

A new high-performance liquid chromatography method determines low production of 9- β -D-arabinofuranosylguanine triphosphate, an active metabolite of nelarabine, in adult T-cell leukemia cells

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Received September 17, 2009; Accepted October 30, 2009

DOI: 10.3892/or_00000661

Abstract. The 9- β -D-arabinofuranosylguanine (ara-G), an active compound of nelarabine, demonstrates potent cytotoxicity specifically on T-cell malignancies. In cells, ara-G is phosphorylated to ara-G triphosphate (ara-GTP), which is subsequently incorporated into DNA, thereby inhibiting DNA synthesis. Because ara-GTP is crucial to ara-G's cytotoxicity, the determination of ara-GTP production in cancer cells is informative for optimizing nelarabine administration. Here, we developed a new, sensitive isocratic-elution HPLC method for quantifying ara-GTP. Samples were eluted isocratically by using phosphate buffer at a constant flow rate. Ara-GTP was clearly separated from other nucleotides by using an anion-exchange column and it was quantitated by its peak area at 254 nm. The standard curve was linear with low variability and a sensitive detection limit (10 pmol). Furthermore, due to ara-G's specificity to T-cells we hypothesized that nelarabine might be effective against adult T-cell leukemia (ATL). The ara-GTP production was compared between T-lymphoblastic leukemia CCRF-CEM and ATL cell lines *in vitro*. When CEM cells were incubated with ara-G, the ara-GTP production increased in a concentration- and time-dependent manner. In contrast, 5 ATL cell lines accumulated lower ara-GTP in the same condition. While ara-G inhibited the growth of CEM cells with a 50% growth inhibition concentration of 2 μ M, the inhibitory-concentration values were >1 mM in 8 of the 12 ATL cell lines. This ineffectiveness appeared to correspond with the low ara-GTP production. The present study is the first to evaluate the

potential of ara-G against ATL cells; our results suggest that nelarabine would not be effective against ATL.

Introduction

The 9- β -D-arabinofuranosylguanine (ara-G), an active compound of nelarabine, 2-Amino-9- β -D-arabinofuranosyl-6-methoxy-9H-purine, is a purine nucleoside analog that has demonstrated potent cytotoxic effects specifically on T-cell malignancies *in vitro* (1-3). Nelarabine has been clinically evaluated for its efficacy against hematological malignancies including T-cell acute lymphoblastic leukemia (T-ALL), T-cell lymphoma, and chronic lymphocytic leukemia (4,5).

Nelarabine is demethylated to ara-G by adenosine deaminase in the plasma after intravenous administration. Ara-G is transported into cancer cells through membrane transporters including the human equilibrative nucleoside transporter 1 (hENT1). Thereafter, ara-G is phosphorylated to ara-G monophosphate by cytoplasmic deoxycytidine kinase and mitochondrial deoxyguanosine kinase and then to ara-G diphosphate and eventually to ara-G triphosphate (ara-GTP) (6-8). Ara-GTP is subsequently incorporated into DNA, thereby terminating DNA elongation and inducing apoptosis (6). In this regard, intracellular ara-GTP is considered to be the most critical determinant of ara-G cytotoxicity. Thus, quantification of intracellular ara-GTP production is required to evaluate the cytotoxicity of ara-G.

The parameter for predicting the clinical efficacy of a given nucleoside analog is not the plasma drug concentration but its triphosphate form concentration in cancer cells (9-11). Pharmacokinetic evaluation of ara-GTP will provide crucial information to optimize ara-G therapy (3-5). Clinical data have already confirmed that response to nelarabine is associated with higher intracellular ara-GTP concentrations in both acute and indolent leukemias (4,5). The sole method for determining intracellular ara-GTP concentrations uses gradient-elution ion-exchange HPLC that was developed by Rodriguez *et al* (12). This method was sensitive and applicable to measuring ara-GTP in leukemic cells from patients receiving nelarabine. However, a gradient mode requires a complicated computerized system equipped with multiple pumps for regulating

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Key words: isocratic HPLC, 9- β -D-arabinofuranosylguanine, ara-G triphosphate, nelarabine, adult T-cell leukemia

two different buffers. Moreover, the gradient-elution HPLC methods usually induce base-line drift, which might interfere with a quantification of the small peak in biological materials. Development of a simple and inexpensive monitoring method is thus helpful to overcome these disadvantages.

