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ON THE CHARACTERISTICS OF INHIBITION OF DEOXYRIBONUCLEIC ACID SYNTHESIS BY 2,2′-ANHYDRO-1-β-D-ARABINOFURANOSYLCYTOSINE*

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Abstract—Anhydro-ara-C, a cyclic anhydride of cytosine arabinoside, is initially a very weak inhibitor of DNA synthesis in L1210 murine leukemia cells. With increasing incubation times, such inhibition markedly increases. No evidence could be found to indicate uptake of unaltered anhydro-ara-C, although the drug appears to be a weak inhibitor of the uptake of cytosine arabinoside. Anhydro-ara-C is, however, gradually hydrolyzed, apparently by a non enzymatic process, to form cytosine arabinoside in the extracellular space. Rapid uptake of the latter compound then results in progressive inhibition of DNA synthesis.

THE ANTINEOPLASTIC drug, 1-β-D-arabinofuranosylcytosine (cytosine arabinoside, ara-C), has been useful in the treatment of malignant disease, especially acute myelogenous leukemias.[1,2] The drug is converted to ara-CTP, the nucleoside-5′-triphosphate, by intracellular enzymes responsible for analogous conversion of deoxycytidine to dCTP; ara-CTP is a competitive inhibitor of mammalian DNA polymerase.[3] One factor which limits the usefulness of ara-C is rapid deamination of the drug to an inactive derivative, ara-U.[4,5] A variety of procedures have been proposed to alleviate this deamination problem and provide the clinician with a a long-acting form of ara-C. A novel approach involves the use of the cyclic anhydride of ara-C: 2,2′-anhydro-1-β-D-arabinofuranosylcytosine (cyclocytidine, anhydro-ara-C). The latter is resistant to the deaminase,[6] and is as effective as ara-C in treatment of murine leukemias.[7] Moreover, infrequent administration of anhydro-ara-C does not reduce its antitumor action,[7] suggesting that the anhydride might be a repository form of ara-C. Anhydro-ara-C is inactive against murine leukemias resistant to ara-C,[8] and is readily hydrolyzed, apparently via a nonenzymatic process, to ara-C.[9] We have compared transport of ara-C and anhydro-ara-C in a murine cell line unable to phosphorylate or deaminate the former compound.[10] Extent of inhibition of DNA synthesis by ara-C and anhydro-ara-C were compared, along with possible enzymatic factors involved in the conversion of anhydro-ara-C to ara-C.

MATERIALS AND METHODS

Chemicals. Thymidine and deoxycytidine, both labeled with ^{14}C, were purchased from New England Nuclear Corp. Ara-C, labeled with tritium (3 Ci/m-mole) in the

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pyrimidine ring, was provided by the National Cancer Institute, and was purified as described in Ref. 11. Anhydro-ara-C, labeled with ^{14}C in the 2-position of the ring, was supplied by the Isotope Synthesis Laboratory of the Stanford Research Institute (0·02 mCi/m-mole). Purity of the anhydride was checked using thin-layer chromatography and a solvent composed of 100 parts of 100 mM phosphate buffer, pH 6·8, 60 parts of saturated ammonium sulfate solution and 2 parts of *n*-propanol.[12] In this system, anhydro-ara-C migrates more slowly than ara-C. Nonlabeled anhydro-ara-C and ara-C were provided by the Drug Development Branch, National Cancer Institute. All solutions of these compounds were preparaed just before use.

Tumor cells. The L1210/CA cell line, which lacks capacity to phosphorylate or deaminate ara-C,[10] was maintained in CDF_1 mice. Cells were collected from animals inoculated 6 days previously with 10^6 tumor cells. The collected cells were freed from contaminating erythrocytes, if necessary, by osmotic shock, suspended in a medium (TES-E) previously described.[13] This mixture, containing 71 mM NaCl, 20 mM KCl, 1·5 mM $MgCl_2$, 1·3 mM $CaCl_2$, 1 mM NaH_2PO_4 and 75 mM *N*-Tris(hydroxymethyl)-2-aminoethane sulfonate (TES) at pH 7·3, approximates the ionic composition of ascitic fluid.[14]

L1210 cells were maintained in culture using MEM–Eagle's medium supplemented with 10% fetal calf serum. Cells were collected, suspended in fresh medium at a concentration of 5–7 × 10^6 cells/ml, and used for studies of drug effects on DNA synthesis.

