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A PROPOSED MECHANISM OF ACTION OF 1-β-D-ARABINOFURANOSYL-CYTOSINE AS AN INHIBITOR OF THE GROWTH OF LEUKEMIC CELLS*

M. Y. CHU and G. A. FISCHER

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut

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Abstract—Cytosine arabinoside inhibited the reproduction of L5178Y leukemic cells by inhibition of the reduction of cytidylic acid to 2′-deoxycytidylic acid. The specificity of the inhibitory action of cytosine arabinoside was indicated by nutritional studies and by the demonstration that the radioactivity of uridylic 2′-deoxyuridylic acid, thymidylic acid and RNA fractions of cells incubated with ^{3}H-UR was little affected, and that the synthesis of DNA continued when 2′-deoxycytidine was added to cells otherwise inhibited by cytosine arabinoside. Data on the sensitivity of FUDR- and thymidine-resistant mastocytoma cells to cytosine arabinoside and FCDR and cross-resistance to other pyrimidine analogs are presented. Nutritional findings are described which suggest additional secondary sites of action of cytosine arabinoside, possibly at a kinase or phosphokinase level.

I. INTRODUCTION

THE metabolic activity of 1-β-D-arabinofuranosyl-cytosine (cytosine arabinoside, CA)† and related compounds on mutants of *Escherichia coli* has been investigated by Pizer and Cohen[1] and by Slechta.[2] Recently, Evans *et al.*[3] reported that CA is an inhibitor of the growth of sarcoma-180, Ehrlich carcinoma and L-1210 lymphoma in mice.

From theoretical considerations we have assumed that an inversion of the 2′-hydroxy functional group of 1-β-D-ribofuranosyl-cytosine 5′-phosphate (CMP) might inhibit the enzyme system[4] which catalyzes the reduction of CMP to 1-β-D-2′-deoxyribofuranosyl-cytosine-5′-diphosphate (dCDP). In support of this assumption, studies with murine lymphoblasts (L5178Y), herein described, have indicated that CA (or a metabolic derivative of it) inhibits the formation of dCDP from CMP, and in this manner prevents the synthesis of DNA and the reproduction of the cells. Previous studies have demonstrated that a phosphorylated derivative of thymidine also inhibits this enzyme which, in media free of 2′-deoxycytidine, is essential for the reproduction of L5178Y cells.[5]

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† CA was obtained through the courtesy of the Upjohn Company, Kalamazoo, Mich.

II. EXPERIMENTAL PROCEDURE

Materials

Murine leukemic (L5178Y) and mastocytoma (P815Y cells[6] were employed. 2′-Deoxycytidine (CdR), cytidine (CR), 5-methyl-2′-deoxycytidine (5-MeCdR), thymidine (TdR), uridine (UR), 2′-deoxyadenosine (AdR), 2′-deoxyguanosine (GdR), 2′-deoxyinosine (HXdR), adenine (A), guanine (G), xanthine (X), and hypoxanthine (HX) were obtained from the California Corporation for Biochemical Research. Tritiated uridine (^{3}H-UR), supplied by the New England Nuclear Corporation, was purified by adsorption on Dowex-1 × 4 (200–400 mesh) formate anion-exchange resin, followed by elution with 0·01 N formic acid; the specific activity (0·40 mc/μmole) was recalculated against a standard reference.

Methods

The medium and culture techniques used in the growth experiments have been described.[6, 7] Cells (approximately 6×10^8) for biochemical studies were harvested from culture populations in the logarithmic phase of growth using a continuous flow centrifuge (Servall Model RC-2) at 0 °C and 13,000 × **g**. The cells were washed twice with cold medium containing 10% horse serum,[7] and resuspended in approximately 60 ml of cold medium containing serum; the number of cells (approximately 10^7/ml) was determined in two aliquots, using a Coulter Particle Counter, Model A. Of this suspension, 10 ml were added to each incubation tube containing either CA or CdR. After pre-incubation at 37 °C for 5 min, ^{3}H-UR ($8{\cdot}9 \times 10^6$ counts/min, 0·025 μmoles) was added to each tube, and the incubation was continued for 40 min at 37 °C. To stop the reaction, the tubes were transferred to an ice-bath and centrifuged at 0 °C for 10 min at about 800 × **g** in 12-ml conical centrifuge tubes. The supernatant fraction was withdrawn with a pipette and most of the residual supernatant fluid was removed by wiping the inner surfaces of the inverted tubes with absorbent paper. The cells were then treated in such manner as to obtain three fractions: (a) cold acid-soluble, (b) RNA* and (c) DNA.*