Determination of intracellular ara-GTP concentrations would help determine the potential therapeutic efficacy of nelarabine against other malignancies that are refractory to conventional chemotherapy. One such T-cell malignancy is adult T-cell leukemia (ATL) (13-15). ATL is associated with human T-cell leukemia virus type 1 (HTLV-1) infection. Despite aggressive chemotherapy, the prognosis of ATL is poor, and new treatments have been sought. Because the cytotoxicity of ara-G has been demonstrated selectively for T-cell malignancies, ATL may be a good candidate for nelarabine therapy.

In the present study, we established a sensitive method for determining intracellular ara-GTP concentrations in cancer cells. We selected an isocratic HPLC procedure for its simplicity and inexpensiveness. We further validated this method by determining the intracellular production of ara-GTP in a T-ALL cell line CCRF-CEM (CEM). Finally, we quantitated the ara-GTP production of cultured ATL cell lines and compared the ara-G sensitivity of 12 cultured ATL cell lines to CCRF-CEM cells *in vitro*.

Materials and methods

Chemicals and reagents. ara-G was purchased from R.I. Chemicals (Orange CA, USA) and dissolved in 100% dimethyl sulfoxide. Standard ara-GTP was provided by GlaxoSmith Kline, Japan.

HPLC apparatus and chromatographic condition. The HPLC apparatus consisted of a pump (CCPM-II; Tosoh Corp., Tokyo, Japan), an autosampler (AS-8020; Tosoh Corp.), an in-line degasser (SD-8022; Tosoh Corp.), and a variable-wavelength detector (UV-8020; Tosoh Corp.). LC-8020 software (Tosoh Corp.) installed on a personal computer was used to control the system and analyze the data (16). Samples were eluted isocratically with 0.06 M Na₂HPO₄ (pH 6.9) containing 20% acetonitrile, at a constant flow rate of 0.7 ml/min and at ambient temperature. Separation was performed by an anion-exchange column, TSK gel DEAE-2 SW column (length, 250 mm; internal diameter, 4.6 mm, Tosoh) and was monitored at 254 nm.

Standard curve and % coefficient of variation (CV). To determine the standard curve and %CV for the assay, a 500- μ l aliquot from each diluted concentration of standard ara-GTP ranging between 10 pmol and 1 nmol (10, 20, 50, 100, 500 pmol and 1 nmol) was applied to the HPLC. The solutions were measured in triplicate on 3 separate days to determine the within-day and inter-day variation. Data were combined and plotted to determine the correlation between the amount of ara-GTP and the peak area. The standard curve was fitted with the weighted least-squares linear regression analysis method by using the equation $y = ax + b$. Ara-GTP in unknown samples was quantified by extrapolating the peak area count into the equation of the standard curve.

Cell culture. We used the T-ALL cell line, CCRF-CEM (CEM), and the following 12 ATL cell lines: ATL-43T, KOB, SO4, M8166, MT-2, MT-4, ED-40515 (+), ED-40515 (-), ST-1-dependent, ST-1-independent, KK1-dependent, and KK1-independent. The MT-2, MT-4, M8166, ED-40515 (+), and ED-40515(-) cell lines were kindly provided by Dr Takashi Uchiyama (Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University). The ATL-43T, KOB, SO4, ST-1-dependent, ST-1-independent, KK1-dependent, and KK1-independent cell lines were kindly provided by Dr Masao Tomonaga (Department of Hematology, School of Medicine, Nagasaki University). All cell lines were maintained in RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO₂-humidified atmosphere. ED (+), ST-1-dependent and KK1-dependent cells were cultured with 500 ng/ml of IL₂.

