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2′-DEOXY-2′-METHYLENE DERIVATIVES OF ADENOSINE, GUANOSINE, TUBERCIDIN, CYTIDINE AND URIDINE AS INHIBITORS OF L1210 CELL GROWTH IN CULTURE

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Abstract—The 2′-deoxy-2′-methylene derivatives of adenosine (MdAdo), guanosine (MdGuo), tubercidin (MdTu), cytidine (MdCyd) and uridine (MdUrd) were synthesized as mechanism-based inhibitors directed at ribonucleotide reductase. It was shown that MdCyd 5′-diphosphate irreversibly inactivated ribonucleotide reductase from *Escherichia coli* (Baker *et al.*, *J Med Chem* **34**: 1879–1884, 1991). In studies reported here, MdAdo/EHNA, MdGuo and MdCyd inhibited L1210 cell growth with IC_{50} values of 3.4, 10.6 and 1.4 μM, respectively. Since MdAdo is a substrate for adenosine deaminase, the presence of EHNA was required to give maximal growth inhibition. 8-Aminoguanosine was not required to maximize the cytotoxic effects of MdGuo. The 2′-deoxy-2′-methylene derivatives of tubercidin and uridine did not inhibit L1210 cell growth at concentrations as high as 50 μM (MdTu) or 100 μM (MdUrd). L1210 cell lines resistant to hydroxyurea (directed at the non-heme iron subunit of ribonucleotide reductase) or deoxyadenosine (directed at the effector binding subunit of ribonucleotide reductase) were not resistant to MdCyd. An L1210 cell line that was highly resistant to dGuo due to the loss of a relatively specific deoxyribonucleoside kinase (Cory *et al.*, *J Biol Chem* **268**: 405–409, 1993) had a 6.6-fold increase in the IC_{50} value toward MdCyd, but showed only a 2-fold increase in resistance to MdGuo. Another L1210 cell line that was markedly deficient in adenosine kinase activity was highly resistant to MdAdo. Analysis by flow cytometry showed that MdCyd slowed the transit of the cells through the G_2/M phase of the cell cycle resulting in the buildup of the G_2/M population. MdAdo, MdGuo and MdCyd inhibited the incorporation of [^{14}C]cytidine into DNA without an effect on RNA synthesis or total cellular uptake of [^{14}C]cytidine. The conversion of [^{14}C]cytidine to deoxycytidine nucleotides was partially inhibited by MdGuo, but not by MdAdo or MdCyd. These data show that the 2′-deoxy-2′-methylene derivatives of adenosine, guanosine and cytidine are activated via specific nucleoside kinases and that the modes of action of these compounds are not identical.

Key words: 2′-deoxy-2′-methyleneadenosine, 2′-deoxy-2′-methyleneguanosine, 2′-deoxy-2′-methylenetubercidin, 2′-deoxy-2′-methylenecytidine, 2′-deoxy-2′-methyleneuridine, ribonucleotide reductase

2′-Deoxy-2′-methylenecytidine (MdCyd§) was synthesized as a potential analog of araC, which would be active as an antitumor agent but which could be resistant to cytidine deaminase [1, 2]. Studies *in vitro* [3, 4] and *in vivo* [3, 4] have shown that MdCyd is an inhibitor of tumor cell growth over a broad spectrum of tumors. The studies of Takenuki *et al.* [1] showed that MdCyd inhibits thymidine incorporation into DNA with no effect on uridine or leucine incorporation into RNA or protein, respectively. Utilizing ribonucleotide reductase purified from *Escherichia coli*, Baker *et al.* [5] showed that MdCDP causes the irreversible inactivation of reductase activity. Other 2′-deoxy-2′-methylenenucleosides have been synthesized [3, 6] and studied for their effects on tumor cell growth in tissue culture and *in vivo* [3]. These compounds included the 2′-deoxy-2′-methylene derivatives of adenosine, tubercidin, guanosine and uridine.

In the studies to be reported here, we compared the effects of the 2′-deoxy-2′-methylene derivatives of cytidine, uridine, adenosine, tubercidin and guanosine on L1210 cell growth in wild-type and kinase-deficient cell lines, on cell-cycle effects, and on [^{14}C]cytidine metabolism via ribonucleotide reductase activity.

