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Note

Separation of 1-β-D-arabinofuranosylcytosine 5′-triphosphate and 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-triphosphate in human leukemia cells by high-performance liquid chromatography

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Both 1-β-D-arabinofuranosylcytosine (ara-C) and 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A) are antileukemic drugs; ara-C is widely used for the treatment of adult acute leukemia [1] while the activity of F-ara-A is being evaluated in Phase I and II trials [2–6]. The primary mechanism of action may be similar for each drug. Each nucleoside analogue is metabolized inside the cell to the active triphosphate, 1-β-D-arabinofuranosylcytosine 5′-triphosphate (ara-CTP) [7] and 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-triphosphate (F-ara-ATP) [8–10], respectively.

The ability of circulating leukemic cells to retain ara-CTP during therapy with high-dose ara-C regimens has been correlated with the probability of achieving complete remission [11,12]. Studies of the cellular pharmacokinetics of F-ara-ATP in leukemic cells suggest that similar relationships with response may be found [2].

Preliminary investigations with human leukemic cells have demonstrated that preincubation with F-ara-A potentiates the accumulation of ara-CTP [13]. Since this appears to depend upon the cellular concentration of F-ara-ATP, a single assay that could separate and quantitate each nucleotide analogue would greatly facilitate the analysis of these events. Because it is likely that this combination will be evaluated clinically, an assay capable of simultaneously quantitating ara-CTP and F-ara-ATP would essentially halve the requirement for tumor cells necessary for cellular pharmacology investigations. Thus, the objective of this paper is to report the development and characterization of a rapid, sensitive procedure for the quantitation of these active nucleotides.

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Chromatographic methods have previously been reported for the separation and quantitation of ara-CTP [14] and F-ara-ATP [9] in leukemic cells. Procedures are also available for detection of ara-CTP and 3-deazauridine 5′-triphosphate [15], and ara-CTP and 2′-deoxycytidine 5′-triphosphate [16]. We describe a method that optimizes the separation of ara-CTP and F-ara-ATP in leukemic cells in culture and in the leukemic cells of patients treated with each pro-drug.

EXPERIMENTAL

Chemicals

Ara-C was purchased from Sigma (St. Louis, MO, U.S.A.). F-ara-A was provided by Dr. V.L. Narayanan (National Cancer Institute, Bethesda, MD, U.S.A.). In the clinic, the 5′-monophosphate of F-ara-A (F-ara-AMP) was used; it was supplied by the National Cancer Institute as a sterile lyophilized powder (200 mg per vial) free of antibacterial preservatives and was reconstituted with 10 ml of sterile water before infusion.

Ara-CTP and other ribonucleotides which were used as standards were obtained from Sigma. F-ara-ATP, used as a standard, was prepared by intraperitoneal injection of F-ara-AMP into mice bearing P388 leukemia. F-ara-ATP was extracted and purified from the P388 cells as described previously [17].

Cell culture

CCRF-CEM cells, originally cultured from the peripheral blood of a patient with acute lymphoblastic leukemia [18], were maintained in exponential growth in suspension culture in RPMI 1640 medium supplemented with 5% fetal calf serum (Grand Island Biological Corp., Grand Island, NY, U.S.A.) [9].

Patient

Peripheral blood samples were obtained, with informed consent, from a 36-year-old female patient with a diagnosis of acute myelomonocytic leukemia in first relapse. She had received extensive chemotherapy (thioguanine, adriamycin, and conventional dose ara-C; vincristine, prednisone, and conventional dose ara-C; continuous infusion of amsacrine; etoposide and *cis*-diamminedichloroplatinum) before treatment with continuous infusion of high-dose ara-C following a single dose of 3 g/m^2 ara-C given intravenously (i.v.) over 2 h [11]. No chemotherapy, however, had been administered in the three weeks before receiving this salvage treatment. Because of the failure of her disease to respond, she was treated with F-ara-AMP (50 mg/m^2 per day for five days i.v. over 30 min) four weeks later. The patient had adequate hepatic and renal function before treatment.

