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Dual Mechanisms of 9-β-D-Arabinofuranosylguanine Resistance in CEM T-Lymphoblast Leukemia Cells

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The guanine nucleoside analog araG is selectively toxic to T-lymphoblasts and has recently shown promise in treatment of lymphoid malignancies of T-cell origin. The molecular mechanism of this tissue-selective cytotoxicity is, however, yet unclear. AraG is phosphorylated, and thereby pharmacologically activated, by the mitochondrial deoxguanosine kinase and the cytosolic/nuclear deoxycytidine kinase. We have recently shown that araG is predominantly incorporated into mitochondrial DNA of cancer cell lines, which suggests a role of mitochondria as its pharmacological target. In the present study, we have generated araG-resistant CEM T-lymphoblast cell lines and show that araG resistance may occur by two separate molecular mechanisms that can occur sequentially. The first mechanism is associated with a decrease of araG incorporation into mitochondrial DNA, and the second event is associated with loss of dCK activity. © 2001 Academic Press

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Inherited deficiency of purine nucleoside phosphorylase (PNP) causes a severe immunodeficiency syndrome due to depletion of T-lymphocytes (1). In these patients, deoxyguanosine accumulates and induces selective cytotoxic effects on T-cells compared to cells of other hematopoietic lineages (2). Pharmacological inhibitors of PNP, that *in vitro* exhibit selective T-cell cytotoxicity, have been investigated for potential use in the treatment of T-cell lymphoid malignancies. However, these studies have so far been largely unsuccessful due to the lack of clinically suitable PNP inhibitors. The guanosine nucleoside analog 9-β-D-arabinofuranosylguanine (araG) is resistant to degradation by PNP (3) and the compound is, similar to deoxyguanosine, selectively toxic to T-cells (4, 5). AraG was synthesized already in 1964 by Reist and Goodman (6), but its poor water solubility and complicated chemical synthesis has long prevented it from being evaluated in clinical trials. Recently, a water soluble prodrug of araG, 2-amino-6-methoxypurine arabinoside (GW506U78), was developed (7), and initial clinical trials have shown promising results in the treatment of several lymphoid malignancies of T-cell origin (7, 8).

AraG is, similar to several other nucleoside analogs, dependent on intracellular phosphorylation to its triphosphate derivative for pharmacological activity. The selective T-cell cytotoxicity of araG appears to be due to a higher level of araG-TP accumulation and a longer retention of the triphosphate in T-cells compared to other cell types (4, 9–11). The initial and rate-limiting phosphorylation step is catalyzed by both deoxycytidine kinase (dCK) and deoxyguanosine kinase (dGK) *in vitro.* Early studies suggested a predominant role of dCK in araG phosphorylation, and the high level of dCK expression in T-lymphoblasts was suggested to be an important factor for its-tissue selective toxicity (5, 10, 11). However, in another study nucleoside kinase measurements showed no significant differences in araG phosphorylation in T- and B-cells that could account for the preferential ara-GTP accumulation in T-cells (9). A major difference between dCK and dGK is their subcellular location; dCK is located in the cytosol/nucleus whereas dGK is located in the mitochondria (12–14). We have recently shown that overexpression of dGK in cancer cell lines enhances araG sensitivity (15) and that araG is predominantly incorporated into mitochondrial DNA (16). These findings suggest that mitochondrial dGK may be the predominant araG phosphorylating enzyme *in vivo* and that araG may elicit its effect via the mitochondria. The role of mitochondria in mediating cytotoxic

Abbreviations used: dGK, deoxyguanosine kinase; dCK, deoxycytidine kinase; TK1, thymidine kinase 1; TK2, thymidine kinase 2; araG, 9-β-D-arabinofuranosylguanine; araC, 1-β-D-arabinofuranosylcytosine; araT, 1-β-D-arabinofuranosylthymine; CdA, 2-chloro-2′-deoxyadenosine; dThd, deoxythymidine; dCyd, deoxycytidine; dFdC, 2′,2′-difluorodeoxycytidine.

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effects of dGuo and araG is supported by a recent report that shows that dGuo selectively accumulates in mitochondria of PNP-deficient mice (17).

