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# Deoxyguanosine-resistant Leukemia L1210 Cells:

## LOSS OF SPECIFIC DEOXYRIBONUCLEOSIDE KINASE ACTIVITY*

**Ann H. Cory**, **Ivan A. Shibley**, **Joseph M. Chalovich**, and **Joseph G. Cory**‡

*From the Department of Biochemistry, East Carolina University School of Medicine, Greenville, North Carolina 27858*

## Abstract

A mouse leukemia L1210 cell line was selected for resistance to deoxyguanosine. The deoxyguanosine-resistant cells (dGuo-R) were 126-fold less sensitive to deoxyguanosine than the wild-type cells. The $IC_{50}$ values for araC and araG were increased, but only 10–12-fold in the dGuo-R cells when compared with the wild-type cells. The dGuo-R cell line showed an increased level of resistance to 2-fluoro-2′-deoxyadenosine and 2-fluoroadenine arabinoside (11–14-fold), but essentially no increase in resistance to deoxyadenosine or adenine arabinoside. Deoxyribonucleoside kinase activity was decreased only slightly (19%) when deoxycytidine was utilized as substrate; when cytosine arabinoside or deoxyguanosine was used as the substrate, the kinase activity in the extracts from the dGuo-R cells was only 10% of the enzyme activity in the extracts from the wild-type cells. The determination of the kinetic parameters, $K_m$ and $V_{max}$, indicated that there were marked decreases in the $V_{max}$ values for deoxyguanosine and cytosine arabinoside as substrates, but not for deoxycytidine as substrate; the $K_m$ values for deoxycytidine and cytosine arabinoside were increased in the extracts from the dGuo-R cells. By use of high-performance liquid chromatography, the kinase activities in the extracts from the wild-type and resistant cells could be resolved. There was the specific loss of kinase activity toward cytosine arabinoside and deoxyguanosine as substrates. These data indicate that the dGuo-R cells have decreased levels of a specific deoxyribonucleoside kinase activity.

Ribonucleotide reductase consists of two nonidentical protein subunits which are both required for enzymatic activity. One of the subunits contains the tyrosyl free-radical and nonheme iron; the other subunit contains at least two allosteric binding sites for the nucleoside 5′-triphosphates which act as positive and negative effectors of enzyme activity. A high affinity site binds ATP and dATP, while the low affinity site binds ATP, dATP, dGTP, and dTTP (1). Previous studies (2,3) had shown that resistance of L1210 cells to deoxyadenosine resulted from the loss of allosteric control by dATP without an effect on ATP activation or dGTP inhibition of CDP reduction supporting the idea that multiple effector-binding sites are responsible for controlling ribonucleotide reductase activity.

In continuing studies directed at exploring the nature of the nucleotide-binding sites on the effector-binding subunit of ribonucleotide reductase, L1210 cells, in culture, were exposed to increasing concentrations of deoxyguanosine to determine if a deoxyguanosine-resistant cell line could be selected in which the ribonucleotide reductase activity would be insensitive to dGTP as a negative inhibitor of CDP, UDP, and GDP reductions.

In the studies to be reported here, we describe the generation of a deoxyguanosine-resistant (dGuo-R) cell line. This dGuo-R cell line was resistant to araC[1] and araG but still sensitive to

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‡ To whom correspondence should be addressed: Dept. of Biochemistry, East Carolina University School of Medicine, Brody Medical Sciences Bldg., Greenville, NC 27858. Tel: 919-551-2675..

dAdo; kinase activity toward dGuo and araC as substrates was markedly decreased; kinase activity toward dCyd as substrate was only minimally decreased. These data indicate that the dGuo-R cell line had decreased levels of a relatively specific deoxyribonucleoside kinase with preferred substrate specificity toward dGuo, araG, and araC.

## EXPERIMENTAL PROCEDURES

### Materials

The labeled nucleosides were purchased from commercial suppliers: [$^3$H]deoxycytidine, 25.3 or 27 Ci/mmol, ICN Radiochemicals, Costa Mesa, CA; [$^3$H]cytosine arabinoside, 30 Ci/mmol, Amersham; and [$^3$H]deoxyguanosine, 4 Ci/mmol, Moravek Biochemicals, Brea, CA. The unlabeled nucleosides and other biochemicals were purchased from Sigma. 2-FdAdo and 2-FaraA were gifts from Dr. John A. Montgomery, Southern Research Institute, Birmingham, AL. EHNA was purchased from Burroughs-Wellcome, Research Triangle Park, NC. 8-Aminoguanosine was purchased from United States Biochemicals.