Nucleotide extraction

Peripheral blood samples (10 ml) were drawn into sterile heparinized tubes and placed in an ice bath. Mononuclear cells were isolated from whole blood by standard step-gradient density centrifugation procedures [14,15]. Cell suspensions of either CCRF-CEM cells or mononuclear cells from whole blood were

counted and the mean cell volume was determined with a Coulter Counter (Coulter Electronics, Hialeah, FL, U.S.A.) equipped with a cell size analyzer (Model C-1000). The cells were washed once in phosphate-buffered saline (PBS, containing per liter: 8.1 g NaCl, 0.22 g KCl, 1.14 g $NaHPO_4$, 0.27 g KH_2PO_4 at pH 7.4) and resuspended in 0.5 ml of water. Nucleotide pools were extracted with an equal volume of 0.8 *M* perchloric acid. After centrifugation at 400 *g* for 5 min the pellet was washed with 0.5 ml of 0.4 *M* perchloric acid and centrifuged at the same speed. The supernatants were combined, neutralized with 10 *M* potassium hydroxide, brought to pH 7 by dropwise addition of 1 *M* potassium hydroxide as determined with universal indicator paper. After cooling in an ice slurry the resulting potassium perchlorate was removed by centrifugation at 400 *g* for 5 min. The supernatants were stored at −20°C until chromatographic analysis [14]. Extraction of replicate samples resulted in less than 10% variation in nucleotide levels. Similarly, greater than 95% of radiolabelled nucleotides added to cell extracts were recovered.

High-performance liquid chromatography (HPLC)

A high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.), consisting of two Model 6000A pumps, a U6K injector, 720 system controller, 730 data module, and Model 481 variable-wavelength detector, was used in these procedures. To select the best running conditions, three different gradient schemes and five different running conditions per gradient scheme were used. For this, an automatic sample injector (Model 710B, Waters Assoc.) attached to the HPLC system was used and each running condition was programmed on a pump set of the Waters 720 system controller. Triplicate samples were injected from one vial for each running condition.

Reference compounds (generally 20 μl) and 50–2000 μl of perchloric acid extracts of human leukemic cells were injected onto a column of Partisil-10 SAX (250 mm × 4.6 mm, 10 μm average particle size, Whatman, Clifton, NJ, U.S.A.). The Model 720 system controller was used to form a gradient from 0.005 *M* $NH_4H_2PO_4$, pH 2.8 (buffer A) and 0.75 *M* $NH_4H_2PO_4$, pH 3.5 (buffer B). The starting percentage of each buffer was different for different running conditions. The scheme that gave the optimal separation started with 70% buffer A and 30% buffer B. A concave gradient was formed following curve 9 on the gradient programmer and reaching the final condition of 100% buffer B in 30 min. All flow-rates were 3 ml/min. This eluted compounds were generally detected by their absorbance at 262 nm. To minimize the baseline drift, the column was purged with 20 ml of 2 *M* $NH_4H_2PO_4$ before injection of 0.1 ml of 0.1 *M* disodium ethylenediaminetetraacetate under 30% buffer B each day before use. The identities of ara-CTP and F-ara-ATP were confirmed by the co-elution of each with authentic compounds, their resistance to periodate oxidation, and the ratio of absorbance (ara-CTP, 280/254 = 3.11 and F-ara-ATP, 262/280 = 3.84).

Peak areas were quantified with a Model 730 data module. Preprogrammed calibration curves were used to convert peak areas to nmol. Dose response curves for ara-CTP and F-ara-ATP were linear between 50 and 800 pmol, the greatest amount analyzed. The largest coefficient of variation for the retention time of

any nucleotide was less than 4% in triplicate determinations. However, differences in retention time became apparent with the extent of use of a column. Little variation was evident in nucleotide retention times between different columns packed with the same lot of anion-exchange resin. The intracellular concentration of nucleotides was expressed as the nmol quantity from a given number of cells of known volume assuming that the nucleotides are uniformly distributed in total cell water.