The cold acid-soluble fraction was prepared by three successive extractions of the packed cells with 1·5 ml of cold 0·2 N perchloric acid. To convert the pyrimidine di- and tri-phosphate derivatives to mono-phosphates, 2 μmoles of non-radioactive CMP and 2′-deoxycytidine-5′-monophosphate (dCMP) were added as carriers, the concentration of the perchloric acid was adjusted to 1 N, and the mixture was placed in a boiling water bath for 10 min. Perchlorate was removed as the potassium salt by precipitation and centrifugation at 4 °C. The CMP and dCMP of the acid-soluble fraction were eluted from Dowex-50 (200–400 mesh; H^+-form), according to the method of Reichard.[8] Of each of the carriers added, approximately 95 per cent was recovered. The effluents (15 ml) and water-wash (15 ml) from the Dowex-50 columns were concentrated under reduced pressure at 45 °C and brought to 1·0 ml with water. Of these fractions, 100 μl were applied to Whatman No. 1 paper, together with appropriate cold carrier compound, and developed for 72 hr in a tetraborate solvent system[9]. Total radioactivity of sections of the chromatogram was determined by gas-flow counting on aliquots of water extractive; this provided an estimate of the radioactivity of UMP, dUMP and thymidine 5′-phosphate (dTMP) Recovery of the radioactivity after elution of the chromatograms ranged from 90 to 96 per cent.

* Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).

After extraction of the acid-soluble portion of the cells, the RNA was obtained by extracting the residue twice with cold 1 N perchloric acid, as described by Stansly and Chu,[10] and twice with hot 0·2 N sodium hydroxide, according to the method of Mantsavinos and Canellakis.[11] The radioactivity of the final extract was negligible. The DNA was obtained by hydrolysis of the residual material with 1 N perchloric acid at 80° for 30 min.

RESULTS AND DISCUSSION

Nutritional studies

Cytosine arabinoside is an effective inhibitor of the reproduction of L5178Y lymphoblasts, 1×10^{-7}M being sufficient to inhibit the rate of growth by 50 per cent. In the presence of CA(16×10^{-7}M), CdR at a concentration of 5×10^{-5}M almost completely prevents the inhibition of growth (Fig. 1), while at the same concentration,

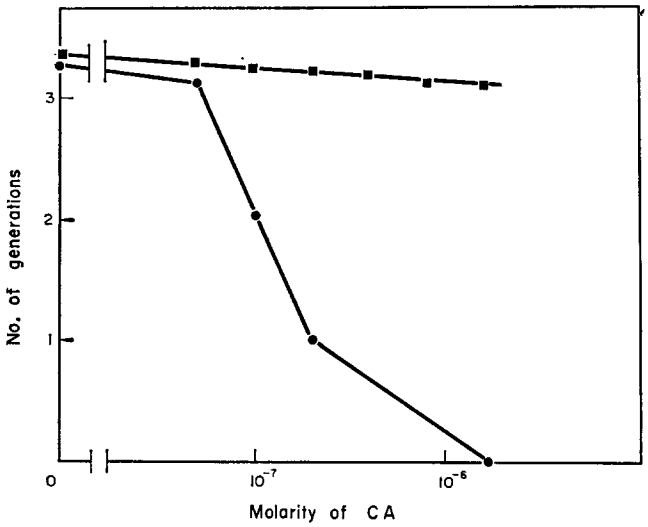

FIG. 1. Inhibition of reproduction of L5178Y cells by 1-β-D-arabinofuranosyl cytosine (CA) and its prevention by 2′-deoxycytidine (CdR). The leukemic cells were grown from an inoculum of 2×10^4 cells per ml for a period of 48 hr in the presence of different levels of CA (●) and CA plus CdR at a molarity of $5{\cdot}0 \times 10^{-6}$ (■). The final numbers of cells were determined in a Coulter Particle Counter, Model A, and the number of cell-generations was determined.

