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Effects of 9-β-D-arabinofuranosyladenine and 1-β-D-arabinofuranosylcytosine on levels of deoxyribonucleic acid precursors in uninfected and herpes simplex virus-infected cells

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Two aranucleosides, 9-β-D-arabinofuranosyladenine (araA, vidarabine) and 1-β-D-arabinofuranosylcytosine (araC, cytarabine), are used currently in humans for treatment of herpetic disorders and certain lymphocytic leukemias, respectively. Both of these compounds are phosphorylated to toxic nucleotides which exert their biological activities through inhibition of DNA polymerase and/or incorporation into DNA (reviewed in Refs. 1 and 2). In either event, the aranucleoside 5′-triphosphate, araATP or araCTP, competes with a natural DNA precursor, dATP or dCTP, respectively. In addition, it was reported by Moore and Cohen [3], and subsequently by Chang and Cheng [4], that araATP inhibites mammalian ribonucleoside diphosphate reductase (rNDP reductase). From this is was suggested that araA may "self-potentiate" its biological activity by decreasing production of the competing deoxyribonucleotide, dATP [4]. AraC would not be expected to display such "self-potentiation" because araCTP is a much weaker inhibitor of rNDP reductase, and no inhibition of this enzyme is achieved at concentrations of araCTP that are attained *in vivo* [3, 4].

It has not been determined whether the effects of araATP upon rNDP reductase are biologically significant because DNA polymerases are much more sensitive to inhibition by araATP than rNDP reductase. Generally, the K_i for inhibition of mammalian DNA polymerases by araATP is ~1 μM [5]. By contrast, Moore and Cohen reported that the inhibition by araATP of rNDP reductase from rat tumor cells required much higher concentrations [3]. Chang and Cheng reported that the K_i values for inhibition of the rNDP reductase from human T-lymphoblast tumor cells was 4–15 μM [4]. However, the intracellular concentration of araATP-treated cells has been shown to exceed 200 μM [6], and under these conditions both DNA polymerase and rNDP reductase should be inhibited. The following experiments were performed to evaluate effects of araA upon levels of DNA precursors and to determine whether such "self-potentiation" might occur and whether it can be detected under conditions where DNA polymerase is also inhibited.

Materials and methods

HeLa F cells and the Miyama strain of herpes simplex virus (HSV) type 1 were grown and maintained as previously described [7] in Joklik-modified Minimum Essential Medium supplemented with 10% horse serum (which contains no detectable adenosine deaminase). All experiments were performed in medium supplemented with 10% dialyzed horse serum in order to minimize synthesis of DNA precursors from exogenous deoxyribonucleosides via salvage pathways. All media and sera were obtained from the Grand Island Biological Co., Grand Island, NY. Mycoplasma assays were routinely performed, and stocks of cells and virus were consistently negative [7, 8].

AraA, araC and non-radioactive deoxyribonucleotides were obtained from the Sigma Chemical Co., St. Louis, MO. *Erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) was provided by H. Schaeffer and G.B. Elion, Wellcome Research Laboratories, Research Triangle Park, NC. Tetrahydrouridine (THU) was provided by G. Neil, The Upjohn Co., Kalamazoo, MI. Radioactive materials, [*methyl*-^{3}H]-dTTP (18.7 Ci/mmole), [5-^{3}H]dCTP (24.8 Ci/mmole), [8-^{3}H]dATP (23.5 Ci/mmole) and [8-^{3}H]dGTP (11.8 Ci/mmole), were all purchased from The New England Nuclear Corp., Boston, MA. *Escherichia coli* DNA polymerase I was obtained from Bethesda Research Laboratories, Inc., Gaithersburg, MD. All other materials for quantitation of dNTPs were as previously described [9].

Cells to be treated with aranucleosides were grown in 100 mm Petri plates and used at a subconfluent density. Medium was removed, cells were washed twice with 5 ml of phosphate-buffered saline [10], and then fresh medium was added containing araA or araC at indicated concentrations. The inhibitor of adenosine deaminase, EHNA, was included (10^{-6} M) in all experiments with araA to prevent its deamination. Similarly, the inhibitor of cytidine deaminase, tetrahydrouridine (THU), was included (10^{-5} M) in experiments with araC to prevent its deamination. For experiments with HSV-infected cells, subconfluent HeLa cells were infected with HSV at an input multiplicity of 10 plaque-forming units (PFU)/cell, and virus was allowed to adsorb for 1 hr as previously described [7]. Following the adsorption period, medium was removed and infected cells were washed and treated with araA + EHNA or araC + THU as described above.