The RPMI 1640 culture medium, horse serum, and sodium bicarbonate were purchased from GIBCO. The wild-type L1210 cell line was originally purchased from the American Type Culture Collection. The MTS was a gift from Dr. Terence C. Owen, Department of Chemistry, University of South Florida, Tampa, FL.

### Methods

**Growth of L1210 Cells—**L1210 cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% horse serum, sodium bicarbonate (2 g/liter), and gentamicin sulfate (50 mg/liter). The cells were grown at 37 °C in a humidified incubator with 5% $CO_2$/95% air. The dGuo-resistant cell line was generated from the wild-type L1210 cell lines by the selective pressure of growing the cells in increasing concentrations of dGuo. No chemical mutagen was used for the selection of this cell line. 8-Aminoguanosine was included to inhibit purine phosphorylase activity (4), but the concentration was held constant at 25 $\mu$M throughout the selection of the dGuo-R cell line. The wild-type L1210 cells were grown in the presence of dGuo (120 $\mu$M) and 8-aminoguanosine (25 $\mu$M)for 24 h. The cells (75 cells/dish) were plated out on soft agar (Sea-Kem agar, 0.275%, with 20% horse serum). The large colonies which grew out were “plucked” and regrown in suspension culture. These cells were then grown in increasing concentrations of dGuo at a constant concentration of 8-aminoguanosine (25 $\mu$M). The cell line which had the highest $IC_{50}$ value toward dGuo was selected for further study. The doubling times of the wild-type and dGuo-resistant cells were the same.

**Cell Growth Assay—**L1210 cell growth was assayed using the method of Cory *et al.* (5) in which MTS was substituted for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. 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 2,000 cells/well (150 $\mu$l) on day 0. The drugs at various concentrations were added 24 h later in a volume of 50 $\mu$l; and 72 h later the MTS/phenazine methosulfate was added, and the plates were read at 492 nm as described (5). The 96-well plates were set up with two rows of control cells (no drugs); before the addition of MTS/phenazine methosulfate, the contents of one row of control cells were removed from the wells, pooled, and counted on a Coulter counter, Model ZF (Coulter Electronics, Hialeah, FL). In this way the absorbance of the formazan product of the control cells could be related

[1]The abbreviations used are: araC, cytosine arabinoside; araG, guanine arabinoside; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; DTT, dithiothrietol; HPLC, high-performance liquid chromatography; 2-FdAdo, 2-fluoro-2′-deoxyadenosine; 2-FaraA, 2-fluoroadenine arabinoside; MAIQ, 4-methyl-5-aminoisoquinoline thiosemicarbazone; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; dGuo, 2′-deoxyguanosine; dCyd, 2′-deoxycytidine; dAdo, 2′-deoxyadenosine.

to an actual cell count. In a typical experiment the cells in the control wells grew to a concentration of 200,000–300,000 cells/well. 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 three or six separate wells. At least five different concentrations of each drug were used to determine the $IC_{50}$. In those studies in which EHNA was used, the EHNA concentration was kept constant at 5 $\mu$M.

**Metabolism of $^3$H-Labeled Nucleosides in Wild-type and dGuo-resistant Cells—** Log-phase wild-type and dGuo-resistant cells were set up in triplicate cultures at $1 \times 10^6$ cells/ml (20 ml) and incubated at 37 °C for 30 min. [$^3$H]Deoxycytidine (27 or 25.3 Ci/mmol, 0.25 $\mu$Ci/ml), [$^3$H]dGuo (4 Ci/mmol, 0.25 $\mu$Ci/ml), or [$^3$H]araC (30 Ci/mmol, 0.25 $\mu$Ci/ml) was added to the cells, and the incubation was continued for 1 h at 37 °C in the $CO_2$/air incubator. After 1 h, the cells were collected by centrifugation, washed with cold phosphate-buffered saline, and recentrifuged. The cell pellet was extracted with 1 ml of 6% perchloric acid, the homogenate was centrifuged, and the pellet was re-extracted with 0.5 ml of 6% perchloric acid. The acid-soluble fractions were pooled, and the acid-insoluble pellet was solubilized in 0.25 M NaOH (1 ml). Aliquots of the acid-soluble and acid-insoluble fractions were taken for $^3$H determination by liquid scintillation counting. Each experiment was carried out twice, and there were triplicate flasks per labeled nucleoside in each experiment.