5-methyl-2′-deoxycytidine only partially prevents the inhibition, and CR has but slight, although significant, activity (Fig. 2). Inactive metabolites include TdR (1×10^{-5}M), UdR (5×10^{-5}M), UR (5×10^{-5}M), AdR (5×10^{-5}M), AR (1×10^{-5}M), GdR (1×10^{-5}M), HXdR (1×10^{-5}M), HX ($1{\cdot}5 \times 10^{-5}$M) and X ($1{\cdot}5 \times 10^{-5}$M). These findings are consistent with the assumption that CA (or a metabolic derivative of it) inhibits the reduction of CMP to a derivative of CdR. During the reproduction of the cells an approximately competitive relationship between CA and CdR was observed throughout a concentration-range of inhibitor of 1 : 1000 (Fig. 3). It is possible that CA and CdR may compete for entry into the cell or that the kinases which phosphorylate CdR or its derivatives similarly modify CA; also, it is possible

that the triphosphate of CA may be formed, and that this serves as a precursor of a CA-containing DNA (or possibly RNA), in this manner inhibiting the reproduction of cells. The slight ability of CR to prevent inhibition produced by CA could be attributed to an increase in the amount of substrate (CMP) for the enzyme system

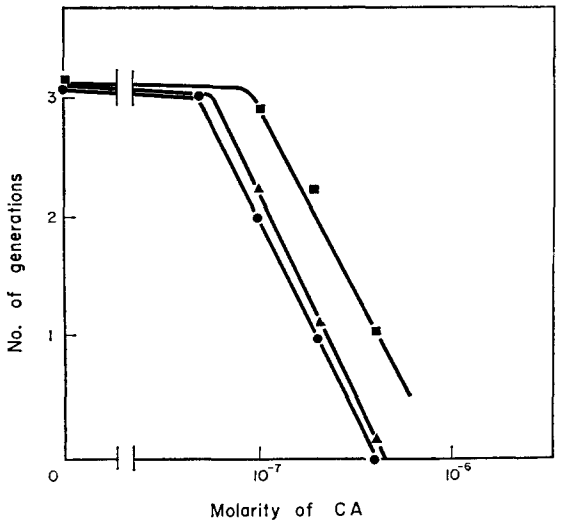

FIG. 2. The slight ability of 5-methyl-2′-deoxycytidine (5-MeCdR) and of cytidine (CR) to prevent the inhibition of growth produced by CA.

The leukemic cells were grown from an inoculum of 2×10^4 cells per ml for a period of 48 hr in the presence of different levels of CA alone (●), CA plus 5-MeCdR, 5×10^{-5} M (■) and CA plus CR, 5×10^{-5} M (▲).

