decline in dNTP levels (including dATP) due to the inhibition of ribonucleotide reductase potentiates the activity of Cl-F(↑)-dATP against DNA polymerases. The mechanism of action of Cl-F(↑)-dAdo is very similar to that of Cl-dAdo. Cl-dAdo is phosphorylated to Cl-dATP, which is also a potent inhibitor of both ribonucleotide reductase and DNA polymerases (Parker et al., 1991). One difference between these compounds is that the incorporation of Cl-F(↑)-dAdo into the 3′ end of a DNA chain results in a greater disruption of DNA elongation than does the incorporation of Cl-dAdo (Parker et al., 1991). The incorporation of Cl-dAdo into DNA has been shown to disrupt various activities that involve DNA (Hentosh and McCastlain, 1991; Hentosh and Grippo, 1994a, 1994b; Hentosh and Tibudan, 1995). Cl-dATP has recently been shown to substitute for dATP in the activation of caspase-3 in a cell-free system (Leoni et al., 1998), and it is possible that these compounds could also substitute for dATP in the activation of this apoptotic pathway. However, the importance of this action of Cl-dAdo and related compounds to their ability to kill cells has yet to be determined.

In the current work, we compared the biochemical pharmacology of Cl-F(↑)-dAdo with that of two compounds that are very similar in structure but much less potent as inhibitors of CEM cell growth and do not exhibit selective antitumor activity in mice. We determined that the reason for the differences in potency in CEM cells was primarily due to differences in the inhibition of ribonucleotide reductase activity by these compounds rather than to differences in their activation. Differences in the interaction of the 5′-triphosphate analogs of these compounds with DNA polymerases could also contribute to the differences seen in the potencies of these agents. However, the fact that most of the differences in cytotoxicity in CEM cells could be explained by the differences in the inhibition of ribonucleotide reductase activity suggested that the DNA-directed activities of these compounds (direct inhibition of DNA elongation due to their incorporation into DNA) are of secondary importance in the inhibition of CEM cell growth. The antitumor activity of hydroxyurea, an inhibitor of ribonucleotide reductase, indicates that the depression of dNTP levels alone is sufficient to result in antitumor activity.

Cl-F(↓)-dAdo was a poorer substrate for dCyd kinase iso-

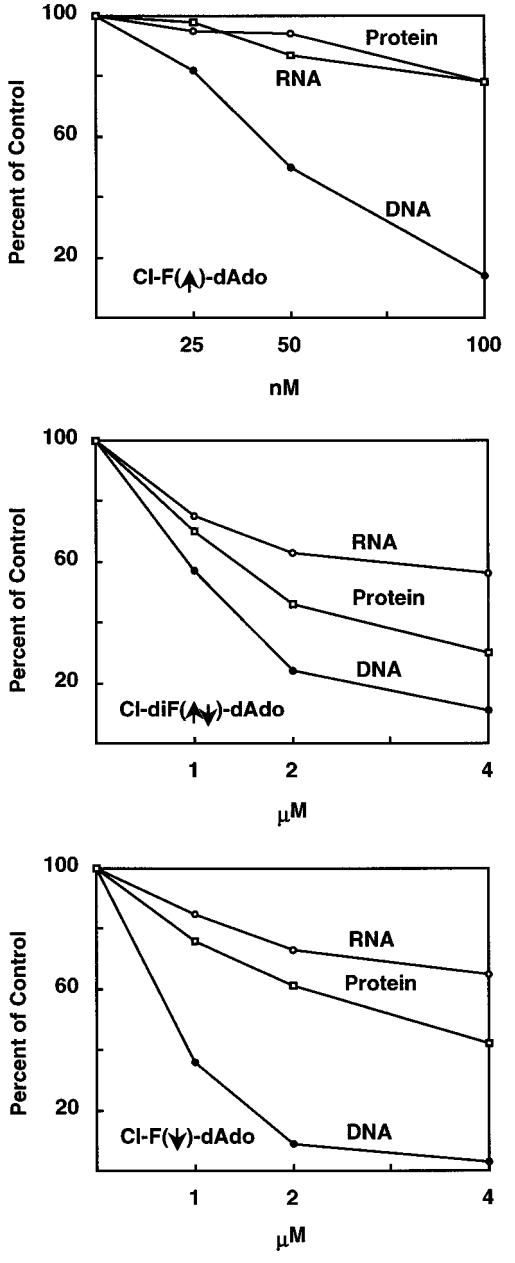


Fig. 5. Effect of Cl-F(↑)-dAdo, Cl-F(↓)-dAdo, and Cl-diF(↑↓)-dAdo on DNA, RNA, and protein synthesis. CEM cells were incubated with varying concentrations of Cl-F(↑)-dAdo, Cl-F(↓)-dAdo, or Cl-diF(↑↓)-dAdo. Radiolabeled precursors of DNA ([*methyl*-^{3}H]dThd), RNA ([5-^{3}H]Urd), or protein ([4,5-^{3}H]leucine) were added to the cell cultures 30 min hours after the addition of the compounds, and cell samples were taken 1, 2, 3, or 4 h after the addition of radiolabel to determine their incorporation into RNA, DNA, or protein as described in *Materials and Methods*. This experiment was repeated one time with similar results.