**Assay of Deoxyribonucleoside Kinase Activity—**Log-phase wild-type and dGuo-resistant L1210 cells were suspended in 0.05 M HEPES, pH 7.5, containing 1 mM DTT at a concentration of $3.33 \times 10^8$ cells/ml of buffer. The cells were homogenized in a glass vessel using a motor-driven Teflon pestle. The homogenates were centrifuged for 1 h at 15,000 RPM, and the supernatant fluids were removed and quick-frozen in small aliquots in a dry-ice acetone bath. These cell-free extracts were used to assay deoxyribonucleoside kinase activity with [$^3$H]dCyd, [$^3$H]dGuo, or [$^3$H]araC as the substrate.

The deoxyribonucleoside kinase activities were assayed with the standard conditions of deoxynucleoside (50 $\mu$M, 0.4 $\mu$Ci), ATP (5 mM), DTT (1.5 mM), magnesium acetate (10 mM), HEPES buffer, pH 7.5 (0.05 M), and extract (150–200 $\mu$g of protein). The reaction mixture had a final volume of 100 $\mu$l. The reaction assays were set up in triplicate; all assays were run at 37 °C. Preliminary experiments showed that product formation was linear for at least 30 min. The usual assay was run for 20 min. The reactions were stopped by heating for 4 min in a boiling water bath. Heated controls served as background counts. The samples were centrifuged in a Microfuge to pellet the protein; aliquots (10 $\mu$l) of the supernatant fluids were spotted on DEAE-cellulose discs. These discs were dried and washed in water (three changes of water). The discs were placed in scintillation vials and 1 ml of 0.1 M HCl added to each vial to elute the nucleotide product from the DEAE-cellulose.

For the determination of the kinetic parameters, $K_m$ and $V_{max}$, the substrate concentrations were varied with a constant level of tritiated substrate. The amount of cell-free extract for the dGuo-resistant cells was doubled in those assays. The $K_m$ and $V_{max}$ values were determined by linear regression analysis of the Lineweaver-Burk plots. The nature of the inhibition and the $K_i$ values were determined from Dixon plots (6).

**Separation of Deoxyribonucleoside Kinase Activities by HPLC—**Cell-free extracts (12 mg of protein/ml) were passed through a 0.22-$\mu$m filter. An aliquot (0.3 ml) was loaded onto the Mono Q column (Pharmacia LKB Biotechnology Inc.), and a gradient was run to elute the protein fractions. The gradient was run in 0.05 M Tris-HCl, pH 7.6, containing 1 mM DTT starting with an initial concentration of 0.09 M KCl to a final concentration of 0.50 M KCl. This gradient was run over 45 min. Fractions (1 ml) were collected, immediately put on ice, and then used for the assay of deoxyribonucleoside kinase activities. The assay mix consisted

of: $^3$H-labeled substrate, 0.2 $\mu$Ci, 10 $\mu$M; ATP, 5 mM; magnesium acetate, 2 mM; DTT, 1.5 mM; HEPES, pH 7.5, 0.05 M; and an aliquot (25 $\mu$l) of the protein fraction off the HPLC column. The final reaction volume was 50 $\mu$l. The assays for each substrate were set up in duplicate. The reactions were run for 1 h at 37 °C. The reactions were stopped by heating for 4 min in a boiling water bath. Aliquots (20 $\mu$l) were spotted on DEAE-cellulose discs and washed as described above.

**Protein Determination—**The protein concentrations in the cell-free extracts were determined by the method of Lowry *et al.* (7) using the bovine plasma γ-globulin (Bio-Rad) as the standard.

