Correlation of Cytotoxicity with Total Intracellular Exposure to 9-β-D-Arabinofuranosyladenine 5'-Triphosphate

Donna S. Shewach and William Plunkett

The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030

ABSTRACT

The mechanism by which 9-β-D-arabinofuranosyladenine produces cell death has been studied extensively, but the details remain controversial. The results presented here describe an evaluation of 9-β-D-arabinofuranosyladenine-induced cytotoxicity in terms of the total amount of the active 5'-triphosphate metabolite, 9-β-D-arabinofuranosyladenine 5'-triphosphate (ara-ATP), which accumulated in the cells, and the duration of the exposure expressed in units of ara-ATP µM-hr. It was demonstrated that a strong correlation exists between these parameters which was not affected by the rate of accumulation of ara-ATP. In addition, inhibition of 9-β-D-arabinofuranosyladenine deamination by 2'-deoxycoformycin did not alter the relationship between cell death and total intracellular exposure to ara-ATP. The consistency of this relationship both within and between experiments indicates that the quantitation of the total cellular exposure to ara-ATP is useful in predicting cytotoxicity.

INTRODUCTION

The development and clinical use of ara-A as an antitumor agent has been documented extensively (3, 8, 11, 19, 40) and antiviral agent (23, 24, 32, 38, 39) and has been studied extensively, but the details remain controversial. The results presented here describe an evaluation of 9-β-D-arabinofuranosyladenine-induced cytotoxicity in terms of the total amount of the active 5'-triphosphate metabolite, 9-β-D-arabinofuranosyladenine 5'-triphosphate (ara-ATP), which accumulated in the cells, and the duration of the exposure expressed in units of ara-ATP µM-hr. It was demonstrated that a strong correlation exists between these parameters which was not affected by the rate of accumulation of ara-ATP. In addition, inhibition of 9-β-D-arabinofuranosyladenine deamination by 2'-deoxycoformycin did not alter the relationship between cell death and total intracellular exposure to ara-ATP. The consistency of this relationship both within and between experiments indicates that the quantitation of the total cellular exposure to ara-ATP is useful in predicting cytotoxicity.

The cytotoxic action of ara-A could be characterized by evaluating the relationship between cell death and each one of the previously mentioned biochemical perturbations resulting from cellular exposure to ara-A and determining which perturbation correlates most strongly with cytotoxicity. At the present time, there are no reports in the literature of such comprehensive correlative studies with ara-A. Furthermore, it would be difficult to analyze such an investigation since the cytotoxicity produced by ara-A may involve 2 or more alterations of cellular function. Alternatively, because accumulation of ara-ATP is a prerequisite for cell lethality (4, 37), the cytotoxicity of ara-A may be characterized more accurately in terms of the intracellular concentration of ara-ATP to which the cells are exposed. It may also be expected that the duration of exposure to ara-ATP is important in determining cytotoxicity. This approach would account for the possibility that the toxicity of the drug is derived from more than one biochemical perturbation. The data presented here describe an analysis of the toxicity of ara-A to CHO cells by determining the relationship between the total intracellular exposure to ara-ATP and cell death. In addition, the effect of a potent inhibitor of adenosine deaminase, dCF, on this relationship has been investigated and is reported here.

MATERIALS AND METHODS

Materials. dCF, produced by Parke, Davis and Co. (Detroit, Mich.), and ara-A, a product of Pfanstiehl Laboratories (Waukegan, Ill.), were obtained through the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute. [2-3H]ara-A (18.7 and 19.8 Ci/mmole) was purchased from ICN Pharmaceuticals, Inc. (Irvine, Calif.), and it was more than 98.0% pure as determined by thin-layer chromatography and HPLC. After recrystallization from water or purification by HPLC, the purity was greater than 99.0%. All other chemicals were reagent grade.

Cell Culture Methods. CHO cells were maintained in monolayer cultures in McCoy’s modified Medium 5A and supplemented with either 20% horse serum or 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). All experiments used exponentially growing cells that were detached from the culture flask surface with 0.05% trypsin and resuspended in McCoy’s modified Medium 5A for suspension culture supplemented with 20% horse serum. In the absence of CHO cells, no detectable deamination of ara-A occurred in the culture medium containing horse serum. Cell number was determined using a model ZBI electronic particle counter (Coulter Electronics, Inc., Hialeah, Fla.). The mean cell volume was calculated by multiplying the number of cells extracted per sample by the mean volume of the cells at that time.