MATERIALS AND METHODS

Materials. RPMI 1640 culture medium, horse serum and sodium bicarbonate were purchased from the Grand Island Biological Co., Grand Island, NY. MTS [7] was a gift from Dr. Terence C. Owen,

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§ Abbreviations: MdCyd, 2′-deoxy-2′-methylenecytidine; MdAdo, 2′-deoxy-2′-methyleneadenosine; MdGuo, 2′-deoxy-2′-methyleneguanosine; MdTu, 2′-deoxy-2′-methylenetubercidin; MdUrd, 2′-deoxy-2′-methyleneuridine; MTS, 3-(4,5-dimethylthiazolyl)-2*H*-tetrazolium, inner salt; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMS, phenazine methosulfate; dFdCyd, 2′-deoxy-2′,2′-difluorocytidine; IMPY, pyrazoloimidazole; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine; DTT, dithiothreitol; MdCDP, 2′-deoxy-2′-methylenecytidine 5′-diphosphate; and dFdCDP, 2′,2′-difluoro-2′-deoxycytidine 5′-diphosphate.

Department of Chemistry, University of South Florida, Tampa, FL. [U-^{14}C]Cytidine (400 mCi/mmol) was purchased from the Research Products International Corp., Mount Prospect, IL. [U-^{14}C]-CDP (517 mCi/mmol) was purchased from the New England Nuclear Corp., Boston, MA. [6-^{3}H]-Deoxyuridine (22 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA. The unlabeled nucleosides and other biochemicals, adenosine deaminase (calf intestinal mucosa) and purine nucleoside phosphorylase (calf spleen) were purchased from the Sigma Chemical Co., St. Louis, MO.

Synthesis of 2'-deoxy-2'-methylenenucleosides. The syntheses of the 2'-deoxy-2'-methylenenucleosides were carried out as previously described [2, 3, 6].

Growth of L1210 cells. The wild-type mouse leukemia L1210 cell line was purchased originally from the American Type Culture Collection, Rockville, MD. The hydroxyurea-resistant (HU) cell line [8], the deoxyadenosine-resistant (Y8) cell line [9], and the deoxyadenosine/IMPY-resistant (ED2) cell line [10] were generated and characterized as previously described. The deoxyguanosine-resistant cell line (dGuo-R) was selected for resistance to deoxyguanosine and characterized with respect to the substrate specificity of the deoxyribonucleoside kinase activities [11]. The L1210 cells were grown in RPMI 1640 culture medium which was supplemented with 10% horse serum, sodium bicarbonate (2 g/L) and gentamycin sulfate (50 mg/L). The cells were grown in a humidified incubator at 37° with 95% air/5% CO_2. The drug-resistant cells were maintained in the same culture medium supplemented with the appropriate drugs (HU, hydroxyurea; Y8, deoxyadenosine/EHNA; ED2, deoxyadenosine/EHNA plus IMPY/Desferal; dGuo-R, deoxyguanosine). Before carrying out the growth studies in the presence of the drugs, the cells were collected by centrifugation, resuspended in fresh culture medium containing no drugs, and grown overnight in the absence of drugs.

Cell growth assay. L1210 cell growth was assayed using the method of Cory *et al.* [7] in which MTS was substituted for MTT. The formazan product of MTS is water soluble and easily measured at 492 nm in a 96-well plate reader. The 96-well plates were set up with 2000 cells/well (150 μL) on day zero. The drugs at various concentrations were added 24 hr later in a volume of 50 μL; 72 hr later, the MTS/PMS was added and the plates read at 492 nm as described [7]. The 96-well plates were set up with two rows of control cells (no drugs); before the addition of MTS/PMS, the contents of one row of control cells were removed from the wells, pooled and counted on Coulter counter, model ZF (Coulter Electronics, Hialeah, FL). In this way the absorbance of the formazan product of the control cells could be related to an actual cell count. In a typical experiment the cells in the control wells grew to a concentration of 200,000 to 300,000 cells/well (approximately seven doublings). The IC_{50} values were determined for each drug in at least three separate experiments. In each experiment, each drug concentration was set up in 3 or 6 separate wells. At least five different concentrations of each drug were used to determine the IC_{50} value.