## RESULTS

### Comparison of the Effects of Various Antitumor Agents on the Growth of Wild-type and dGuo-R L1210 Cells

The effects of a selected group of antitumor agents on the growth of wild-type and dGuo-R L1210 cell lines were studied. The data for the $IC_{50}$ values of these compounds are compared in Table I. The L1210 cell line selected for resistance to dGuo showed a 126-fold increase in the $IC_{50}$ toward dGuo. This dGuo-R cell line was also resistant to araC and araG, but the level of resistance was increased only 10–12-fold. These data suggested that the resistance was due to the loss of deoxyribonucleoside kinase activity. For comparative purposes, studies with other compounds were carried out. The dGuo-R cell line showed only a modest decrease in sensitivity to dAdo/EHNA (3-fold) or araA/EHNA (2-fold). On the other hand, the dGuo-R cell line showed an 11-fold decrease in sensitivity to 2-FdAdo/EHNA and at least a 14-fold decrease in sensitivity to 2-FaraA. The ribonucleotide reductase inhibitors, hydroxyurea and MAIQ, which do not have to be activated through a kinase-mediated reaction, were also compared in these two cell lines. The dGuo-R cell line showed a 2-fold decrease in sensitivity toward hydroxyurea but essentially no change in sensitivity toward MAIQ.

### Uptake and Metabolism of [$^3$H]Deoxycytidine, [$^3$H]Deoxyguanosine, and [$^3$H]Cytosine Arabinoside in Wild-type and dGuo-R L1210 Cells

The uptake and incorporation of tritiated dCyd, dGuo, or araC into the acid-soluble and acid-insoluble fractions of wild-type and dGuo-R L1210 cells were determined. As seen from the data in Table II, the dGuo-R cells showed a marked decrease in the total uptake of the labeled nucleosides into the cell as measured in the acid-soluble and acid-insoluble fractions. The dGuo-R cell line metabolized [$^3$H]dGuo to only 9% of the wild-type cells; the uptake and metabolism of [$^3$H]dCyd and [$^3$H]araC were decreased to 27 and 21%, respectively, in the dGuo-R cells.

### Deoxyribonucleoside Kinase Activity in Cell-free Extracts from Wild-type and dGuo-R Cells

Cell-free extracts were prepared from log-phase wild-type and dGuo-R cells. The deoxyribonucleoside kinase activity in these extracts was assayed using [$^3$H]dCyd, [$^3$H]dGuo, or [$^3$H]araC as substrate at substrate concentrations of 50 $\mu$M. As seen from the data in Table III, the dCyd kinase activity was only slightly decreased in the drug-resistant cell lines at this substrate concentration. On the other hand, the kinase activity or activities which phosphorylated dGuo and araC was/were reduced to approximately 10% of the wild-type level. Studies were then carried out to determine the kinetic parameters $K_m$ and $V_{max}$ from Lineweaver-Burk plots with dCyd, dGuo, or araC as substrate using the cell-free extracts from the wild-type and dGuo-R cell lines. These data are summarized in Table IV. With dCyd as substrate, the $V_{max}$ was 1.37-fold larger in extracts from the dGuo-R cells than in the extracts from the wild-type cells, while the $K_m$ for dCyd was 8-fold larger for the kinase activity from the dGuo-R cells. In the extracts from the dGuo-R cells, the $V_{max}$ with dGuo as substrate was

only 4% of that seen in the extracts from the wild-type cells; the $K_m$ for dGuo was decreased approximately 4-fold with the kinase in the dGuo-R cells. The $V_{max}$ with araC as substrate was decreased in the extracts from the dGuo-R cells to 35% of the value obtained in the extracts from the wild-type cells. In addition, this decrease in $V_{max}$ was accompanied by a 15-fold increase in the $K_m$ for araC for the kinase in the dGuo-R cells.

The effect of araC on dCyd kinase activity was determined as well as the effect of dCyd on araC kinase activity. Using Dixon plots, it was found that araC was a competitive inhibitor of the phosphorylation of dCyd with a $K_i$ of 390 $\mu_M$. Conversely, dCyd was a competitive inhibitor of araC phosphorylation with a $K_i$ of 0.3 $\mu_M$. However, dCyd and araC at concentrations as high as 1500 and 2500 $\mu_M$, respectively, did not inhibit the phosphorylation of dGuo.