The reproducibility of the CHO cells was determined by cloning. Cells were harvested from the drug-containing medium and resuspended in fresh medium. After appropriate dilution, 1 ml of cell suspension was added to 4 ml of warm medium (containing either 10% fetal calf or 10% newborn calf serum) in triplicate 60-mm Petri dishes.
and incubated for 7 days, at which time the macroscopic colonies were counted. Approximately 100 colonies were grown in control dishes; cloning efficiency varied from 80 to 95%. Multiple dilutions were performed with cells obtained from drug-treated cultures to ensure between 50 and 100 colonies/Petri dish.

Quantitation of Intracellular ara-ATP. After the appropriate incubation period with ara-A in the absence or presence of dCF, cells were harvested and extracted with HClO4 as described previously (27). The nucleotides in the neutralized acid-soluble fraction were analyzed by HPLC using a Waters Associates, Inc. (Milford, Mass.), ALC-204 high-pressure liquid chromatograph equipped with 2 Model 6000A pumps, a Model 660 gradient programmer, and a column (250 x 4 mm) containing Partisil-10 SAX anion-exchange resin (Whatman, Inc., Clifton, N. J.). Samples of 0.5 to 8.0 x 10⁶ cell equivalents were injected onto the column by means of the U6K-LC injection system. Nucleotides were eluted from the column initially with 50% 0.005 M NH₄H₂PO₄, pH 2.8, and 50% 0.75 M NH₄H₂PO₄, pH 3.7. The buffer concentration was increased to 100% 0.75 M NH₄H₂PO₄, pH 3.7, using a concave gradient (Curve 8 on the gradient programmer) over a period of 30 min. Eluted compounds were detected by their absorbance at 254 nm by the Model 440 detector. The [³H]ara-ATP eluate was fractionated at 0.5-min intervals and collected into scintillation vials containing 0.3 ml H₂O and 11 ml Aquasol (New England Nuclear, Boston, Mass.). The radioactivity in each vial was determined by liquid scintillation counting. Counting efficiency for ¹⁴C was 35%. The intracellular ara-ATP concentration was calculated by dividing the pmol ara-ATP in a cell extract, measured by HPLC, by the total volume of the cells analyzed as determined prior to extraction with HClO4.

Calculation of Total Intracellular Exposure to ara-ATP. Cellular exposure to ara-ATP was defined as the product of the intracellular concentration of ara-ATP (μM) and the duration of exposure (hr) to that concentration, expressed as ara-ATP μM-hr. The total cellular exposure to ara-ATP was defined as the sum of these products for all possible concentrations of ara-ATP after the addition of ara-A to the culture. Graphically (Chart 1), this quantity is equivalent to the area under the curve describing the change in the intracellular ara-ATP concentration with time, which can be calculated as the sum of two areas: (a) the area under the ara-ATP accumulation curve from the time of ara-A addition until drug washout (Chart 1, area A); and (b) the area under the curve describing the decay of ara-ATP after drug washout (Chart 1, area B). The area under the accumulation curve (AUCₐ) was calculated by weighing triplicate cutouts from photocopies of the actual graphic representation of that area and dividing that weight by the graph paper weight of a known amount of ara-ATP μM-hr. The intracellular concentration of ara-ATP was determined at 1-, 2-, or 3-hr intervals. When the AUCₐ was calculated based on measurements of intracellular ara-ATP levels at 2- or 3-hr intervals and compared to the AUCₐ derived from hourly determinations of the analog triphosphate concentration, the values did not differ significantly.