RESULTS AND DISCUSSION

Initial determinations were directed at achieving an optimal separation among standard samples of the major cellular nucleoside triphosphates and the analogue triphosphates. Three concave gradient curves, Nos. 7, 8 and 9 on the gradient programmer, were used for different elution schemes. Five different starting buffer concentration schemes were run on each curve. The five initial buffer concentrations, with respect to buffer B, were: 25, 30, 35, 40, and 45%, respectively. Each elution scheme reached a final condition of 100% buffer B. The initial concentration of buffer B was plotted against the retention time for each nucleoside triphosphate to facilitate comparison of the separations that were achieved (data not shown). Using curve 7, resolution of peaks was less than desirable as all six compounds eluted within 10 min at each of the different initial conditions using this gradient shape. When the gradient was formed following curve 8, the six nucleotides were eluted in three distinct groups; CTP and ara-CTP, UTP and ATP, followed by F-ara-ATP and GTP. Separation of all nucleotides was optimized when curve 9 was used at initial conditions of 30 or 35% buffer B. At 40 or 45% buffer B initial conditions, ATP, F-ara-ATP, and GTP peaks separated well, but CTP, ara-CTP, and UTP were not well resolved. An elution gradient from initial conditions of 30% buffer B formed by curve 9 was finally selected as the optimal gradient scheme.

The separation of major ribonucleoside 5′-triphosphates from CCRF-CEM cells by this elution scheme is shown in Fig. 1A. The peaks were CTP, UTP, ATP, and GTP. In addition, the endogenous deoxynucleotides, deoxyadenosine triphosphate and deoxyguanosine triphosphate, were separated at elution times of 27.5 and 31.5 min, respectively. Nucleotide concentrations in these cells were as follows: CTP, 490 μM; UTP, 1570 μM; ATP, 4140 μM; GTP, 690 μM. A separate culture of cells was incubated with 300 μM F-ara-A for 4 h, washed, and incubated with 10 μM ara-C for 1 h before cellular nucleotides were extracted and separated. Two peaks not present in control cells were evident (Fig. 1B), between CTP and UTP and between ATP and GTP. These two peaks were identified as ara-CTP and F-ara-ATP, respectively, by UV absorbance characteristics, co-chromatography with authentic ara-CTP and F-ara-ATP, and resistance to periodate oxidation. The cellular concentrations of nucleotides were CTP, 610 μM; ara-CTP, 140 μM; UTP, 1540 μM; ATP, 2770 μM; F-ara-ATP, 310 μM; GTP, 530 μM.

The potential simultaneous use of both the drugs in the clinical setting directed us to evaluate this separation method using patient samples. The ability of the elution scheme to separate ribonucleotides, along with ara-CTP and F-ara-ATP,