Determination of intracellular ara-GTP in human leukemia CEM cells *in vitro*. We used cultured leukemia cells to validate the applicability of our method to biologic materials. CEM cells (1x10⁶ cells/ml, 20 ml), with or without ara-G treatment, were washed twice with fresh media and centrifuged (400 x g, 10 min, 4°C) in a microcentrifuge tube. The cells were then resuspended into 200 μ l of 0.3 M cold perchloric acid and vortexed for 10 sec before allowed to stand for 15 min at 4°C. The acidic supernatant was isolated by centrifugation of the sample (15,600 x g, 20 sec, 4°C), followed by neutralization with 100 μ l of 0.5 N potassium hydroxide. After centrifugation (15,600 x g, 20 sec, 4°C), the neutralized supernatant was obtained as an acid-soluble fraction (ASF), a nucleotide pool (17). Water was added to each ASF sample to achieve a total volume of 700 μ l, from which a 500- μ l aliquot was applied to the chromatographic analysis. The intracellular concentration of ara-GTP was expressed as pmol/10⁷ cells. The samples were stored at -80°C until analyzed. Five ATL cell lines also were applied to this HPLC measurement of intracellular ara-GTP concentrations.

Growth inhibition assay. To evaluate the proliferation of each cell line, a sodium 3'-(1-[(phenylamino)-carbonyl-3,4-tetrazolium])-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay was performed according to the manufacturer's instructions (Roche, Indianapolis, IN, USA) with slight modifications (18). In brief, 1 ml of the cells (5x10⁴/ml) was incubated for 24 h in a 24-well plate, followed by the addition of a 10- μ l aliquot of ara-G solution at various concentrations. The cells were incubated for the next 72 h and a 100- μ l aliquot was transferred to a 96-well microplate. The cells were then mixed with 50 μ l XTT and incubated for 4 h at 37°C. The samples were applied to spectrophotometric measurement at the absorbance of 480 nm by using a fluorescent microplate reader (Spectra Max 250, Molecular Device Japan, Ashiya, Japan). The concentration needed for 50% inhibition of cell growth (IC₅₀) was extrapolated from a growth inhibition curve drawn for each cell line.

Statistical analyses. The standard curve was obtained by using GraphPad Prism software (version 5.0, GraphPad Software Inc., San Diego, CA, USA). The mean, standard deviation and

%CV for the ara-GTP measurements were obtained by using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

Results

Standard curve and validation. To determine the standard curve, 5 different concentrations of standard ara-GTP solutions were applied in triplicate to the present HPLC scheme on 3 separate days. A linear relationship was obtained between the amount of ara-GTP and the corresponding peak areas (Fig. 1). The within-day variability and inter-day variability were determined for each amount of ara-GTP. The variations were low; all %coefficient of variation (%CV) values were <10% (Table I). The detection limit, which is the lowest amount of ara-GTP that yielded a %CV of <10%, was 10 pmol. Furthermore, this limit was more sensitive than that of the previous HPLC assay method, which was 13 pmol (12). Thus, our HPLC method was precise and sensitive for quantifying ara-GTP.

Separation of ara-GTP. To confirm the separation of ara-GTP, a mixture of standard nucleotides (CTP, UTP, ATP, GTP, and ara-GTP) was injected onto the HPLC. The ara-GTP peak was clearly separated from the other nucleotides (Fig. 2a), with a retention time of 108 min. The retention times of standard deoxyribonucleotides (dATP, dCTP, dTTP, dGTP), which were almost identical to those of the corresponding ribonucleotides (data not shown), did not overlap the ara-GTP peak.

To verify if our HPLC assay was applicable to the measurement of ara-GTP in biological samples, we tested nucleotide pools extracted from leukemic cells. Fig. 2b represents a blank chromatogram of an ASF extracted from untreated CEM cells. As shown in Fig. 2b, endogenous nucleoside triphosphates (CTP, UTP, ATP, and GTP) were clearly separated in the same way, as had been demonstrated using the mixture of standard nucleotides (Fig. 2a). Moreover, the chromatogram did not contain any peaks soon after the GTP peak, which might otherwise have interfered with the ara-GTP peak. When the ASF was co-eluted with the standard ara-GTP solution, the peak of ara-GTP was clearly independent of the other endogenous peaks of the leukemic nucleotides (Fig. 2c), confirming the applicability of the method to biological samples. Furthermore, an ASF sample from CEM cells treated with 10 μ M ara-G was applied to the present HPLC scheme, and the accumulation of ara-GTP in leukemic cells was clearly demonstrated in the chromatogram (Fig. 2d). These results suggest that the present HPLC condition offered appropriate ara-GTP separation in biological materials.