### Separation of Deoxyribonucleoside Kinase Activities in Extracts from Wild-type and dGuo-R Cells by HPLC

Cell-free extracts prepared from the wild-type and dGuo-R L1210 cells were separated on a Mono Q column by HPLC. Aliquots of the fractions were assayed for kinase activity utilizing [$^3$H] dCyd, [$^3$H]dGuo, and [$^3$H]araC as substrates. These data are shown in Fig. 1, *A* and *B*. Fig. 1*A* shows the kinase activities in the extracts from the wild-type cells in the fractions obtained from the HPLC column; Fig. 1*B* shows the kinase activities in the extracts from the dGuo-R L1210 cells in the fractions from HPLC column. As seen in Fig. 1*A*, the peak of dCyd kinase activity occurred at fraction 31, with the peak of dGuo and araC kinase activities being at tube 30; there was a small shoulder of dCyd and araC kinase activities. Deoxyguanosine kinase activity showed two distinct peaks. As seen in Fig. 1*B*, there was a marked decrease in kinase activities toward dGuo and araC as substrates, with the major loss of activities corresponding to the larger peak of activity (tube 30). The kinase activities toward dCyd, dGuo, and araC were resolved into two peaks in the extracts from the dGuo-R cells due to the decrease of the main peak of activities. Fig. 1*C* shows the UV profile of the protein eluting from the the HPLC column. The extracts from the wild-type and dGuo-resistant cells gave identical protein elution profiles. The peak of enzyme activities eluting from the column did not correlate with the peak of protein at tube 25.

## DISCUSSION

As a result of efforts to generate a deoxyguanosine-resistant cell line which had an altered dGTP binding site on ribonucleotide reductase, we isolated an L1210 cell line which was highly resistant to dGuo, but this was due to the rather selective loss of a deoxyribonucleoside kinase. This deoxyribonucleoside kinase-deficient cell line has some interesting and selective cross-resistant patterns.

Using the $IC_{50}$ values, the sensitivity of the dGuo-R cell line to a series of nucleosides and nucleoside analogs was compared with the wild-type L1210 cell line. The dGuo-R L1210 cell line was highly resistant to dGuo (126-fold). This cell line also showed modest resistance to araC and araG (10–12-fold). Shewach *et al.* (8) reported on a MOLT-4 T-lymphoblast cell line which was highly resistant to araG (600-fold) with a relatively modest 36-fold increase in resistance to dGuo and only a 4-fold increase in resistance to araC. Whether the difference in the levels of araC and araG resistance relative to the level of resistance to dGuo in the dGuo-R L1210 cells is due only to the loss of a relatively specific deoxyribonucleoside kinase activity or is also due to the altered ability of the mutant L1210 cell line to maintain araGTP, araCTP, or dGTP levels is not known. Clearly, the level of incorporation of [$^3$H]dGTP into DNA was decreased by 91% in the dGuo-R cells (Table II). The decreased level of [$^3$H]dCyd incorporation into DNA in the dGuo-R cells compared to the wild-type cells (27% of the wild-type cells) in spite of the relatively small decrease in dCyd kinase activity may be related to the 8-fold increase in $K_m$ (Table IV) and the fact that in the cell-labeling studies, the substrate