[^{14}C]*Cytidine metabolism in the presence of 2'-deoxy-2'-methylenenucleosides.* The L1210 cells in log-phase were collected by centrifugation and resuspended in fresh culture medium (10 mL) at 2.5×10^6 cells/mL. The 2'-deoxy-2'-methylene-nucleosides were added in a volume of 1 mL. The cells were incubated for 2 hr in the presence and absence of MdCyd, MdAdo or MdGuo. [^{14}C]-Cytidine (400 mCi/mmol, 2 μCi/flask) was added and the incubation continued for 1 hr. The cells were collected by centrifugation and subjected to the Schmidt–Thannhauser procedure [12] to separate the nucleotide pool, RNA and DNA fractions. Aliquots of each fraction were taken for radioactivity measurements. The flasks were set up in duplicate. The acid-soluble nucleotide pool fraction was neutralized with KOH and centrifuged to remove $KClO_4$; then the supernatant fluid was lyophilized. The lyophilized material was dissolved in Tris–HCl buffer, 0.1 M, pH 9.0 (50 μL). Carrier dCMP (0.3 μmol) and magnesium acetate (0.1 μmol) in Tris–HCl buffer, 0.1 M, pH 9.0 (250 μL) and snake venom (4 mg/200 μL) were added, and the reaction was carried out for 4 hr at 37°. The reactions were stopped by heating in a boiling water bath. The tubes were cooled and centrifuged. The supernatant fluids were loaded onto Dowex-1-borate columns to separate deoxycytidine from cytidine [13].

[6-^{3}H]*Deoxyuridine incorporation into DNA.* The L1210 cells in log-phase were collected by centrifugation and resuspended in fresh culture medium (10 mL) at a final concentration of 2.5×10^6 cells/mL. The 2'-deoxy-2'-methylenenucleosides were added in a volume of 1 mL. The cells were incubated for 2 hr in the presence and absence of drugs. [6-^{3}H]Deoxyuridine (3.6 μCi/flask, 22 Ci/mmol) was added and the incubation continued for an additional hour. The cells were collected by centrifugation and extracted with 6% perchloric acid to separate the acid-soluble and acid-insoluble fractions. The acid-insoluble fraction was dissolved in 1.0 mL of 0.25 M NaOH, and an aliquot (100 μL) was taken for radioactivity measurements by liquid scintillation counting.

Ribonucleotide reductase activity in cell-free extracts. The wild-type L1210 cells, in log-phase, were incubated in culture in the presence and absence of MdCyd (7 μM) overnight. The control and drug-treated cells were collected by centrifugation. The cells were washed with cold phosphate-buffered saline and recentrifuged (two times). The cell pellets were resuspended in 0.02 M Tris–HCl buffer, pH 7.0, with 1 mM DTT, at a concentration of 5.0×10^8 cells/mL and swelled for 5 min on ice. The cell suspension was homogenized with a motor-driven Teflon pestle (20 strokes). The homogenate was centrifuged for 1 hr at 21,000 *g* at 4°. To remove endogenous nucleotides, the supernatant fluid was passed through a Dowex-1-acetate column that had been prewashed with 0.02 M Tris–HCl buffer, pH 7.0, containing 1 mM DTT. The enzyme extract was eluted from the column with 2 vol. of Tris–HCl/DTT. To the eluant, enough ammonium sulfate was added to bring the sample to 80% saturation. The

precipitate was collected by centrifugation, dissolved in Tris–HCl/DTT buffer, and dialyzed against the same buffer for 3 hr with three changes of buffer. Aliquots of the dialyzed extracts were quick-frozen in an acetone–dry ice bath and stored at −90°.

CDP reductase activity was assayed by the method of Steeper and Steuart [14] in a volume of 0.15 mL containing: [^{14}C]CDP, 0.03 μCi, 1.87 nmol; 5'-adenylylimidodiphosphate, 150 nmol; DTT, 900 nmol; magnesium acetate, 600 nmol; sodium phosphate buffer, pH 7.0, 1000 nmol; and enzyme extract [15]. The CDP reductase reaction was carried out for either 15 or 30 min at 37° and terminated by heating in a boiling water bath for 4 min. After snake venom phosphodiesterase treatment to convert nucleotides to nucleosides [13], deoxycytidine was separated from cytidine on Dowex-1-borate columns.

Protein determination. The protein concentrations in the cell-free extracts were determined by the method of Lowry *et al.* [16] using bovine plasma gamma globulin (Bio-Rad, Richmond, CA) as the standard.