Quantitation of ara-GTP concentrations in the T-ALL CEM cells in vitro. We used the present HPLC assay to evaluate the production of ara-GTP in CEM cells. ASF samples that were extracted from CEM cells after incubation with various concentrations of ara-G for the indicated time periods were then applied to the HPLC analysis. Intracellular ara-GTP production increased in a time- and concentration-dependent manner (Fig. 3a and b). Thus, the present HPLC method enabled us to quantitate ara-GTP in biological samples.

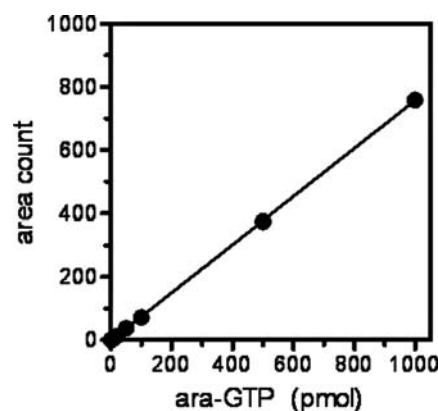


Figure 1. Standard curve for ara-GTP measurement. Various concentrations of standard ara-GTP (10, 20, 50, 100, 500, and 1,000 pmol) in aqueous solution were applied in triplicate to the present HPLC scheme on 3 separate days. A linear relation was obtained between the ara-GTP amount and peak area, using all the data sets. Parameters for the curve were: slope = 0.76 ± 0.001 ($r^2 = 0.9998$, $P < 0.0001$, for the linear regression line).

Table I. Within-day and inter-day variation for standard ara-GTP injection.

Ara-GTP (pmol)	Within-day (day 1)			Inter-day		
	Mea	SD	%CV	Mea	SD	%CV
10	7.1	0.1	1.4	6.2	0.5	7.9
20	13.1	0.3	2.2	12.1	0.4	3.7
50	36.6	0.8	2.3	38.6	0.7	1.8
100	75.0	0.9	1.3	70.9	0.6	0.8
500	374.2	3.5	0.9	371.8	6.6	1.8
1000	757.0	7.2	1.0	762.2	5.6	0.7

Standard ara-GTP solutions were measured by the present HPLC in triplicate on 3 separate days. The within-day variation is determined by the values measured on day 1. The inter-day variation was calculated from the mean of each day's peak area count. CV, coefficient of variation.

Ara-GTP production in ATL cells in vitro. Because the cytotoxicity of ara-G is specific toward T-cell leukemia/lymphoma cells, it may be informative to determine if ATL cells are sensitive to ara-G. We used intracellular ara-GTP production as a surrogate marker for the cytotoxicity of ara-G in 5 ATL cell lines. When the cells were incubated with 10 μ M ara-G, the intracellular ara-GTP production in the ATL cell lines was approximately one fourth of the production in the T-ALL CEM cell line (Fig. 4a). The difference was more obvious when cells were incubated with the highest concentration (1 mM) of ara-G (Fig. 4b). The results suggest that ATL cells have only a small capacity to produce ara-GTP compared with T-ALL CEM cells.

Growth inhibition effect of ara-G. To further examine the sensitivity of ATL cells to ara-G, we determined the ara-G-