was not at a saturating concentration. Bhalla *et al.* (9) have shown that a highly araC-resistant HL-60 cell line which had only 1.2% of the dCyd kinase activity of the wild-type HL-60 cell line was as sensitive to dAdo or dGuo as the wild-type HL-60 cells. Interestingly, while the dGuo-R L1210 cell line showed only a 2–3-fold increase in $IC_{50}$ toward dAdo or araA, there was a marked increase in the level of resistance of the dGuo-R cells to the fluorinated derivatives (2-FdAdo and 2-FaraA). These data indicate that different kinases may be utilized by the cells in activating dAdo *versus* 2-FdAdo and araA *versus* 2-FaraA. Krenitsky *et al.* (10) had previously shown that the dCyd kinase from calf thymus had much higher substrate efficiencies ($V_{max}/K_m$) for 2-FaraA and 2-FdAdo than for the non-fluorinated compounds, araA and dAdo. Hershfield *et al.* (11) and Verhoef *et al.* (12) have shown that dAdo is phosphorylated by both dCyd kinase and Ado kinase. Furthermore, Dow *et al.* (13) have shown that a CCRF-CEM cell line deficient in dCyd kinase activity was highly resistant to 2-FaraA but not araA. Our data, in terms of the specificity of resistance to 2-FdAdo and 2-FaraA, can be explained by these observations of Krenitsky *et al.* (10), Hershfield *et al.* (11), and Dow *et al.* (13). The dGuo-resistant cells did not show cross-resistance to compounds such as hydroxyurea or MAIQ, inhibitors of ribonucleotide reductase, that do not have to be activated via a kinase-mediated reaction. This would suggest that the nature of the alteration in the mutant cells was relatively specific. When the deoxyribonucleoside kinase activities were compared in cell-free extracts prepared from the wild-type and dGuo-R cells under a standard assay condition of substrate at 50 $\mu_M$, it was found that dCyd kinase activity in the dGuo-R cells was 81% of the wild-type cells. However, kinase activity toward dGuo and araC as substrates was selectively reduced to 10% of the wild-type levels. In this regard, Richel *et al.* (14) isolated a rat leukemia cell line which was resistant to araC. Deoxyribonucleoside kinase activity toward dCyd as substrate was the same in both the wild-type and the araC-resistant cell line. With araC as the substrate, there was no kinase activity in the resistant cell line (14). In our experiments the levels of enzyme activity measured in the cell-free extracts (Table III) utilizing a substrate concentration of 50 $\mu_M$, agree extremely well with the values calculated using the $V_{max}$ and $K_m$ values determined (Table IV) in the Michaelis-Menten expression [$\upsilon = V_{max}(S)/K_m + (S)$] taking into account the changes in $V_{max}$ and $K_m$ observed in the dGuo-resistant cell line. The $K_m$ values observed in this study agree well with the published values obtained for murine systems. Habteyesus *et al.* (21) reported a $K_m$ (dCyd) of 1.8 $\mu_M$, while Myers and Kreis (15) reported a $K_m$ (dCyd) of 9.3 $\mu_M$; in our studies we obtained a $K_m$ (dCyd) of 5 $\mu_M$. For araC, a $K_m$ (araC) of 30 $\mu_M$ was reported (21), while we obtained a $K_m$ (araC) of 16 $\mu_M$. Neither Habteyesus *et al.* (21) nor Myers and Kreis (15) reported a $K_m$ for deoxyguanosine as substrate. The relative $V_{max}$ values for dCyd and araC as substrates were in excellent agreement; *i.e.* the $V_{max}$ for araC was larger than the $V_{max}$ for dCyd as reported by Habteyesus *et al.* (21) and this study (Table IV). The resolution of the kinase activities on the Mono Q column suggests that there are multiple kinase activities (Fig. 1, *A* and *B*). Other investigators have reported on the separation of the kinase activities. Meyers and Kreis (15) and Lewis and Link (16) have shown that dGuo kinase activity could be separated from dCyd kinase activity. In the wild-type L1210 cells, there is a major peak of kinase activity which is capable of phosphorylating dCyd, dGuo, and araC. In the dGuo-R cells there was a marked decrease in the kinase activity with dGuo and araC as substrates but only a small decrease in the kinase activity which phosphorylates dCyd. In the extract from the dGuo-R cells, there was a clear resolution of the dCyd and araC kinase activities into two peaks. These data would suggest that there was the specific loss of a kinase with preferred substrate specificity for dGuo and araC. Furthermore, the ratio of $V_{max}$ values for dGuo and araC as substrates was not the same in the extracts from the wild-type and dGuo-R cell lines. In the wild-type cells the ratio was 2.94, while the ratio was 0.35 in the dGuo-R cells, indicating a greater loss of the deoxyguanosine kinase activity.

An interesting kinetic feature of dCyd kinase has been observed in the study of dCyd kinase from several different types of cells (17,18). For example: the $K_m$ for a substrate such as dCyd was not approximately equivalent to its $K_i$ as a competitive inhibitor of a substrate such as

dGuo or dAdo; the $K_i$ for araC as a competitive inhibitor varied 100-fold depending on whether the substrate was dCyd or dGuo (18); and the $K_m$ for araC was 30,000 times greater than its $K_i$ with respect to dCyd as substrate (17). Our results are in agreement with these observations. The second substrate acted as a competitive inhibitor, but the $K_i$ values did not approximate the $K_m$ values. The $K_i$ for araC as a competitive inhibitor of dCyd was 390 $\mu$M compared to a $K_m$ of 15 $\mu$M for araC as a substrate; dCyd as a substrate had a $K_m$ of 5 $\mu$M and a $K_i$ of 0.3 $\mu$M with respect to araC as substrate. AraC and dCyd, at concentrations as high as 2.5 and 1.5 mM, respectively, did not inhibit the phosphorylation of dGuo. These kinds of results have been interpreted as being due to allosteric effects which result in multiple forms of the enzyme (18). Taken together, the kinetic data and the resolution of the deoxyribonucleoside kinase activities on the Mono Q column are consistent with the possibility that the L1210 cells have a deoxyribonucleoside kinase with a substrate preference for deoxyguanosine over the other deoxyribonucleosides and arabinonucleosides.