Cell-cycle analysis. The wild-type L1210 cells were grown in culture to log-phase. MdAdo, MdCyd or MdGuo was added to the cells at a concentration two times their respective IC_{50} values. The cells were incubated for either 12 or 24 hr in the presence of the drugs. The flasks were set up in triplicate. Cell counts were made and aliquots of the cell cultures were taken so that 1×10^6 cells could be processed for cell-cycle analysis by flow cytometry using propidium iodide staining [17]. The cells were collected by centrifugation and the cell pellets were resuspended in sodium citrate (3 mM, 0.495 mL) by vigorous vortexing, and filtered propidium iodide (5 μL, 5 mg/mL) was added. The cells were incubated on ice for 20 min in the dark; ribonuclease (90 U/mL) was added and the cells were incubated at room temperature for 30 min in the dark; the cells were then kept overnight at 4°. Immediately prior to cell-cycle analysis the cells were passed through a 25-gauge needle. Flow cytometry was carried out on a Becton-Dickinson Facscan (Mountain View, CA) using an argon laser at 488 nm. The data were analyzed by the Cell Fit Cell-Cycle Analysis Program, Version 2.01.2.

Assays of enzyme activities. Adenosine deaminase activity was determined spectrophotometrically using the conditions previously described [18]. Purine nucleoside phosphorylase activity was assayed by HPLC by measuring the formation of guanine from MdGuo or dGuo. The assay conditions were as previously described [19]. Guanine was separated from MdGuo or dGuo on an ODS column (Zorbax) using a solvent of 5% methanol/2.5 mM potassium phosphate, pH 6.9 [20] with a flow rate of 1.0 mL/min. The elution times for guanine, MdGuo and dGuo were 4.2, 14 and 12 min, respectively.

RESULTS

Effects of MdAdo, MdGuo, MdCyd, MdTu and MdUrd on the growth of L1210 cells. MdAdo, MdGuo and MdCyd inhibited the growth of L1210 cells in culture. The IC_{50} values are summarized in Table 1. Since MdAdo was a substrate for adenosine deaminase (44% as effective as deoxyadenosine, data not shown), EHNA (5 μM) was required to give optimal growth inhibition by MdAdo. In the absence of EHNA, the IC_{50} value was >25 μM. EHNA, alone, at 5 μM had no effect on the growth of the L1210 cells. On the other hand, 8-AGuo (an inhibitor of purine nucleoside phosphorylase) did not potentiate the growth inhibitory effects of MdGuo. In the presence of 8-AGuo, the IC_{50} value was 34.1 ± 4.2 μM. MdCyd was the most effective compound in this series. L1210 cell growth was not inhibited by MdTu (50 μM) or MdUrd (100 μM). An L1210 cell line (ED2) which was markedly deficient in adenosine kinase (<2% of wild-type Ado kinase activity) with slightly decreased deoxyadenosine kinase activity (45% of wild-type dAdo kinase activity) showed a 29-fold increase in resistance to MdAdo/EHNA, but only a 3-fold increase in resistance to MdCyd. Another L1210 cell line that had been selected for resistance to deoxyguanosine (dGuo-R) due to a decrease in deoxyguanosine kinase activity [11] showed a 6.6-fold increase in resistance to MdCyd but only a 2-fold increase in resistance to MdGuo. Hydroxyurea-resistant cells (HU) [8] and deoxyadenosine-resistant cells (Y8) [9], which had alterations at the ribonucleotide reductase site, were as sensitive to MdCyd as were the wild-type cells.

Table 1. Effects of 2'-deoxy-2'-methylenenucleosides on L1210 cell growth

Cell type	Nucleoside	IC_{50} (μM)
WT	MdAdo	>25 (4)
WT	MdAdo + EHNA*	3.4 ± 0.04 (8)
WT	MdCyd	1.4 ± 0.2 (5)
WT	MdGuo	10.6 ± 2.7 (6)
WT	MdGuo + 8-AGuo†	34.1 ± 4.2 (3)
WT	MdTu‡	⩾50 (1)
WT	MdUrd§	⩾100 (1)
ED2‖	MdAdo + EHNA	98.5 ± 1.5 (3)
ED2	MdCyd	4.6 ± 1.4 (4)
dGuo-R¶	MdGuo	20.6 ± 1.0** (2)
dGuo-R	MdCyd	9.2 ± 0.6 (4)

Values are means ± SD of the number of experiments given in parentheses, unless noted otherwise.

* EHNA, 5 μM.

† 8-AGuo, 25 μM.

‡ No inhibition of cell growth at 50 μM.

§ No inhibition of cell growth at 100 μM.

‖ The ED2 cell line lacks adenosine kinase activity (<2% of wild-type), but has deoxyadenosine kinase activity (45% of wild-type) [21].