Transport studies. The procedures are outlined in Ref. 10. A suspension of L1210/CA cells (7 × 10^6/ml) in TES-E medium was incubated with radioactive substrates and other nonradioactive additions, as specified for each experiment. Incubations were terminated by collection of cells by centrifugation (30 sec in a microcentrifuge). The pellets were blotted dry and radioactivity was measured by liquid scintillation counting of solubilized cell pellets. A correction[10] was made for contamination of cell pellets by extracellular radioactivity.

Some incubations involving labeled CdR or ara-C were terminated by addition of 0·2 mM persantin, a drug which instantly abolishes further movement of these compounds across the cell membrane in either direction.[15] The cells were then washed with 0·9% NaCl containing 0·2 mM persantin without loss of intracellular pools of CdR or ara-C.

In some experiments, a sample of the extracellular fluid was retained after incubations and subjected to thin-layer chromatographic analysis to determine the extent of hydrolysis of anhydro-ara-C. No significant hydrolysis (< 1 per cent) was found during these procedures.

Studies on DNA synthesis. Incorporation of labeled thymidine into DNA was measured as outlined in Ref. 16. A suspension of L1210 cells was warmed to 37°, then mixed with a specified level of ara-C or anhydro-ara-C. At intervals thereafter, 1-ml portions of this suspenison were removed and 0·05 μCi of labeled thymidine (final concentration, 1 μM) was added. The incubation was continued for 5 min longer; the cells were then collected by centrifugation, washed several times with 0·3 M $HClO_4$ and then with ethanol. The acid-insoluble material was solubilized and radioactivity measured by liquid scintillation counting.

In other studies, cell suspensions were mixed with solutions of anhydro-ara-C which had previously been held at 37° for specified times. At intervals thereafter, cell

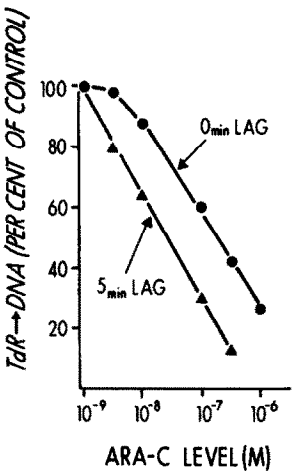

FIG. 1. Inhibition of incorporation of 10-min pulses of labeled thymidine into DNA by ara-C. The ara-C level is shown. Zero-min lag, ara-C and TdR added simultaneously; 5-min lag, ara-C added 5 min before TdR. Control values represent 3500 ± 500 cpm/mg cells of incorporation of label.

samples were removed, incubated with labeled thymidine and otherwise treated as outlined above.

RESULTS

Transport studies. We have shown[10,15] that CdR or ara-C, at a concentration of 0·05 mM, rapidly equilibrates with the intracellular space of L1210 cells, regardless of the temperature of incubation (range, 0–37°). Addition of nonradioactive nucleosides inhibited uptake or loss of ara-C and CdR. In some cases, both uptake and loss were inhibited. In the present study, addition of 1 mM nonlabeled ara-C inhibited uptake of labeled CdR or ara-C by about 10 per cent. A 20-fold higher level of nonlabeled anhydro-ara-C was required to produce this level of inhibition. Addition of nonlabeled anhydro-ara-C to cells preloaded with radioactive ara-C or CdR did not slow the loss of label from the cells. These data suggest that anhydro-ara-C

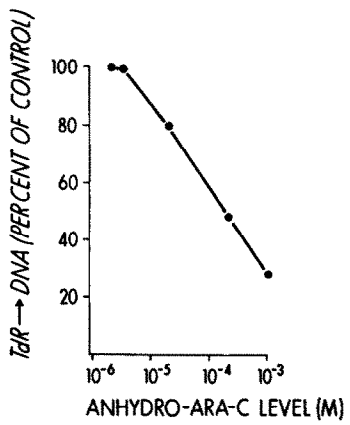

FIG. 2. Inhibition of incorporation of 10-min pulses of labeled thymidine into DNA by anhydro-ara-C. The anhydro-ara-C level is shown. Drug and TdR were added simultaneously.