The molecular basis for the loss of this deoxyribonucleoside kinase activity in the L1210 cell line is not known. The recent results of Clottiner *et al.* (19) using human cell lines have shown that the level of the mRNA for dCyd kinase was decreased in cell lines resistant to araC. Detailed analysis of two human cell lines that had decreased deoxyribonucleoside kinase activities toward dCyd, dAdo, and dGuo showed that the molecular bases for the decreased dN kinase activities in these two cell lines were multiple and different (20). One cell line (ara-C-8D) had two mutations: (*a*) one allele had a deletion of exon 5 (nucleotides 710–825) of the *dCK* gene; and (*b*) the second allele had a G → A transition at nucleotide 242 (kinase ATP binding domain). The second cell line (ddC50) had a point mutation resulting in a A → G transition at nucleotide 626 with the other allele not being expressed. In both cell lines the dCK activity was markedly reduced, but to a much larger extent in the ara-C-8D cell lines. It is possible that the changes observed in the dN kinase activities in the dGuo-R cell line is the result of two different mutated alleles or the selective silencing of one of the kinase alleles. Habteyesus *et al.* (21) have shown that the purified dCyd kinases from human and mouse tissues have different substrate specificity properties which make extrapolation from the studies in human cells to the mouse cells not necessarily appropriate.

Clearly, one level of resistance of the dGuo-R cells resides in the loss of a relatively specific deoxyribonucleoside kinase activity. There may be, in addition, other factors at the cellular level such as the steady-state concentrations of the active inhibitory species (dGTP, araCTP, araGTP) which are maintained and which influence the cross-resistance patterns in the dGuo-R cells. Whether the decreased kinase activity is due, at the molecular level, to decreased mRNA levels or to a mutated mRNA which codes for a kinase with altered enzyme activities or decreased half-life remains to be determined. The dGuo-R cell line will be useful in studying the role of specific deoxyribonucleoside kinases in the activation of nucleoside analogs as antitumor agents.

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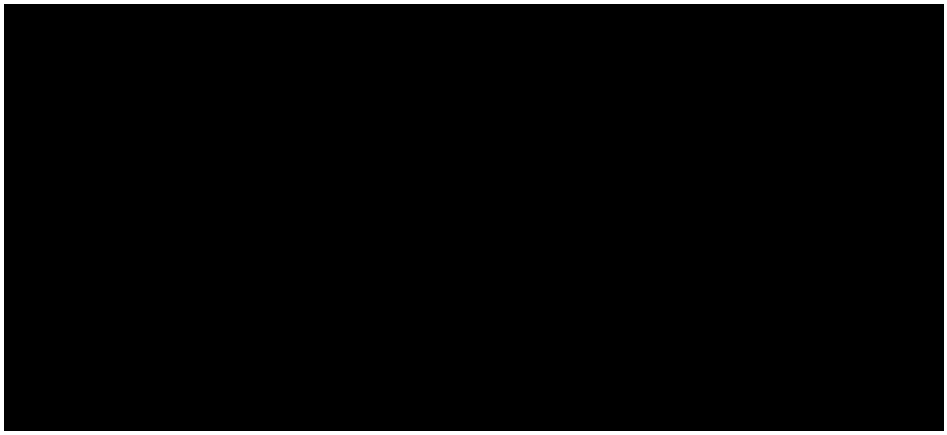

**Fig. 1. Deoxyribonucleoside kinase activities in fractions separated by HPLC**

The dCyd kinase (•), araC kinase (○), and dGuo kinase (▪) activities were determined in the fractions off the HPLC column. Equal amounts of extract protein from the wild-type and dGuo-R cells were put on the column. *A* shows the kinase activities in the extract from the wild-type cells; *B* shows the kinase activities in the extract from the dGuo-R cells; *C* shows the UV profile of the protein eluting from the column (the extracts from the wild-type and dGuo-R cells gave the same profile). Note that the *y* axis for *A* and *B* is a log plot. Extracts from the wild-type cells were run over the HPLC column in four different experiments; extracts from the dGuo-R cells were run over the HPLC column in two separate experiments. For dCyd, dGuo, and araC as substrates, 10,000 dpm of product on the filter paper were equivalent to 0.012, 0.027, and 0.013 nmol/h.