The exposure to ara-ATP after drug washout was quantitated from the area under the curve describing the decay of ara-ATP in the absence of exogenous ara-A. We have demonstrated previously (35) that the intracellular concentration of ara-ATP in CHO cells grown under these conditions declines exponentially in ara-A-free medium, with a slope of -0.41 hr⁻¹. Thus, the concentration of ara-ATP at any time after washout of ara-A can be described by the equation

\[ C(t) = C(0)e^{-0.41t} \]

where \( t \) is the time in hr after the cells were resuspended in ara-A-free medium and \( C \) is the concentration of ara-ATP at time \( t \). The area under the ara-ATP decay curve (AUCₖ) is then given by the definite integral of the preceding equation:

\[ AUCₖ = \int_0^∞ C(0)e^{-0.41t}dt = -\frac{C(0)e^{-0.41t}}{0.41} \bigg|_0^∞ \]

When the AUCₖ was estimated using the preceding equation during a specific period of time and compared to the AUCₐ determined from actual measurements of the cellular ara-ATP concentration over the same time period, the 2 values differed by less than 8%. Thus, the total intracellular exposure to ara-ATP is AUCₐ + AUCₖ, in units of ara-ATP μM-hr.

RESULTS

Since dCF enhanced both the accumulation of ara-ATP from ara-A and the cytotoxicity of the nucleoside analog (35, 36), it was also of interest to determine the effect of dCF on the relationship between ara-ATP μM-hr and cell viability. A preliminary experiment was designed to determine if there was a correlation between increasing total exposure to ara-ATP and decreasing cell survival. Chart 2 illustrates the results of an experiment in which CHO cells were incubated with various concentrations of ara-A alone or in the presence of 10 μM dCF for 3 hr. The deaminase inhibitor alone was not toxic to this cell line at the concentration used, and it inhibited the cellular deamination of ara-A completely for more than 30 hr (35). Cell viability decreased exponentially with increasing total exposure to ara-ATP (Chart 2). Although the presence of dCF during the drug incubation period enhanced the accumulation of ara-ATP from ara-A nearly 2-fold, the inclusion of the deaminase inhibitor did not alter the relationship between ara-ATP μM-hr and cytotoxicity.

In a separate experiment in which cells were continuously incubated with 50 μM ara-A in the presence of 10 μM dCF (not shown), viability and total exposure to ara-ATP were determined periodically. After 6.25 hr, the cells incubated in the presence of dCF had been exposed to 1600 μM-hr ara-ATP, whereas a 17-hr incubation period was necessary to achieve a similar exposure in the absence of dCF. The consequent reduction in cell viability was approximately 45% in each culture, differing from each other by less than 2%. Thus, although the intracellular exposure to ara-ATP differed by more than 10 hr, there was no substantial difference in the effect of this treatment on cell viability.

The data in Chart 2 indicate that cell death was quantitatively related to increasing total exposure to ara-ATP and that the same relationship exists between these parameters when ara-ATP was accumulated from ara-A alone or in the presence of dCF. Consequently, further studies of this relationship were
carried out using CHO cells, incubated with ara-A in the presence of dCF to allow greater total exposure to ara-ATP. The results presented in Chart 2 were extended in an experiment designed to determine the range of ara-ATP μM-hr that would reduce viability by from 0 to 99%. During incubation of CHO cells with 20, 50, 100, or 200 μM ara-A in the presence of 10 μM dCF for 10 hr, the decrease in cell viability was both time and dose dependent, as illustrated in Chart 3. Fewer than 20% of the cells exposed to 20 μM ara-A were killed, whereas the incubation with 200 μM ara-A was toxic to more than 95% of the cells after 10 hr. In this experiment, the intracellular concentration of ara-ATP was also measured when cell viability was determined so that the total exposure to ara-ATP could be calculated and evaluated with respect to the consequent cytotoxicity of the incubation. As illustrated in Chart 4, there was a strong correlation between increasing ara-ATP μM-hr and decreasing cell survival (r = -0.94). A statistical analysis indicated that there was little probability that no association exists between the 2 variables (p < 0.001 according to Student’s t test for the significance of the correlation coefficient).

The reduction in the percentage of surviving cells from 80 to 2% of the initial value corresponded to a 25-fold increase in ara-ATP μM-hr. Cell viability decreased by less than 20% when the total intracellular exposure to ara-ATP increased from 0 to 600 μM-hr. It was determined in a separate experiment that a cellular exposure to at least 400 μM-hr ara-ATP was necessary to effect a statistically significant decline in CHO cell viability. In contrast, fewer than 10% of the cells survived exposure to 6000 μM-hr ara-ATP. This experiment also demonstrates that the relationship between ara-ATP μM-hr and cytotoxicity was the same for each concentration of ara-A to which the cells were exposed.