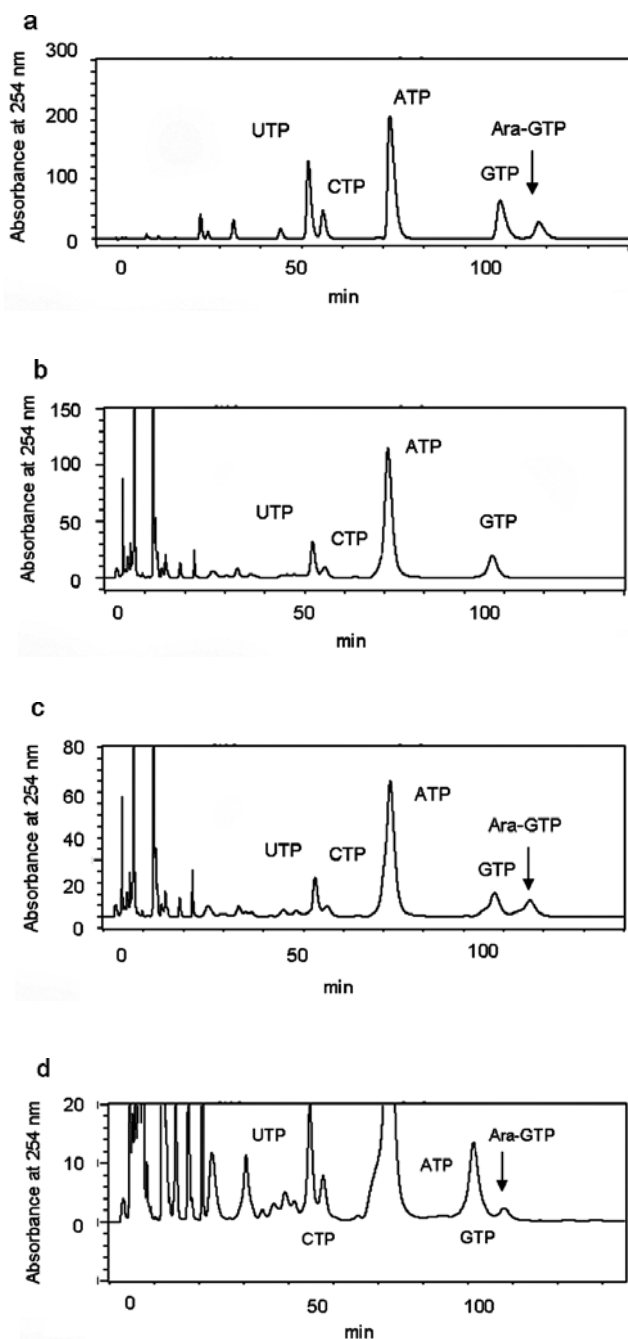


Figure 2. HPLC profiles of nucleotides. (a) Standard nucleotides (ATP, CTP, UTP, GTP, and ara-GTP) dissolved in aqueous solution were applied to the present HPLC scheme; (b) an acid-soluble fraction was extracted from untreated leukemic CEM cells (1×10^7 cells) and applied to the HPLC; (c) the same acid-soluble fraction was co-eluted with the standard ara-GTP solution; (d) an acid-soluble fraction was extracted from CEM cells after a 5-h incubation with $10 \mu\text{M}$ ara-G and applied to the HPLC.

induced inhibition of cell proliferation in 12 cultured ATL cell lines. As determined from the XTT assay, the IC_{50} values of 8 of the 12 cell lines were $>1 \text{ mM}$ (Fig. 5a, Table II). There was no difference in the ara-G cytotoxicity between the IL_2 -dependent and IL_2 -independent cell lines (Fig. 5a, Table II). In contrast, ara-G was quite effective for inhibiting the growth of T-cell leukemia CEM cells (Fig. 5b, Table II). Taken together with the results of the ara-GTP production, the present findings indicate that ara-G is ineffective against ATL cells.