Studies on cell lines selected for resistance to araG have shown partially conflicting results as to the molecular mechanism of resistance. Fridland *et al.* reported araG resistance associated with loss of dCK activity (9). However, araG resistance has been reported to occur with retained dCK activity in other cell lines (5, 18). In the present study we have further investigated the molecular mechanisms of araG resistance in the CEM T-lymphoblast cell line. In summary, we showed that araG resistance can occur as a two step process—the first level of resistance is associated with a decrease in araG incorporation into mitochondrial DNA whereas the second level of resistance is associated with loss of dCK expression.

MATERIALS AND METHODS

Cell culture. The CEM/wt cells were cultured at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium supplemented with heat-inactivated 10% fetal calf serum (Life Technologies Inc.), 100 units/ml penicillin and 0.1 mg/ml streptomycin.

Selection of araG-resistant cells. The CEM/wt cells were exposed to 5 μM araG (R. I. Chemical Inc.) in a 5-ml cell culture bottle. Initial cell number was approximately 3×10^5 cells/ml. The drug-exposed cell culture was subcultured once or twice a week depending on the cell density. The araG concentration was kept constant during the subcultivations until the cells were at least doubled in density. The araG concentration was then increased in a stepwise fashion (usually by 2-fold) until an araG concentration of 40 μM was reached for both the CEM/araG-1 and the CEM/araG-2 cells. The CEM/araG-3 cells were created from the CEM/araG-1 cells that were further selected in the same way as described above until they reached a final concentration of 400 μM. The cultures were then kept at the highest araG concentration for at least five additional subcultivations. Removal of araG from the extracellular medium did not affect the degree of araG resistance when the cells were further maintained in the absence of the drug.

Inhibition of tumor cell growth by drugs. Approximately 2.5×10^5 to 3×10^5 cells/ml were seeded in 200 μl-wells of 96-well microtiter plates in the presence of serial 5-fold dilutions of the test compounds. The cells were then allowed to proliferate at 37°C for 72 h. After this time period, control cells (in the absence of test compounds) were almost at the end of the exponential growth phase. The cell number was determined by use of a Coulter counter type ZM (Coulter Electronics, Harpenden Hertz, UK).

Enzyme assays. The cells were suspended at approximately 60×10^6 cells/ml in an extraction buffer containing 50 mM Tris–HCl, pH 7.6, 2 mM dithiothreitol, 5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol and 0.5% Nonidet P40. The cells were further disrupted by three freeze–thaw cycles in liquid nitrogen, then centrifuged at 4°C and 9000 rpm for 10 min, whereupon the supernatants were retrieved and stored at −80°C until used. The protein concentrations were determined using the Bio-Rad protein assay reagent with bovine serum albumin as a protein concentration standard.

The enzymatic assays were performed as described previously (19). Briefly, they were performed in 50 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM ATP, 2 mM dithiothreitol, 15 mM NaF, 100 mM KCl, 0.5 mg/ml bovine serum albumin, 40–60 μg protein extract and finally labeled (hot) and unlabeled (cold) substrate were added to a total volume of 32 μl. In the different assays the mixture of hot and cold substrates were as follows: 3 μM [8-^{3}H]araG (Moravek Biochemicals Inc.) and 1 μM unlabeled araG; 3 μM [methyl-^{3}H]thymidine (DuPont) and 7 μM unlabeled thymidine; 2.5 μM [8-^{3}H]CdA (Moravek Biochemicals Inc.) and 2.5 μM unlabeled CdA either with or without the addition of 500 μM deoxycytidine (Moravek Biochemicals Inc.); 5 μM [methyl-^{3}H]araT (Moravek Biochemicals Inc.) and 5 μM unlabeled araT. Ten microliters of the reaction mixtures was spotted on Whatman DE-81 filter paper disks after 10-, 20-, and 30-min incubation in 37°C. The filters were then washed three times in 5 mM ammonium formate. The filter-bound nucleoside analog monophosphate product of the reaction was then eluted from the filter with 0.1 M KCl and 0.1 M HCl, and the radioactivity quantified by scintillation counting.