¶ The dGuo-R cell line [11] lacks deoxyguanosine kinase activity (10% of wild-type), but has deoxycytidine kinase activity (80% of wild-type).

** Mean ± range.

Effects of MdCyd, MdAdo and MdGuo on the cell cycle of L1210 cells. The effects of MdCyd, MdAdo and MdGuo on the cell cycle of L1210 cells were studied by flow cytometry. For proper comparison of the effects of the 2'-deoxy-2'-methylene nucleosides on the cell cycle, the L1210 cells were incubated

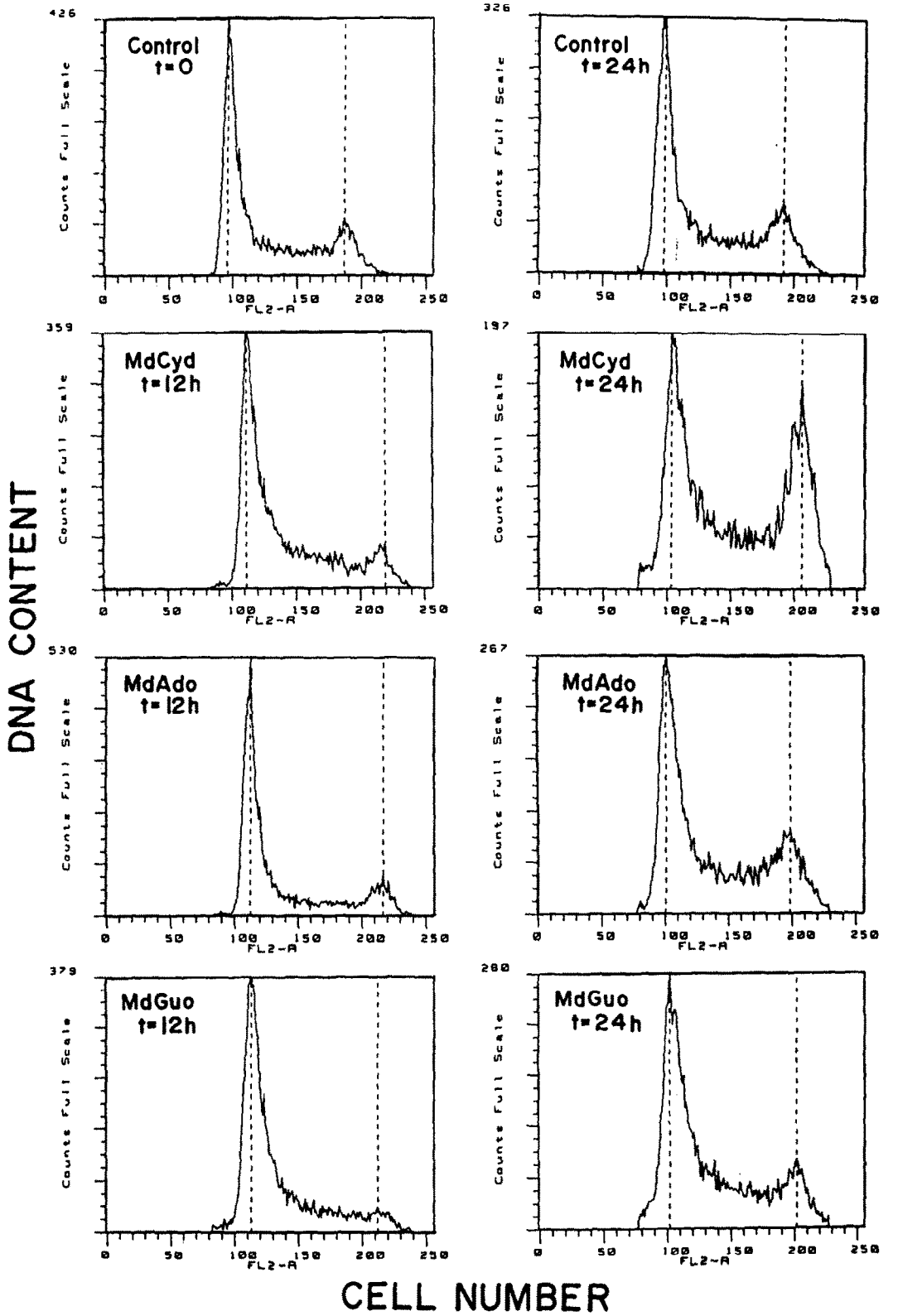

Fig. 1. Cell-cycle analysis of L1210 cells treated with MdCyd, MdAdo and MdGuo. L1210 cells were treated in culture with MdCyd, MdAdo or MdGuo at drug concentrations equal to two times their respective IC_{50} values. The cells were incubated for either 12 or 24 hr in the presence of drug. Cells were collected by centrifugation and treated as described in Materials and Methods for cell-cycle distribution. These data are representative of two separate experiments in which in each experiment the flasks were set up in triplicate.