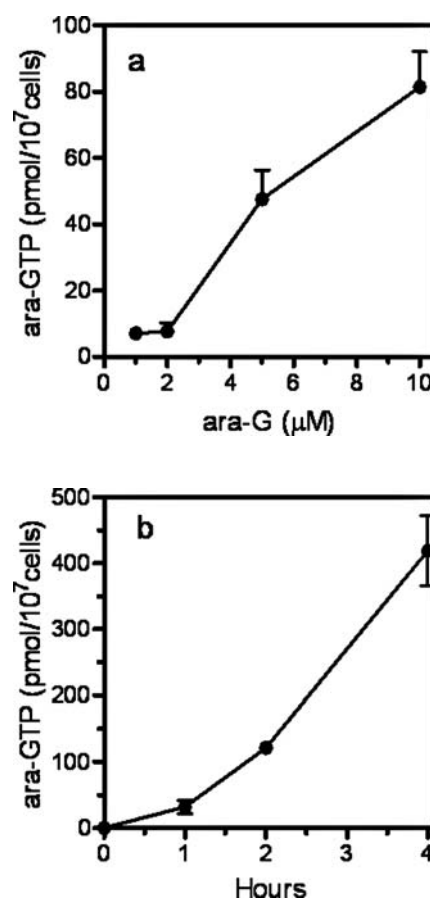


Figure 3. CEM cells were incubated with indicated concentrations of ara-G for 5 h (a) or with $100 \mu\text{M}$ ara-G for indicated time periods (b), followed by HPLC analysis for ara-GTP production.

Discussion

Nucleoside analogs belong to one of the most clinically useful and most often used class of drugs for the treatment of cancer including hematological malignancies. Nelarabine, a prodrug of ara-G, is a new purine analog that has been approved for the treatment of T-ALL and T-lymphoblastic lymphoma in Japan. The anticancer spectrum of nelarabine is narrowly specific to T-cell malignancies; thus, nelarabine should be used in selected patients or subtypes of cancer. Given that the intracellular ara-GTP production is crucial to the cytotoxicity of ara-G, pharmacokinetic studies of ara-GTP concentrations in cancer cells may be indispensable for optimizing nelarabine therapy.

Here, we have developed a simple and sensitive analytical method for quantifying ara-GTP in leukemic cells by using isocratic HPLC. Ara-GTP was clearly separated from adjacent nucleotides (Fig. 2), and the assay variation was low, with the $\%CV < 10\%$ (Table I). The applicability of the method to biological materials was demonstrated by measuring ara-GTP concentrations in CEM cells *in vitro* (Fig. 3). The previously reported HPLC method for determining intracellular ara-GTP concentrations employed a concave gradient elution and 2 different buffers (12). Although this method uses less running time than our method, it requires a computerized system to regulate two pumps for the elution. In contrast, our isocratic HPLC method uses a single buffer and can be controlled by a computerized system or a conventional, mechanical single

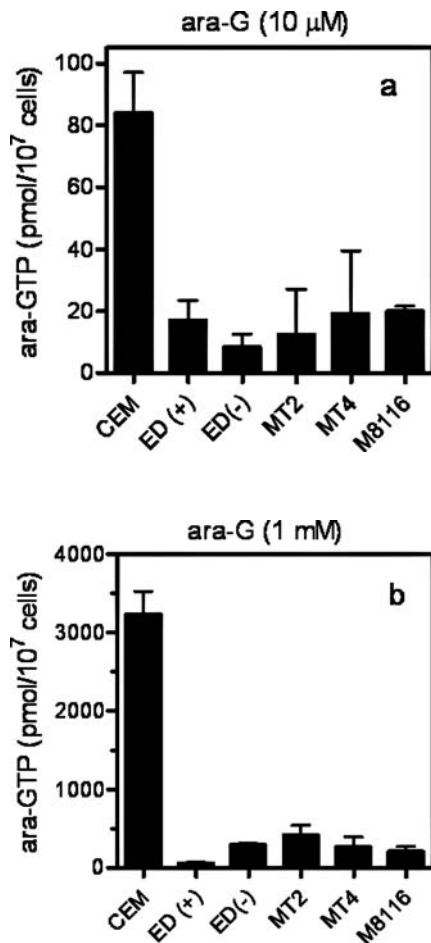


Figure 4. Intracellular ara-GTP production. Cells were incubated with 10 μ M (a) or 1 mM (b) ara-G for 5 h, followed by HPLC analysis for ara-GTP production.