Nucleoside transporter assay. The uptake of [8-^{3}H]-araG into CEM/wt and CEM/araG-1 cells was measured as previously described (20). In brief, the assays were conducted at room temperature in either sodium-containing transport buffer (20 mM Tris–HCl, 3 mM K_2HPO_4, 1 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2$, 5 mM glucose and 130 mM NaCl, pH 7.4) or sodium-free transport buffer (20 mM Tris–HCl, 3 mM K_2HPO_4, 1 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2$, 5 mM glucose and 130 mM *N*-methyl-D-glucosamine–HCl, pH 7.4). Approximately 10^6 cells were washed once with the appropriate transport buffer before [8-^{3}H]-araG was added to a final concentration of 1 μM. The analog uptake was stopped by using cold Dilazep dihydrochloride (Sigma) as the stopping reagent.

Autoradiography. Exponentially growing cells were harvested and resuspended in 100 μl cell culture medium at a final concentration of 5×10^5 cells/ml. Another 100 μl medium containing 3 μM [methyl-^{3}H]thymidine (DuPont), 1.8 μM [5-^{3}H]araC (Moravek Biochemicals Inc.), 1.5 μM [8-^{3}H]araG (Moravek Biochemicals Inc.) or 1.5 μM [methyl-^{3}H]araT (Moravek Biochemicals Inc.) were added. The cells were incubated for 24 h at 37°C in a humidified 5% CO_2 atmosphere. Then the cells were washed in PBS buffer and resuspended in 20 μl PBS buffer, transferred to the microscope slides (Bio-Rad Lab.), and allowed to attach for 1 h in a moist chamber. The slides were rinsed with PBS, fixated 10 min in methanol:acetic acid (3:1), and washed three times with ice-cold 10% TCA, once with water and once with methanol. Then the slides were coated with Hypercoat photoemulsion (Amersham) and kept at 4°C in the dark. The slides with [methyl-^{3}H]thymidine-treated cells were developed after 3 days, the [8-^{3}H]araG- and the [methyl-^{3}H]araT-treated cells were developed after 7 days, and [5-^{3}H]araC-treated cells were developed after 14 days using D-11 developer (Kodak).

RESULTS

Generation of araG-resistant cell lines. CEM T-lymphoblast cells were grown in the presence of increasing concentrations of araG to generate drug-resistant cells. Two araG-resistant cell lines, CEM/araG-1 and CEM/araG-2, were obtained from separate experiments. The highest araG concentration used in culture was 40 μM for both generated cell lines. The araG IC_{50} of the CEM/wt cells was determined to 1.2 μM, whereas it was increased by 132-fold for CEM/araG-1 and by 260-fold for CEM/araG-2. The sensitivity of the two araG-resistant cell lines to several nucleoside analogs was determined and compared to the drug-sensitivity of the wild-type cells (Table I). The CEM/araG-1 cells demonstrated cross-resistance to the guanosine analog dFdG (19-fold) and the thymidine analog araT (>28-fold). The CEM/araG-1 cells were not cross-resistant to the cytosine analogs araC and dFdC.

TABLE I

Sensitivity (IC_{50}) of the CEM Wild-Type Cells (CEM/wt) and the Two araG-Resistant Cell Lines (CEM/araG-1 and CEM/araG-2) to Nucleoside Analogs

		CEM/araG-1		CEM/araG-2	
Compound	CEM/wt: IC_{50} (μM)	IC_{50} (μM)	araG-1/wt	IC_{50} (μM)	araG-2/wt
araG	1.22	161	132	317	260
araT	17.6	>500	>28	>500	>28
araC	0.037	0.085	2.3	51.4	27
dFdC	0.0023	0.0028	1.2	6.01	2613
dFdG	0.021	0.39	19	1.04	50
FdUrd	0.026	0.030	1.1	0.025	1
BdUrd	12.5	61.2	4.9	44.4	3.6

Note. The IC_{50} values of CEM/araG-1 and CEM/araG-2 were compared to the corresponding IC_{50} values of CEM/wt (araG-1/wt and araG-2/wt).

The CEM/araG-2 cells differed from the CEM/araG-1 cells by exhibiting a higher level of resistance to araG and dFdG. The CEM/araG-2 cells also exhibited a marked cross-resistance to araC (1400-fold) and dFdC (2100-fold). A small decrease (<5-fold) in sensitivity to BdUrd was observed in both araG-resistant cell lines whereas no change in sensitivity to FdUrd was detected. The CEM/araG-1 and the CEM/araG-2 cells showed no difference in sensitivity to tubercidine, ribavirine, methotrexate or mycophenolic acid compared to the wild-type parent cell line (data not shown).