TABLE 1. INHIBITORY EFFECT OF ANHYDRO-ARA-C SOLUTIONS ON DNA SYNTHESIS*

Prior treatment of anhydro-ara-C	Inhibition (%)
None†	20
30 min, 37°‡	60
30 min, 37° + cells§	60

* Cell suspensions were incubated in MEM–Eagle's medium, pH 7·3, with 50 μM anhydro-ara-C together with labeled thymidine for 10 min at 37°. Incorporation of label into DNA was measured and compared with control (no drug) values. Controls represent 3500 ± 500 cpm/mg wet cells of activity.

† Freshly prepared solution of anhydro-ara-C.

‡ Drug previously incubated for 30 min at 37° in MEM–Eagle's medium.

§ Drug previously incubated for 30 min at 37° in medium containing 10^7 L1210 cells/ml.

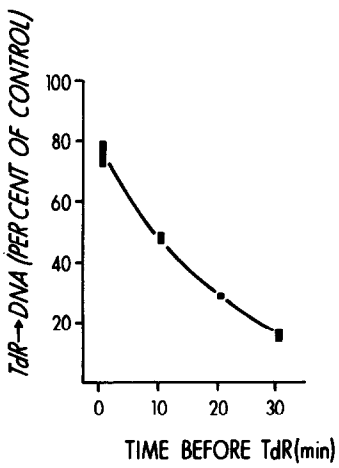

FIG. 3. Inhibition of incorporation of 10-min pulses of labeled thymidine into DNA by anhydro-ara-C. Drug (50 μM) was added at specified intervals before labeled TdR.

TABLE 2. FORMATION OF ARA-C FROM ANHYDRO-ARA-C*

Incubation time (min)	Ara-C (%)
0	< 1
30	5
60	10
90	15

* Solutions of 50 μM labeled anhydro-ara-C were incubated at 37° for specified intervals in MEM–Eagle's medium (pH 7·3), and hydrolysis of the drug to ara-C was measured chromatographically.

can compete, although poorly, with ara-C and CdR for uptake by L1210 cells, but that exodus of ara-C and CdR is not impeded by anhydro-ara-C.

In other studies, we could not demonstrate uptake of labeled anhydro-ara-C by L1210/CA cells. The only radioactivity present in cell pellets after incubation in medium containing labeled drug (0·05 mM, 1–5 min, 0–37°) could be attributed to contamination with extracellular fluid.

Drug effects on DNA synthesis. Addition of ara-C to cell cultures caused a concentration-dependent inhibition of incorporation of labeled thymidine into DNA which increased with time of exposure of cells to the drug (Fig. 1). Anhydro-ara-C was also effective, but at considerably higher levels (Fig. 2). When solutions of anhydro-ara-C were first warmed to 37°, then mixed with cell suspensions, the inhibitory effect on DNA synthesis was thereby enhanced (Table 1). The data show that fresh solutions of anhydro-ara-C could be contaminated with at most 0·1% of ara-C. As the time of exposure of cells to anhydro-ara-C increased, inhibition of DNA synthesis was correspondingly greater (Fig. 3). Prior incubation of solutions of anhydro-ara-C at 37° (pH 7·3), in either the presence or absence of L1210 cells, resulted in substantial "activation" of the drug (Table 1). Chromatographic analysis of ^{3}H-anhydro-ara-C solution after incubation at 37° showed a gradual increase in the level of ^{3}H-ara-C present (Table 2).

DISCUSSION

These data show no evidence of entrance of anhydro-ara-C into L1210/CA cells. The anhydride does appear to exert a slight inhibition on inward transport of ara-C and CdR, but no effect on exodus of these nucleosides. Such a pattern has been observed before, e.g. deoxyguanosine and adenosine inhibited uptake, but not exodus of ara-C.[15] The ether linkage involved in the anhydro-ara-C structure stringently limits the structure of the compound[17] and apparently results in a configuration with almost no affinity for the nucleoside transport sites on the cell surface.

Studies involving measurement of inhibition of DNA synthesis by anhydro-ara-C show the compound to be slow-acting. When solutions of the anhydride are held at 37°, "activation" is observed. This corresponds with findings by Wang *et al.*,[9] who arrived at the same conclusion using different methodology. The presence of L1210 cells did not promote "activation" of anhydro-ara-C at 37°. These data suggest to us that the L1210 cells have no capacity for catalyzing enzymatic conversion of anhydro-ara-C to ara-C.

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