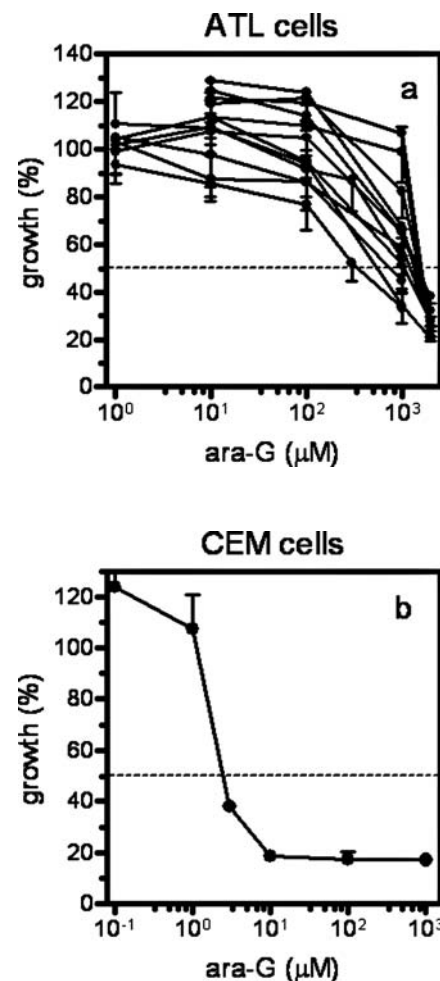


Figure 5. Twelve ATL cell lines (a) and CEM cell line (b) were incubated with various concentrations of ara-G for 72 h, followed by evaluation of growth inhibition using the XTT assay.

pump. Moreover, isocratic elution does not induce base-line drift (Fig. 2), thereby allowing accurate measurements of small amounts of ara-GTP in crude biological samples. Thus, the present method is advantageous for its simplicity, sensitivity, inexpensiveness and clinical applicability.

ATL is an aggressive malignancy of mature activated CD4⁺ T-cells that is associated with HTLV-1 infection (13-15). ATL develops in 1 to 3% of infected individuals after more than two decades of viral persistence. Once the disease is manifested, the prognosis of patients is poor. After standard chemotherapy for aggressive lymphoma, the median survival time is 8 months and the 4-year survival rate is only 12%. New active agents and/or novel combination chemotherapies are needed to improve the clinical outcome. Thus, we evaluated the potential of ara-G for ATL cells *in vitro*. The intracellular accumulation of ara-GTP was greatest in cells of T-lymphoid lineage compared with other cell types, thereby conferring ara-G cytoreductive effects specifically on T-cell malignancies (1-3,6-8). Clinical response to nelarabine in hematologic malignancies was associated with higher intracellular ara-GTP concentrations (4,5). A recent study also revealed that the clinical response to nelarabine was directly related to the maximal concentration of intracellular ara-GTP in patients with indolent leukemia (19). Therefore, we expected that ara-G would be cytotoxic against ATL

Table II. Sensitivity of ATL cell lines to ara-G.

Cell lines	IC ₅₀ (μ M)
ATL-43T	1530
KOB	690
SO4	1540
M8166	360
MT-2	1040
MT-4	1500
ED (+)	490
ED (-)	1100
ST-1-dependent	930
ST-1-independent	1400
KK1-dependent	1300
KK1-independent	1300
CCRF-CEM	2

Cells were incubated with various concentrations of ara-G for 72 h. The IC₅₀ was then determined by using the XTT assay.

cells and that the measurement of ara-GTP levels in ATL cells might provide crucial information for the decision to use nelarabine for treating ATL patients. However, the production of ara-GTP in ATL cells was much less than that in T-ALL CEM cells (Fig. 4), suggesting that ATL cells had only a small capability to yield ara-GTP. Moreover, the standard regimen for nelarabine administration is a 2-h drip infusion of 1,500 mg/m², which achieves a maximal plasma concentration of the active compound, ara-G, of 105 μ M (3). This clinically achievable concentration is nearly one-tenth of the IC₅₀ concentrations for ATL cell lines that were determined by using the XTT assay (Fig. 5, Table II). Because the drug resistance in a given type of cancer cells is usually multifactorial, the low production of ara-GTP might not entirely explain the insensitivity to ara-G against ATL (20). Nevertheless, our *in vitro* results disappointingly suggest the clinical ineffectiveness of nelarabine for treating ATL patients.

In conclusion, we developed a new and simpler HPLC method that we used to quantify the low production of intracellular ara-GTP in ATL cell lines *in vitro*. The growth inhibition effects of ara-G were insufficient against ATL cell lines. The present study is the first to evaluate the potential of ara-G against ATL cells and our results suggest that nelarabine is ineffective against ATL.

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