for 12 and 24 hr in the presence of the drugs at concentrations equal to two times their respective IC_{50} values. As seen in Fig. 1, MdCyd caused the buildup of the L1210 cells in the G_2/M phase of the cell cycle after incubation of the cells for 24 hr (approximately two doubling times). Similar accumulations of the cells in G_2/M were not observed for MdAdo and MdGuo.

Effects of MdCyd, MdAdo and MdGuo on [^{14}C]*-cytidine metabolism in L1210 cells.* The effects of MdCyd, MdAdo and MdGuo on [^{14}C]cytidine metabolism in L1210 cells as measured by total [^{14}C]-cytidine uptake into the cells, nucleotide pool levels, deoxycytidine nucleotide formation and incorporation of cytidine into RNA and deoxycytidine into DNA were determined. As shown in Fig. 2, MdAdo, MdCyd and MdGuo inhibited the incorporation of [^{14}C]cytidine into DNA without an effect on RNA. There was no effect of these 2′-deoxy-2′-methylene nucleosides on total cytidine uptake or on the pools of cytidine nucleotides. MdCyd and MdAdo had no effect on the conversion of [^{14}C]cytidine to [^{14}C]deoxycytidine nucleotides in the acid-soluble fraction. However, MdGuo caused a partial decrease in the conversion of cytidine to deoxycytidine nucleotides in the acid-soluble pool.

Effects of MdCyd, MdAdo and MdGuo on [6-^{3}H]*-deoxyuridine incorporation into DNA.* The effects of MdCyd, MdAdo and MdGuo on [6-^{3}H]deoxyuridine incorporation into DNA were studied. The IC_{50} values for the inhibition of [6-^{3}H]deoxyuridine incorporation into DNA were 2.4, 2.5 and 11.4 μM for MdCyd, MdAdo and MdGuo, respectively.

Ribonucleotide reductase activity in extracts from control and MdCyd-treated L1210 cells. Since it had been reported that MdCDP was an irreversible inhibitor of ribonucleotide reductase from *E. coli* [5], studies were carried out to determine if the treatment of L1210 cells with MdCyd resulted in the inhibition of ribonucleotide reductase activity. Wild-type L1210 cells in log-phase were treated in the presence and absence of MdCyd (7 μM) for 18 hr. Cell counts made before and after the 18-hr incubation period in the presence of 7 μM MdCyd showed that cell growth was inhibited 79%. Cell-free extracts were prepared, and CDP reductase activity was determined. CDP reductase activity was 0.15 nmol/30 min/mg protein in the cell-free extract prepared from the control cells; the extract prepared from the MdCyd cells had CDP reductase activity of 0.32 nmol/30 min/mg protein. This represented a 2-fold increase in reductase activity in the cell-free extract from the MdCyd-treated cells.