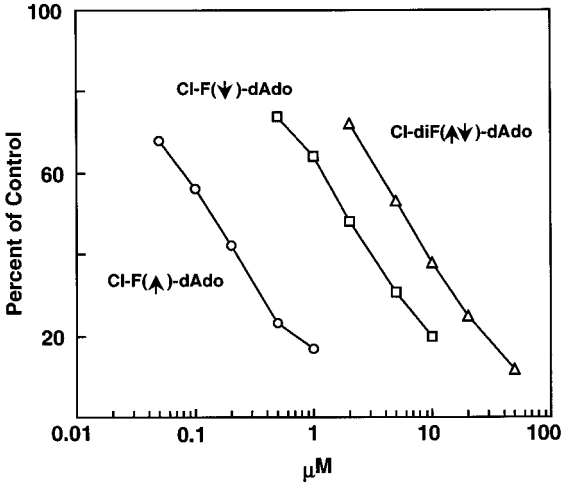


Fig. 6. Inhibition of [^{3}H]Cyd incorporation into DNA. CEM cells were incubated with 10 nM [^{3}H]Cyd plus various concentrations of the nucleoside analogs. After 10 min, the incorporation of [^{3}H]Cyd into the alkali-stable/acid-insoluble fraction (DNA) was determined as described in *Materials and Methods*. The results shown are from a representative experiment from a total of three that were done.

lated from MOLT-4 cells than was Cl-F(↑)-dAdo. There were no large differences in either the accumulation or degradation of Cl-F(↑)-dATP and Cl-F(↓)-dATP in intact CEM cells, and there was only a 2-fold difference in the inhibition of dCyd kinase activity in crude CEM cell extracts. The reason for these small discrepancies between the purified enzyme and studies with intact cells or crude extracts is not known. We conclude from all of these studies that decreased metabolism of Cl-F(↓)-dAdo may play a small role in its low potency in CEM cells but that the primary reason for the relatively low potency of Cl-F(↓)-dAdo is still due to its poor inhibition of ribonucleotide reductase activity.

2′,2′-diF-dCyd (gemcitabine) also inhibits ribonucleotide reductase activity due to the interaction of 2′,2′-diF-dCDP with the substrate active site, instead of allosteric inhibition by 2′,2′-diF-dCTP (Heinemann et al., 1990). 2′,2′-diF-dCDP is approximately 100-fold less potent than either Cl-F(↑)-dATP or Cl-dATP in its ability to inhibit ribonucleotide reductase activity (Heinemann et al., 1990; Parker et al., 1991). These studies suggest that Cl-diF(↑↓)-dAdo may inhibit ribonucleotide reductase activity as a diphosphate analog instead of the triphosphate analog. However, it is important to point out that dATP is an important negative regulator of ribonucleotide reductase activity, whereas dCTP is not (Nutter and Cheng, 1984).

The results of this work indicated that the cytotoxicity of Cl-F(↑)-dAdo in CEM cells is primarily due to its inhibition of DNA synthesis, whereas Cl-F(↓)-dAdo and Cl-diF(↑↓)-dAdo inhibited RNA and protein synthesis at concentrations similar to those required to inhibit DNA synthesis. Because nonreplicating, as well as replicating cells, synthesize RNA and proteins, these results suggest that Cl-F(↓)-dAdo and Cl-diF(↑↓)-dAdo would be toxic to nonreplicating host cells, which could explain why these two agents have poor antitumor activity in vivo. The enzyme or enzymes inhibited by these agents (or one of their metabolites) that results in the inhibition of RNA and protein synthesis are not known. Xie and Plunkett (1995) have found that Cl-F(↑)-dAdo can be incorporated into RNA at approximately 1% of the rate of its incorporation into DNA, which suggests that the incorporation of these molecules into RNA could be responsible for the inhibition of RNA and protein syntheses. It is also possible that these agents interfere with some ATP-requiring enzyme. The concentrations of compound required to inhibit protein and RNA synthesis were similar for all three compounds. The primary difference in the mechanism of action of these molecules is the ability of Cl-F(↑)-dAdo to inhibit DNA synthesis at concentrations much lower than those required to inhibit either protein or RNA synthesis.

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