Although the cultures incubated with 50, 100, or 200 μM ara-A were exposed to approximately 900 μM-hr ara-ATP after 4, 2, or 1 hr of incubation, respectively (Chart 4), there was no significant difference in the subsequent viability observed in each culture (~55%). Exposure to approximately 2000 μM-hr ara-ATP was achieved after 10, 4, or 2 hr of incubation with 50, 100, or 200 μM ara-A, respectively, and the percentage of viable cells in the cultures (~30%) differed from each other by less than 4%. This analysis indicates that, following incubation of CHO cells with ara-A, cell survival correlated with the cellular concentration of ara-ATP and the duration of that exposure, regardless of the initial exogenous ara-A concentration. Moreover, evaluating cytotoxicity on the basis of total cellular exposure to ara-ATP allows a direct comparison of the effect of incubation with various concentrations of ara-A for different periods of time.

It has been reported that the Ki for the inhibition of DNA polymerase by ara-ATP is approximately 1 μM (10, 12), which suggests that a cellular exposure to less than 1 μM ara-ATP does not contribute to the observed cytotoxicity. This possibility was taken into consideration by refining the quantitation of ara-ATP μM-hr so that the portion of the ara-ATP accumulation and decay curves representing an intracellular concentration of less than 1 μM was excluded from the calculation. However, this area was relatively small (less than 100 μM-hr), and its deletion did not alter the relationship between cytotoxicity and...
ara-ATP μM-hr. In addition, subtracting the experimentally determined noncytotoxic exposure to ara-ATP of 400 μM-hr from ara-ATP μM-hr. In addition, subtracting the experimentally determined noncytotoxic exposure to ara-ATP of 400 μM-hr from ara-ATP instead of to time of incubation with ara-ATP. In the experiments presented in Chart 5, both the concentration of ara-A and the duration of drug incubation were the same in each study. Thus, at each time point, the total exposure to ara-A was the same in both cultures, but the corresponding cytotoxicities were significantly different. This variation in cell survival possibly could be explained by nonspecific differences in cell metabolism between the experiments. However, the relationship between total exposure to ara-ATP and cytotoxicity was the same in both studies, indicating that ara-ATP is indeed the cytotoxic metabolite of ara-A.

The consistency of the relationship between intracellular exposure to ara-ATP and cytotoxicity suggests that accumulation of ara-ATP μM-hr is an important indicator of ara-A-induced cell lethality. In view of the rapid metabolism of ara-A in CHO cells (35) and the numerous enzymatic reactions affected by ara-ATP (8, 9), it is possible that cell death results from a combination of 2 or more biochemical perturbations produced by ara-A. Thus, the evaluation of cytotoxicity on the basis of total intracellular exposure to ara-ATP is useful in that it accounts for any number of events involving ara-ATP that may contribute to cell lethality.

The presence of dCF in the cell cultures did not alter the relationship between ara-ATP μM-hr and cytotoxicity (Chart 2). Previous studies have indicated that, although dCF facilitates the accumulation of higher cellular concentrations of ara-ATP, the cytotoxicity of ara-C. However, such studies have been carried out with ara-C, indicating that inhibition of DNA synthesis (13, 14), chromatid breakage (16), and incorporation of 1-β-δ-arabinofuranosylcytosine 5'-monophosphate into DNA (17) are important factors in determining cell death after ara-C exposure, yet no single event that is most important in the cytotoxic action of this drug has been identified. Moreover, none of these studies attempted to correlate more than one biochemical event with the cytotoxicity of ara-C.