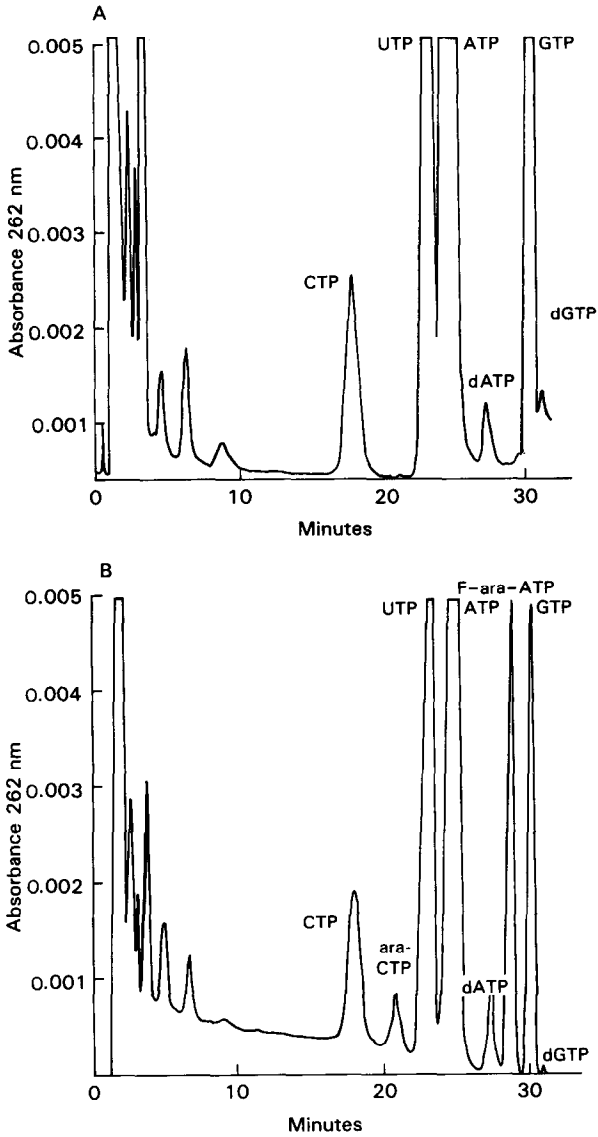

Fig. 1. Separation of nucleoside triphosphates extracted from CCRF-CEM cells. Cells were incubated in drug-free medium (A) or first with 300 μM F-ara-A for 4 h, washed, and then with 10 μM ara-C for 1 h (B). Nucleotides were extracted and separated by HPLC procedures described in Experimental. (A) The equivalent of $1.4 \cdot 10^6$ control cells; (B) the equivalent of $1.2 \cdot 10^6$ drug-treated cells.

was studied in peripheral blood cells obtained from a patient who was first treated with ara-C and later received F-ara-AMP therapy. Perchloric acid-soluble extracts from the equivalent number of cells after each treatment were mixed before HPLC analysis (Fig. 2). Both ara-CTP and F-ara-ATP peaks were observed, in addition to the four peaks of ribonucleoside 5′-triphosphates. The quantities of the nucleotides in the combined cell extract from the equivalent of $2 \cdot 10^7$ cells per treatment were: CTP, 1.4 nmol; ara-CTP, 0.8 nmol; UTP, 12.5 nmol; ATP, 41

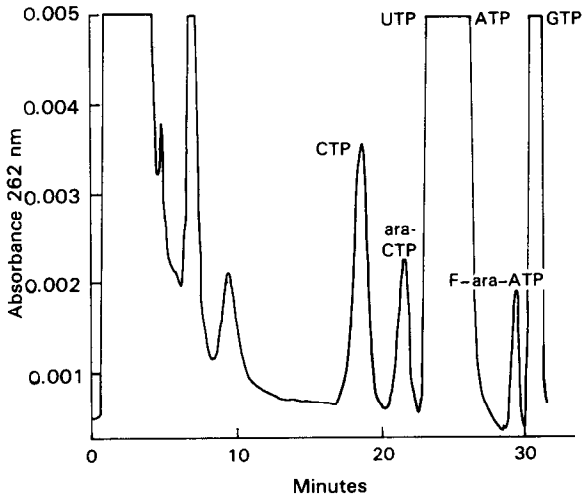

Fig. 2. Separation of nucleoside triphosphates from circulating leukemic blasts after therapy with ara-C and with F-ara-AMP. Leukemic blasts were isolated and nucleotides were extracted 2 h after infusion of ara-C (3 g/m^2 i.v. over 2 h) and on a subsequent treatment protocol, 2 h after administration of F-ara-AMP (50 mg/m^2 i.v. over 30 min). Portions of each extract equivalent to the perchloric acid-soluble material from $5 \cdot 10^7$ cells were combined and 20% of this material was analyzed as described in Experimental.

nmol; F-ara-ATP, 0.1 nmol; GTP, 6 nmol. Because the volumes of the leukemic cells isolated after treatment first with ara-C and then with F-ara-AMP were identical, it is possible to calculate the cellular concentrations of these triphosphates as 210, 120, 1900, 6260, 16, and 880 μM, respectively.

The pharmacokinetic characteristics of ara-CTP in the leukemic cells of patients with relapsed acute leukemia have been correlated with clinical response [11]. Further, preliminary observations indicate that the cellular pharmacokinetics of F-ara-ATP may hold similar importance for response to F-ara-AMP therapy [2]. In vitro studies on the chronic myelogenous leukemia cell line, K562, indicate that ara-CTP accumulation does not exceed more than 100 μM at saturating concentrations of ara-C. However, ara-CTP accumulation is greatly potentiated in cells that contain F-ara-ATP [13]. The higher ara-CTP concentration may result in an increased sensitivity to ara-C. The conduct of future studies is critically dependent upon quantitative, sensitive, rapid, and reproducible chromatographic techniques for detecting the active nucleotides of these pro-drugs. The procedure described demonstrates the separation of both ara-CTP and F-ara-ATP from the major ribonucleoside triphosphates in extracts of human leukemic cells. Thus, the methodology is at hand for the analysis of the active metabolites of these antileukemic drugs during combination drug therapy.

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