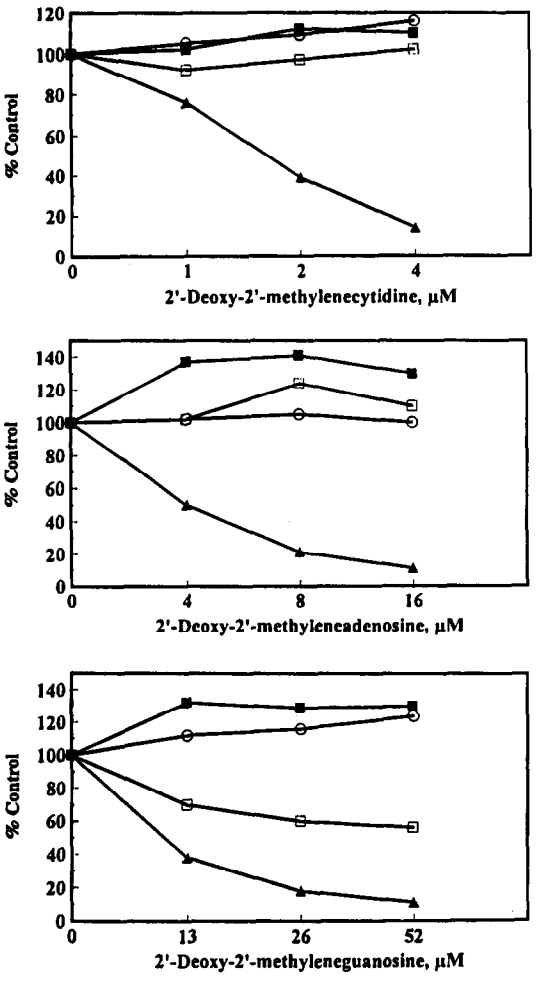

Fig. 2. Effects of MdCyd, MdAdo or MdGuo on [^{14}C]-cytidine metabolism in L1210 cells. The L1210 cells were incubated in the presence of MdCyd, MdAdo or MdGuo for 2 hr; [^{14}C]cytidine was added and the incubation continued for an additional hour. The cells were collected by centrifugation and the acid-soluble, RNA and DNA fractions separated [13]. Deoxycytidine was separated from cytidine on Dowex-1-borate columns [14]. Radioactivity in the acid-soluble fraction (■), and deoxycytidine nucleotides in the acid-soluble (□), RNA (○) and DNA (▲) fractions were determined. The control values for the acid-soluble, RNA, DNA and deoxycytidine fractions were: 18,400, 24,200, 3,100 and 200 cpm/10^6 cells, respectively.

DISCUSSION

The syntheses of 2′-deoxy-2′-methylene derivatives of nucleosides were reported by Takenuki *et al.* [1] and by Robins and coworkers [2, 3, 6]. These compounds have included the 2′-deoxy-2′-methylene analogs of cytidine, uridine, adenosine, tubercidin and guanosine. MdCyd can be viewed as an analog of araC [4] or as an analog of 2′-deoxy-2′,2′-difluorocytidine [5]. MdCyd was reported to be as potent as araC as an inhibitor of tumor cell growth both in culture [3] and *in vivo* against several murine tumors and human xenografts [4]; however, MdCyd was less active than dFdCyd [22]. MdCyd has an advantage over araC since it is a very poor substrate for cytidine deaminase [4]. MdCyd inhibited the incorporation of thymidine into DNA without an effect on the incorporation of uridine and leucine into RNA and protein, respectively [4]. MdCDP irreversibly inhibited ribonucleotide reductase from *E. coli* [5]. MdGuo and MdAdo were shown to inhibit the growth of mouse and human tumor cell lines in culture [3]. In the studies presented here we

show that MdCyd, MdAdo and MdGuo inhibited the growth of wild-type L1210 cells in culture. MdCyd was the most active of these nucleoside analogs with an IC_{50} value of 1.4 μM. MdAdo in the presence of EHNA (5 μM), to block adenosine deaminase activity [23], inhibited wild-type L1210 cell growth with an IC_{50} of 3.4 μM. MdAdo was 44% as efficient as deoxyadenosine as a substrate for adenosine deaminase. MdGuo inhibited L1210 cell growth with an IC_{50} value of 10.6 μM. The addition of 8-aminoguanosine, an inhibitor of purine nucleoside phosphorylase [24], did not potentiate the effects of MdGuo. In fact, in the presence of 8-aminoguanosine there was a 3.4-fold increase in the IC_{50} value even though MdGuo is a very weak substrate for purine nucleoside phosphorylase (Cory JG and Moncla PR, unpublished results). At concentrations of MdTu and MdUrd as high as 50 and 100 μM, respectively, there was no inhibition of L1210 cell growth. These compounds are probably not substrates for the available kinases; MdTu was not a substrate for adenosine deaminase. The effects of the active 2′-deoxy-2′-methylenenucleosides on the growth of variant L1210 cell lines were studied in order to determine the nature of the nucleoside kinase(s) required for the activation of the drugs. The ED2 cell line which lacks adenosine kinase activity and which is resistant to pyrazofurin (139-fold) and 6-methylmercaptopurine riboside (>4000-fold) showed a 29-fold increase in the IC_{50} toward MdAdo/EHNA compared to the wild-type cells. The ED2 cell line also showed some resistance to araA/EHNA (31-fold) and FaraA (8-fold). These data indicate that adenosine kinase plays a role in the phosphorylation of MdAdo although other kinases must also be involved since the level of resistance to MdAdo seen in the ED2 cell line did not approach the levels of resistance seen for pyrazofurin and 6-methylmercaptopurine riboside. Brockman *et al.* [25] have reported that deoxycytidine kinase is also involved in the phosphorylation of araA and FaraA so that it is clear that multiple kinases can be involved. Arner *et al.* [26] have reported that there are undefined kinases in the cell which may have the appropriate substrate specificities for various nucleoside analogs. The ED2 cell line also showed a 3-fold increase in the IC_{50} value for MdCyd. The dGuo-R cell line [11], which has large decreases in deoxyguanosine kinase and araC kinase activities but retains deoxycytidine kinase activity, had only a 2-fold increase in the IC_{50} value for MdGuo, but a 6-fold increase in the IC_{50} value for MdCyd. It has been reported that deoxycytidine kinase-minus CEM cells had a 1300-fold increase in the IC_{50} value for MdCyd but only a 23-fold increase in the IC_{50} for MdGuo [3]. These data of Robins *et al.* [3] and our current data suggest that MdCyd and MdGuo are phosphorylated by different kinases; otherwise the same level of resistance toward these two compounds would have been observed in the kinase-deficient CEM and L1210 cell lines.