Nucleoside kinase activity in crude cell extracts. To determine the level of nucleoside kinase activity in the cell lines, we measured the total nucleoside phosphorylating activity in crude protein cell extracts (Fig. 1). The enzyme activity assays showed that the total araG phosphorylating activity was similar in the wild-type CEM cell line and the CEM/araG-1 and CEM/araG-2 cell lines (2–4 pmol/mg/min). This result suggested that the araG resistance was not due to a deficiency in the araG phosphorylating nucleoside kinase(s). We also determined the levels of CdA phosphorylation, that is catalyzed mainly by dCK but also by dGK (21). The CdA phosphorylation was ≈8-fold lower in the CEM/araG-2 cells compared to the CEM/araG-1 and the CEM/wt cells. Addition of 500 μM dCyd to the CdA phosphorylation assay has been shown to inhibit dCK-mediated phosphorylation of CdA whereas the dGK-catalyzed CdA phosphorylation is unaffected (19, 21). When the assay was performed with CdA and 500 μM dCyd the CdA phosphorylating activity, hence catalyzed only by dGK, was similar in all the extracts of all three cell lines. These data suggest that the CEM/araG-2 cells are deficient in dCK activity whereas the level of dCK activity in the CEM/araG-1 cells is unaffected. We further determined the dThd phosphorylation, catalyzed by both TK1 and TK2, in the cell extracts. As shown in Fig. 1, the phosphorylating activity for dThd was similar in all cell extracts. 1-β-D-arabinofuranosylthymine (araT) is predominantly a substrate of TK2 (19, 22, 23). The araT phosphorylating activity was ≈4-fold lower in the CEM/araG-2 cells compared to the wild type cells, whereas the CEM/araG-1 cells exhibited similar activity as the wild-type cells.

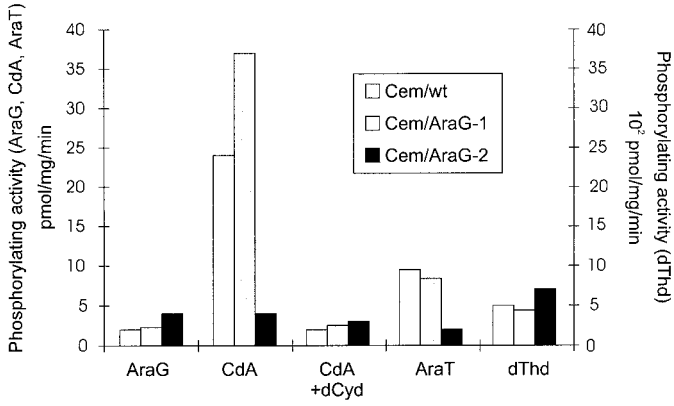

FIG. 1. Nucleoside phosphorylating activity in crude cell extracts.

We also investigated the expression level of dGK in the CEM/wt and the CEM/araG-1 cells by Western blot analysis using an affinity-purified anti-dGK antibody (15). The Western blot indicated similar levels of dGK expression in the CEM/wt and the CEM/araG-1 cells (data not shown). The experiments support the conclusion from the enzymatic assays that the level of dGK protein in not altered in the resistant cell lines. In summary, we detected no decrease in phosphorylation of either of the investigated nucleosides that suggested a loss of a nucleoside kinase activity in crude cell extracts of CEM/araG-1. In contrast CEM/araG-2 showed a marked decrease in CdA phosphorylation, suggesting loss of dCK activity.

Study of the extracellular uptake of araG. Mutations in plasma membrane nucleoside transporter pro-

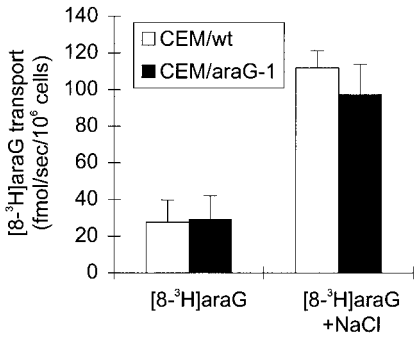

FIG. 2. Uptake of [8-^{3}H]araG from the extracellular space in the presence or absence of NaCl.

teins have been shown to cause resistance to nucleoside analogs (20, 24, 25). To analyze if araG resistance in the CEM/araG-1 cells was due to an altered uptake of araG from the extracellular space, we performed a nucleoside transporter assay (Fig. 2). The assay showed no difference between the CEM/araG-1 and the CEM/wt cells in the import of araG from the extracellular space.