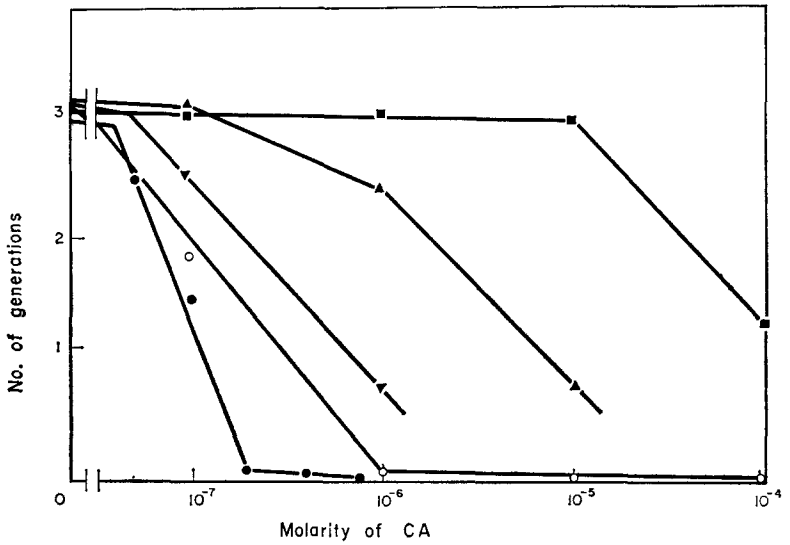

FIG. 3. The competitive relationship between CA and CdR for the reproduction of L5178Y cells. The leukemic cells were grown from an inoculum of 2×10^4 cells per ml for a period of 48 hr at different concentrations of CA (●). To the same concentrations of CA, CdR was added at 5×10^{-7} M (○), 5×10^{-6} M (▼), 5×10^{-5} M (▲) and 5×10^{-4} M (■).

which catalyzes the reduction of CMP to dCDP. 5-MeCdR, which, although much less active than CdR, also decreases the toxicity of CA, might compete with CA for an enzyme site which converts CA to an inhibitory derivative, since CdR was not detected as a contaminant of the 5-MeCdR used.

Cytosine arabinoside also is an effective inhibitor of the growth of a strain of neoplastic mast cells (P815/FUdR) which, unlike the parent strain (P815Y), is deficient in thymidine kinase activity[5] and is cross-resistant to the toxic effect of high levels of TdR, 5-iodo-2′-deoxyuridine (IUdR), 5-bromo-2′-deoxyuridine (BUdR)* and 1-β-D-arabinofuranosyl-thymine† (TA). However, the thymidine-resistant line was not cross-resistant to 5-fluoro-1-β-D-arabinofuranosyl-uracil (FUA)† or to 5-fluoro-2′-deoxycytidine‡ (FCdR) (Table 1). These nutritional studies may indicate

TABLE 1. MOLARITY OF PYRIMIDINE

	P815Y	P815/FUDR
CA	$1{\cdot}1 \times 10^{-7}$	2×10^{-7}
FCdR	6×10^{-10}	1×10^{-9}
FUdR	4×10^{-9}	3×10^{-6}
FUA	3×10^{-6}	4×10^{-6}
TA	$1{\cdot}5 \times 10^{-5}$	1×10^{-4}

Analog required to inhibit by 50 per cent the reproduction of mastocytoma cells, P815Y and P815/FUdR. These cells were grown from an inoculum of 2×10^4 cells per ml for a period of 48 hr in the presence of different levels of the analog and the final number of cells was determined in a Coulter Particle Counter, Model A.

that CA, FUA and FCdR are converted by an enzyme other than thymidine kinase, in either sensitive or resistant lines, to an inhibitory phospho-derivative, while thymidine kinase may phosphorylate 5-FUdR and TA, as well as IUdR and BUdR, in the sensitive cells. Using cell-free extracts of L5178Y cells, Delamore and Prusoff have found that IUdR inhibits the phosphorylation of thymidine, presumably because it also can be phosphorylated by thymidine kinase.[12] However, other explanations may be found for these patterns of cross-resistance. Possibly those analogs which inhibit the resistant lines act as inhibitors of cell reproduction in forms other than the phosphorylated derivatives. Although the resistance to amethopterin of certain strains of L5178Y cells appears to be a reflection only of the extent to which a single enzyme (folic acid reductase) is formed,[13] the cross-resistance observed in the line resistant to FUdR may be attributable to the simultaneous loss of an enzyme, other than thymidine kinase, which converts the analog to an inhibitory derivative.

* J. W. Cramer, N. R. Morris and G. A. Fischer, unpublished results.

† TA and 5-FUA were obtained through the courtesy of Dr. Jack Fox of the Sloan-Kettering Institute, New York City.