The study of the effects of these 2′-deoxy-2′-methylenenucleosides on *in situ* ribonucleotide reductase activity, and on RNA and DNA syntheses showed that the incorporation of cytidine via deoxycytidine nucleotides into DNA was inhibited by MdCyd, MdAdo and MdGuo without effects on the incorporation of cytidine into RNA or on the total uptake of cytidine into the cells. Even with short-term experiments the IC_{50} values for the inhibition of cytidine incorporation into DNA approximated the IC_{50} value for cell growth. The incorporation of [6-^{3}H]dUrd into DNA was also inhibited by MdCyd, MdAdo and MdGuo with IC_{50} values very similar to those obtained for the inhibition of incorporation of cytidine into DNA. Although MdCDP inhibits ribonucleotide reductase from *E. coli* in a stoichiometric manner [5], neither the conversion of cytidine to deoxycytidine nucleotides was inhibited in the intact L1210 cells by MdCyd nor was there a decrease in ribonucleotide reductase activity in the cell-free extracts prepared from MdCyd-treated cells as would be expected if MdCyd were inhibiting irreversibly. There was, however, a partial inhibition of ribonucleotide reductase activity by MdGuo, as measured *in situ* [13]. The formation of deoxycytidine nucleotides in the acid-soluble fraction was decreased approximately 40% by MdGuo (26 μM). MdCyd slowed the transit of the cells through the G_2/M phase of the cell cycle leading to a marked increase in the G_2/M population. Similar effects were not seen with MdAdo and MdGuo. The cell-cycle effects seen with MdCyd would also support the experimental data obtained in the intact cell which indicated that ribonucleotide reductase is not the major site of action for this drug. Differences between our results and the irreversible inhibition of *E. coli* ribonucleotide reductase by MdCDP could arise for several reasons. It is possible that in the intact mammalian cell the steady-state concentration of MdCDP is not sufficient to inhibit reductase activity. By analogy with dFdCyd, the intracellular concentration of dFdCTP greatly exceeds the concentration of dFdCDP [22, 27]. It is also possible that the subtle differences between the *E. coli* ribonucleotide reductase and the mammalian reductase renders MdCDP a less effective inhibitor of the mammalian enzyme. There are previous reports which show that the *E. coli* enzyme is much more sensitive to caracemide than is the mammalian enzyme [28–30]; conversely, the mammalian enzyme is more sensitive to 3,4-dihydroxybenzohydroxamic acid than is the *E. coli* enzyme [31].

Although these studies do not define the exact mechanisms of action of these 2′-deoxy-2′-methylenenucleosides, the results indicate that these compounds are not activated by the same kinases and do not act by the same mechanism. That is, the mechanism by which MdCyd inhibits L1210 cell growth differs from that of MdAdo and MdGuo (Fig. 1), and based on the data in Fig. 2, MdAdo and MdGuo also do not act by the same mechanism. Our data indicate that MdCyd acts as an analog of araC rather than as an analog of dFdCyd which inhibits ribonucleotide reductase activity in the intact cell [22, 27] as one of its modes of action. The potency of these 2′-deoxy-2′-methylenenucleosides as inhibitors of tumor cell growth and their individual modes of action suggest that they may be useful for further study as single agents or in combination.

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