Qualitative studies of incorporation of radiolabeled compounds into the DNA of CEM/wt and CEM/araG-1 cells. We have previously shown that araG is predominantly incorporated into mitochondrial DNA of CEM cells (16). In the present study, we used an autoradiography method to visualize the incorporation of radiolabeled nucleosides into nuclear and mitochondrial DNA of the CEM/wt and CEM/araG-1 cells (Fig. 3). Radiolabeled dThd and araC were efficiently incorporated into the nuclear DNA in both the CEM/araG-1 and the CEM/wt cells, as seen by dark staining in a restricted area of the cells corresponding to the nucleus (Fig. 3). The cells incubated with araG or araT, both substrates of the mitochondrial enzymes, showed a dotted autoradiography pattern distributed throughout the entire cells, indicating incorporation of the nucleoside analogs into mitochondrial DNA (Fig. 3). However, the level of araG and araT incorporation in the resistant CEM/araG-1 cell line was markedly lower compared to the level of araG and araT incorporation into the CEM/wt cells.

Generation of cells with higher level of araG resistance from the CEM/araG-1 cells. Both CEM/araG-1 and CEM/araG-2 exhibited resistance to dFdG and araT in addition to araG. However, a major difference between the CEM/araG-1 and the CEM/araG-2 cells was the cross-resistance of CEM/araG-2 to the cytosine analogs araC and dFdC. Furthermore, the CEM/araG-2 cells also exhibited a higher level of resistance to araG and dFdG than the CEM/araG-1 cells. We hypothesized that both cell lines had acquired resistance to araT and dFdG by a single molecular mechanism and that the CEM/araG-2 cells in addition had acquired araC and dFdC resistance by another mechanism. In an attempt to test this hypothesis, we continued the culturing of CEM/araG-1 cells in an increasing araG concentration up to 400 μM to find out whether a resistance phenotype similar to CEM/araG-2 could be generated. The new cell line obtained (CEM/araG-3) was 546-fold resistant to araG (Table II). CEM/araG-3 showed, similar to CEM/araG-2, resistance to the cytidine analogs dFdC and araC. These findings favors the hypothesis stated above.

DISCUSSION

We have shown that resistance to the nucleoside analog araG in a CEM T-lymphoblast cell line is asso-

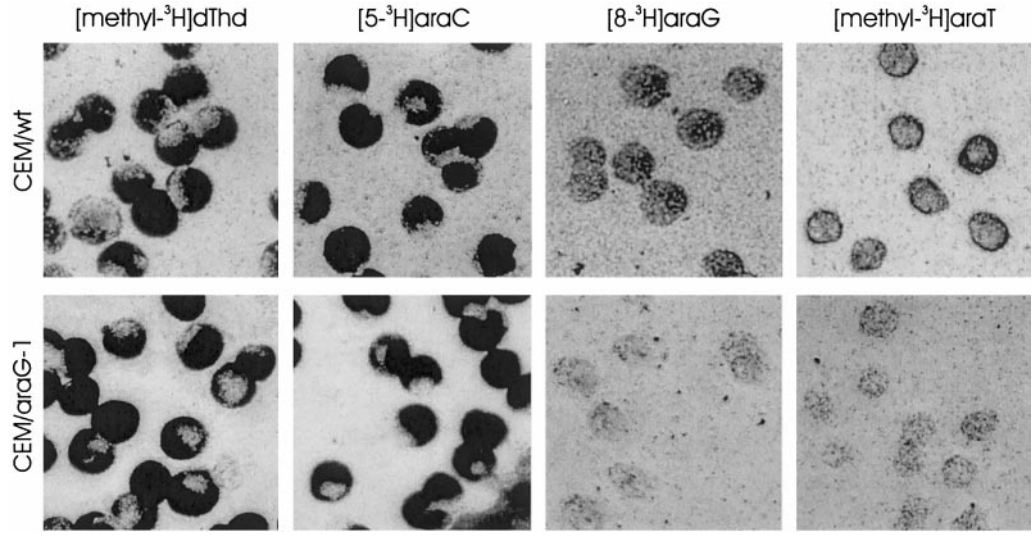

FIG. 3. Autoradiography of CEM/wt and CEM/araG-1 T-lymphoblast cells lines incubated with [methyl-^{3}H]dThd, [5-^{3}H]araC, [methyl-^{3}H]araT, or [8-^{3}H]araG.