**Table I**

Effects of nucleosides and other compounds on the growth of wild-type and deoxyguanosine-resistant L1210 cells

| Compound | $IC_{50}$[a] | | Ratio dGuo-R/WT |
|---|---|---|---|
| | WT | dGuo-R | |
| | *μM* | | |
| dGuo/8AGuo | 11.8 ± 1.3 | 1520 ± 470 | 126 |
| araG | 30.8 ± 3.2 | 384 ± 62 | 12 |
| araC | 60.0 ± 6.8 | 595 ± 68 | 10 |
| dAdo/EHNA | 15.0 ± 2.4 | 42 ± 2.6 | 3 |
| 2-FdAdo/EHNA | 2.9 ± 0.05 | 32 ± 1 | 11 |
| araA/EHNA | 1.8 ± 0.17 | 4.1 ± 1.2 | 2 |
| 2-FaraA | 6.9 ± 0.88 | >100 | >14 |
| Hydroxyurea | 76.7 ± 14.4 | 138 ± 48 | 2 |
| MAIQ | 2.3 ± 0.53 | 2.7 ± 0.4 | 1 |

[a] The $IC_{50}$ values are the average of at least three separate determinations.

## Table II

Uptake and metabolism of [$^3$H]deoxycytidine, [$^3$H]deoxyguanosine, and [$^3$H]cytosine arabinoside in wild-type and deoxyguanosine-resistant L1210 cells

| Labeled nucleoside | Fraction | Wild-type | dGuo-R |
|---|---|---|---|
| | | *cpm/10$^6$ cells*[a] | |
| [$^3$H]dCyd | Acid-soluble | 4,150 | 1,800 (0.43)[b] |
| | Acid-insoluble | 9,950 | 2,090 (0.21) |
| | Total | 14,100 | 3,900 (0.27) |
| [$^3$H]dGuo | Acid-soluble | 20,330 | 2,010 (0.10) |
| | Acid-insoluble | 41,390 | 3,870 (0.09) |
| | Total | 61,720 | 5,880 (0.09) |
| [$^3$H]araC | Acid-soluble | 4,070 | 800 (0.20) |
| | Acid-insoluble | 350 | 50 (0.21) |
| | Total | 4,430 | 850 (0.21) |

[a] Average of duplicate experiments in which there were three flasks for each cell type set up in each experiment. There was less than 10% difference between the two separate experiments.

[b] Ratio of the labeled fraction in the dGuo-R cells versus the wild-type cells.

## Table III

Deoxynucleoside kinase activities in wild-type and deoxyguanosine-resistant L1210 cells

| Substrate | dN kinase activity[a] | |
|---|---|---|
| | WT | dGuo-R |
| | *nmol/min/mg protein* | |
| [$^3$H]dCyd | 0.106 | 0.086 (0.81)[b] |
| [$^3$H]dGuo | 0.220 | 0.024 (0.11) |
| [$^3$H]araC | 0.153 | 0.016 (0.10) |

[a] dN kinase activity was measured utilizing the respective substrate at 50 $\mu$M final concentration.

[b] Ratio of dN kinase activity in the dGuo-R *versus* the wild-type L1210 cells.

## Table IV

**Kinetic parameters for deoxynucleoside kinase activities in wild-type and deoxyguanosine-resistant cells**

Average of two separate determinations of $K_m$ and $V_{max}$; in each experiment, each substrate concentration was set up in triplicate and at least five different substrate concentrations were used.

| Cell type | *S* | $K_m$ | $V_{max}$ |
|---|---|---|---|
| | | *μM* | *nmol/min/mg protein* |
| Wild-type | DCyd | 5 | 0.159 |
| | DGuo | 144 | 0.763 |
| | AraC | 16 | 0.259 |
| dGuo-R | Dcyd | 40 | 0.222 |
| | DGuo | 39 | 0.032 |
| | AraC | 250 | 0.092 |