The available evidence strongly indicates that ara-ATP is the active metabolite of ara-A that inhibits several key cellular metabolic functions, and hence the present study evaluated the cytotoxicity of ara-A in terms of the total intracellular exposure to ara-ATP. This investigation attempted to characterize the relationship between cytotoxicity and the cellular exposure to the active metabolite in quantitative terms while not dealing with the likelihood that more than one ara-ATP-induced alteration in cellular function contributes to cell death. We have taken advantage of recent refinements in HPLC technology which enable the quantitation of intracellular concentrations of ara-ATP and have expressed our findings in terms of the classical pharmacokinetic parameter of drug concentration times the duration of exposure (ara-ATP μM-hr). The results of this investigation demonstrated that the decrease in cellular viability from 0 to greater than 97% was exponentially related to the increasing total intracellular exposure to ara-ATP (Charts 2, 4, and 5B). It was also demonstrated that the rate of accumulation of ara-ATP did not alter the strong correlation between increasing ara-ATP μM-hr and decreasing viability; i.e., when the total exposure to ara-ATP was the same, the subsequent cytotoxicity was also similar whether the ara-ATP accumulated slowly or rapidly in the cells. In addition, the relationship describing exposure to ara-ATP μM-hr and cytotoxicity was similar between experiments (Chart 5B), so that the effect of ara-A on cell viability, determined in separate studies, could be compared.

In the experiments presented in Chart 5, both the concentration of ara-A and the duration of drug incubation were the same in each study. Thus, at each time point, the total exposure to ara-A was the same in both cultures, but the corresponding cytotoxicities were significantly different. This variation in cell survival possibly could be explained by nonspecific differences in cell metabolism between the experiments. However, the relationship between total exposure to ara-ATP and cytotoxicity was the same in both investigations, indicating that ara-ATP is indeed the cytotoxic metabolite of ara-A.

The consistency of the relationship between intracellular exposure to ara-ATP and cytotoxicity suggests that accumulation of ara-ATP μM-hr is an important indicator of ara-A-induced cell lethality. In view of the rapid metabolism of ara-A in CHO cells (35) and the numerous enzymatic reactions affected by ara-ATP (8, 9), it is possible that cell death results from a combination of 2 or more biochemical perturbations produced by ara-A. Thus, the evaluation of cytotoxicity on the basis of total intracellular exposure to ara-ATP is useful in that it accounts for any number of events involving ara-ATP that may contribute to cell lethality.

The presence of dCF in the cell cultures did not alter the relationship between ara-ATP μM-hr and cytotoxicity (Chart 2). Previous studies have indicated that, although dCF facilitates the accumulation of higher cellular concentrations of ara-ATP,
it does not affect either the net intracellular degradation of ara-ATP or the salvage phosphorylation of ara-A metabolites in CHO cells (35). Thus, dCF appears to enhance the cytotoxic effects of ara-A by preserving higher concentrations of the nucleoside analog in the medium (35, 36), which penetrates the cells and is phosphorylated to ara-ATP in a dose-dependent manner (Chart 4). The greater cytotoxicity of ara-A observed in the presence of dCF may thus be understood and predicted in terms of an increased total intracellular exposure to ara-ATP. Finally, the intracellular concentration of ara-ATP can be quantitated in the leukemic cells in peripheral blood and bone marrow from patients with leukemia receiving ara-A therapy (26). It will be important to quantitate the total intracellular exposure of leukemic cells to ara-ATP and relate this parameter to the clinical response of patients. If a correlation exists between reduction in the leukemic cell burden and the amount of ara-ATP to which these cells are exposed, it may be possible to predict treatment efficacy and patient response by monitoring the cellular ara-ATP μM-hr exposure during ara-A administration. The drug regimen might then be altered so that the cellular ara-ATP exposure would be sufficient to produce clinical improvement in patients with leukemia. In addition, this approach may be useful in evaluating the therapeutic effectiveness of protocols using other antimetabolites with similar mechanisms of action, such as ara-C and arabinosyl-2-fluoroadenine. Such studies are being carried out in this laboratory at the present time (1, 2, 26, 30).

REFERENCES


Correlation of Cytotoxicity with Total Intracellular Exposure to 9- β-d-Arabinofuranosyladenine 5′-Triphosphate

Donna S. Shewach and William Plunkett


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/42/9/3637

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/42/9/3637. Click on “Request Permissions” which will take you to the Copyright Clearance Center's (CCC) Rightslink site.