Extracts from uninfected cells were prepared for analysis of deoxyribonucleoside 5′-triphosphates (dNTPs) by the two-step procedure which was described previously [9]. This involved an initial extraction with 60% methanol (−20°) and then, after lyophilization, re-extraction with 0.5 N perchoric acid (ice cold). However, these methods proved unsuitable for analysis of dNTPs from HSV-infected cells. During extraction of HSV-infected cells with 60% methanol, dTTP and dCTP were degraded to their respective monophosphates. This did not occur during extraction of uninfected cells and is presumably due to the deoxypyrimidine triphosphatase reported by Wohlrab and Francke [11]. This problem was alleviated when the order of the two-step extraction procedure was reversed so that cells were extracted first with 0.5 N perchloric acid and then, after neutralization and lyophilization as previously described [9], re-extracted with 60% methanol. Otherwise the details of extraction procedures and for analysis of dNTPs with the enzymatic assay are as previously described [9]. With uninfected cells results of dNTP assays were not affected by the order of the two extractions. All results represent data from at least two separate experiments. Because intracellular levels of dNTPs at the beginning of treatment were somewhat variable, and because dNTP levels change following HSV infection [12, 13], levels of dNTPs in untreated controls were determined in parallel, and values are reported relative to those obtained in these parallel controls.

Results and discussion

To use the enzymatic assay for analysis of dNTPs in extracts from cells treated with aranucleosides, it was first

necessary to ascertain that aranucleotides do not interfere with the assay. It has been reported that araATP and araCTP do not inhibit *E. coli* DNA polymerase I [5, 14], which is used in this assay. In agreement with this, araATP and araCTP (40 μM, which is higher than levels of these aranucleotides in extracts prepared below) do not inhibit DNA polymerase or severe as substrates under conditions used for analysis of dNTPs. Moreover, these aranucleotides did not affect quantitation of dNTPs in the range tested (0.01 to 0.4 μM, data not shown).

Effects of araA upon levels of dNTPs in HeLa cells are shown in Fig. 1. Figure 1A shows results of treatment of these cells with 10^{-4} M araA and 10^{-6} M EHNA, which inhibits DNA synthesis by >99%. During the first few hours after this treatment, levels of all four dNTPs were reduced. Maximal depression of dNTP pools occurred by 6 hr, at which time levels of all four dNTPs were less than 40% of control levels. Interestingly, the dATP level was decreased most dramatically by this treatment; it fell to 12% of the control level by 6 hr. This decrease in dATP concentration is supportive of the "self-potentiation" concept through an effect of araATP upon rNDP reductase, as previously proposed [4]. However, we cannot yet conclude that this decrease in dATP is solely attributable to an effect upon this enzyme, since other enzymes of DNA precursor metabolism might be affected by araA. None of these effects are due to the inhibitor of adenosine deaminase because EHNA alone (10^{-6} M for 24 hr) had no effect on dNTP levels in these cells (data not shown).

At later times after treatment with araA, levels of all four dNTPs increased and actually accumulated to levels several-fold higher than in untreated controls (Fig. 1A). Since DNA synthesis is completely blocked (>99%) under these conditions, any dNTPs formed would not be used. Thus, although there was an early decline in dNTP levels, there must still be a slow provision of them, at least at the later times. Whether this is due to incomplete inhibition of rNDP reductase, degradation of DNA, or salvage of deoxyribonucleosides has not been determined (although the use of dialyzed sera should minimize contributions of the latter).

At a lower concentration of araA (10^{-5} M + 10^{-6} M EHNA), there was much less effect on dNTP levels (Fig. 1B). The dATP pool was not decreased as much or for as long a duration. At 3 hr this treatment decreased dATP to 68% of the control level, but by 6 hr dATP was higher than controls and by 9 hr it was more than three times the control level. Moreover, dTTP levels were not decreased at all by this treatment (Fig. 1B). Thus, this lower concentration of araA still inhibited DNA synthesis (>90%) but had little or no effect on DNA precursor levels.

In contrast to this apparent "self-potentiation" of araA in uninfected HeLa cells, such an effect was not seen in HSV-infected cells. As shown in Fig. 2, treatment of HSV-infected cells with 10^{-4} M araA (+ 10^{-6} M EHNA) caused expansion of all four dNTP pools. Interestingly, dATP was increased much more than the other three dNTPs; it reached a level ten to fifteen times that of the control by 9 hr after infection. Thus, rather than self-potentiation, araA treatment of HSV-infected cells actually leads to an increased concentration of the competing nucleotide, dATP. These data suggest that an inhibition of rNDP reductase by araATP does not contribute to the antiviral activity of araA. Accordingly, the antiviral activity of araA may be increased if the means to block this dATP increase can be devised. Recently, it was reported that HSV induces a rNDP reductase that is not inhibited by araATP [15]. The results represented here are consistent with that report. Treatment of HSV-infected cell with a lower concentration of araA (10^{-5} M + 10^{-6} M EHNA) also resulted in expansion of all four dNTP pools, but the expansions were less dramatic: none of the dNTPs expanded more that 2-fold relative to untreated, HSV-infected cells (data not shown).