TABLE II

Comparison of the Sensitivity (IC_{50}) of the CEM/araG-1 Cell Line and the Same Cell Line after Further Selection for araG-Resistance (CEM/araG-3) to Nucleoside Analogs

	IC_{50} (μM)	
Compound	CEM/araG-1	CEM/araG-3
araG	161	546
dFdG	0.39	2.38
araC	0.085	1.74
dFdC	0.0028	0.0251
FIAU	97.9	173

ciated with either decreased incorporation of araG into mitochondrial DNA or by loss of dCK activity. We have further shown that these two molecular events can occur sequentially, where the decrease of araG incorporation into mitochondrial DNA is followed by loss of dCK activity at higher levels of drug resistance. A role of mitochondria in the cytotoxicity of araG is suggested by recent data showing that the compound is predominantly incorporated into mitochondrial DNA of several cancer cell lines (16). Incorporation of araG into mitochondrial DNA is probably caused by the intramitochondrial phosphorylation of the compound catalyzed by mitochondrial dGK and the subsequent trapping of the phosphorylated nucleoside analog in the mitochondrial matrix. The inner mitochondrial membrane prevents free transport of phosphorylated nucleoside analogs from the mitochondria to the cytosol and several studies suggest that phosphorylated nucleoside analogs become trapped in this compartment (26–28). The selective accumulation of dGTP the mitochondrial matrix have been suggested to induce apoptosis in PNP-deficient mice (17), and it is possible that araG induces cell death by a similar mechanism.

The present study demonstrating two separate mechanisms of araG resistance are consistent with earlier studies reporting that araG resistance in some cell lines is associated with loss of dCK activity (9, 29), while other cell lines exhibiting araG resistance have retained dCK activity (5, 18). At high concentrations of araG (100 μM), the nucleoside analog is predominantly incorporated in S-phase CEM cells, and the S-phase cells exhibit highest sensitivity to araG induced apoptosis (30). These data suggest that at least part of the cytotoxic effect caused by araG is due to its incorporation into nuclear DNA during S-phase. However, we do not yet know the relative importance of the effects on nuclear and mitochondrial DNA in mediating the cytotoxicity of araG *in vivo*. Neither do we know the relative importance of the different phosphorylating enzymes for araG cytotoxicity. It is possible that a small amount of the araG that is phosphorylated by dGK within the mitochondria leaks out, or is transported by unknown mechanisms, to the cytosol and affects nuclear DNA during S-phase also in cells that lack dCK activity. We are currently initiating studies to resolve these issues, since it will be important to define the physiological relevant target of the compound in order to understand its mechanism of action. In a recent phase I trial with the prodrug of araG, GW506U78, most of the patients with T-ALL responded well to the treatment despite the fact that these patients were refractory to other kinds of treatments (8). The initial treatments of patients with leukemia usually involve a combination of compounds that include the front-line anti-leukemic agent araC (31, 32). Patients that have acquired resistance to araC may have lost the expression of dCK (31, 33). The fact that patients with araC resistance can respond to treatment with araG indicates that araG has a different activation pathway that may involve another activating enzyme than dCK also *in vivo*.

We do not yet know the molecular basis of the decrease in araG incorporation into mitochondrial DNA. The total level of araG phosphorylation in the CEM/araG-1 cells was similar to the wild-type cells which argues against altered kinase activity, as was suggested to be the reason for araG resistance in a MOLT cell line in an earlier study (5). Neither were we able to detect any deficiency in transport of araG across the plasma membrane. Interestingly, the CEM/araG-1 cells also exhibited a concomitant decrease in mitochondrial incorporation of araT. One possible explanation could be a deficiency in nucleoside transport across the mitochondrial membrane. Nucleoside transport activities have been demonstrated in the mitochondrial membranes (34), but little is known about the properties of these transporters. Studies are ongoing to elucidate the molecular mechanisms of araG resistance in the cell lines exhibiting a decreased mitochondrial incorporation of araG. These studies may contribute to clarify the different mechanisms of action for araG and thereby provide a basis for a rational use of this compound.

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