In contrast to araATP, physiologically significant concentrations of araCTP do not inhibit rNDP reductase [3, 4]. Thus, if the decrease in dNTP levels following treatment with araA is due to inhibition of the reductase by araATP, araC is not expected to exert such an effect. Table 1 shows the effects of araC (10^{-4} M + 10^{-5} M THU) upon dNTP levels in HeLa cells. This treatment inhibits DNA synthesis by 99%. Levels of all four dNTPs were increased by this treatment with araC, in agreement with results previously reported by others [16, 17]. THU alone (10^{-5} M for 24 hr) had no effect on dNTP levels in these cells (data not shown). Thus, araC does not display the apparent "self-potentiation" observed with araA.

In summary, the ability of araA but not araC to decrease dNTP pools in HeLa cells correlated with the ability of araATP, but not of araCTP, to inhibit ribonucleotide reductase. These data provide the first *in vivo* evidence in

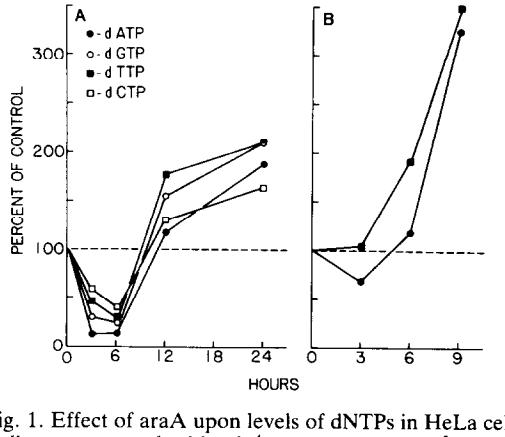

Fig. 1. Effect of araA upon levels of dNTPs in HeLa cells. Cells were treated with 10^{-4} araA (A), or 10^{-5} M araA (B), each in the presence of the inhibitor of adenosine deaminase, EHNA (10^{-6} M). At indicated times cell extracts were prepared and dNTP determinations were performed as described in Materials and Methods. Values of dNTPs in untreated controls (in pmoles/10^6 cells) were: dATP = 30 ± 3, dTTP = 47 ± 3, dGTP = 14 ± 2, and dCTP = 49 ± 6.

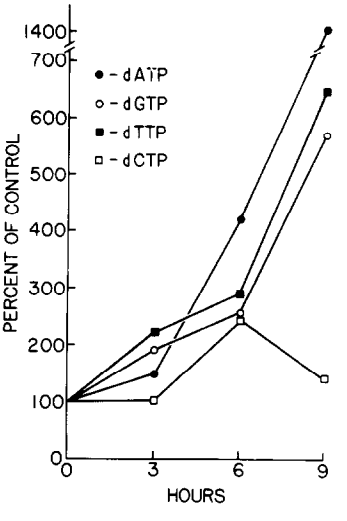

Fig. 2. Effect of araA upon dNTP levels in HSV-infected HeLa cells. Cells were infected with HSV, treated with 10^{-4} M araA + 10^{-6} M EHNA, and prepared for dNTP analysis as described in Materials and Methods.

Table 1. Effect of araC on dNTP pools

Length of treatment* (hr)	dNTP levels (% control) dATP	dTTP	dGTP	dCTP
3	144	113	108	135
6	143	127	185	157
12	276	204		

* HeLa Cells were treated with 10^{-4} M araC + 10^{-5} M THU and, at indicated times, extracts were prepared and dNTP determinations performed as described in Materials and Methods.

support of the "self-potentiation" concept whereby araA activity is increased by a decrease in dATP, which competes with the active form of the drug, araATP. This "self-potentiation" was not observed in HeLa cells that were infected with herpes simplex virus.

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Department of Biochemistry and Pharmacology
Tufts University Schools of Veterinary Medicine and Medicine
Boston, MA 02111, U.S.A.

THOMAS W. NORTH

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Interactions of combretastatin, a new plant-derived antimitotic agent, with tubulin

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A cytotoxic principle extracted from the South African tree *Combretum caffrum* and named combretastatin (NSC-348103) has been purified by Pettit *et al.* [1]. These workers determined the structure of the drug, shown in Fig. 1. Combretastatin has significant activity in reversing the differentiation of AC glioma cells into astrocytes, a model which has demonstrated good selectivity for antimitotic agents [2]. Moreover, the structure of the drug is reminiscent not only of colchicine, but especially of the active colchicine analog 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (MTPT, also shown in Fig. 1) synthesized by Fitzgerald [3]. Both its ability to reverse astrocyte differentiation and its structure prompted us to examine combretastatin for antimitotic and antitubulin activity.

The preparation of purified tubulin and heat-treated microtubule-associated proteins from calf brain and the sources of all other materials have been described elsewhere [4, 5]. The binding of [^{3}H]colchicine to tubulin and tubulin-dependent GTP hydrolysis were measured as described previously [6, 7]. Tubulin polymerization was followed turbidimetrically [8] in a Gilford model 250 recording spectrophotometer. Temperatures were maintained with a Gilford "Thermoset" electronic temperature controller. Baselines were established with the reaction mixtures at 0°. At zero time the instrument was set at 37°, and the point at which the instrument readout reached 37° is indicated in the appropriate figures by an arrow on the abscissa (the time axis). Drug cytotoxicity studies and slide