‡ 5-Fluoro-2′-deoxycytidine was obtained through the courtesy of Dr. Robert Duschinsky of Hoffman La-Roche, Inc.

Biochemical studies

Biochemical studies also have supported the assumption that CA inhibits the reduction of CMP to dCMP. Thus, in the presence of CA, the CMP and dCMP of the cold acid-soluble fraction (Table 2) demonstrate that the conversion of ^{3}H-UR to ^{3}H-dCMP was effectively reduced, although the formation of ^{3}H-CMP (the precursor of ^{3}H-dCMP) was but little affected. In the presence of both CdR and CA, the levels

TABLE 2. INHIBITION OF SYNTHESIS OF dCMP BY CA

Compounds added	Experiment number	Total counts/min CMP	dCMP
None	1	27×10^4	$3{\cdot}4 \times 10^4$
	2	15×10^4	$2{\cdot}9 \times 10^4$
	3	26×10^4	$3{\cdot}6 \times 10^4$
CA	1	20×10^4	$1{\cdot}1 \times 10^4$
	2	17×10^4	$0{\cdot}8 \times 10^4$
	3	23×10^4	$1{\cdot}2 \times 10^4$
CA + dCR	1	24×10^4	$1{\cdot}4 \times 10^4$
	2	14×10^4	$1{\cdot}0 \times 10^4$

In each experiment, approximately 5×10^7 cells were incubated for 1 hr at 37 °C in a volume of 10 ml of growth medium containing, in all cases, $8{\cdot}9 \times 10^6$ counts/min ^{3}H-uridine ($2{\cdot}91 \times 10^6$ counts/min per μmole). CA at $1{\cdot}2 \times 10^{-6}$ M and CdR at 5×10^{-5} M were included where indicated, and the total radioactivity of the CMP and dCMP fractions were determined in a gas flow counter.

TABLE 3. FAILURE OF CA TO AFFECT TOTAL COUNTS IN UMP, dUMP AND dTMP

Compound added	Counts per min UMP	dUMP	dTMP
None	$2{\cdot}2 \times 10^4$	190	240
CA	$1{\cdot}6 \times 10^4$	180	230

The total radioactivity in each fraction was determined by counting aliquots in a gas-flow counter. In each experiment, approximately 5×10^7 cells were incubated for 1 hr at 37 °C in a volume of 10 ml of growth medium containing, in all cases, $8{\cdot}9 \times 10^6$ counts/min ^{3}H-uridine (2·91 counts/min per μmole), and CA at $1{\cdot}2 \times 10^{-6}$ M where indicated. The values given represent the averages of two experiments, determined in a gas flow counter.

of ^{3}H-dCMP were low, a finding which suggests that, during the synthesis of DNA and reproduction, the CA-induced inhibition of the synthesis of dCMP was maintained.

The effluents of the acid-soluble fraction from the Dowex-50 columns (Table 3) indicated that, in the presence of CA, radioactivity in the UMP, dUMP and TMP fractions remained about the same as in the controls incubated with ^{3}H-UR, in the absence of

CA. Thus, it is possible that under these conditions CA did not affect the reduction of UMP to dUMP, nor the conversion of dUMP to TMP. The failure of UdR or of TdR to prevent the inhibition of growth induced by CA is in agreement with the assumption of a specific interference in the synthesis of dCMP from CMP by this analog of cytidine.

Analysis of the RNA and DNA from cells incubated with either ^{3}H-UR alone or ^{3}H-UR in the presence of inhibitory levels of CA, also support the concept of specificity of inhibition of CA, since the synthesis of RNA was not affected, while in the presence of CA and ^{3}H-UR, the radioactivity of the DNA was effectively reduced (Table 4). This latter circumstance is presumed to reflect the limited supply of dCMP derivatives essential for the synthesis of DNA. In the presence of CA and appropriate levels of CdR, the radioactivity of the DNA was intermediate between that of the inhibited cells and the CA-free controls (Table 2). The higher radioactivity of the DNA, in the

TABLE 4. FAILURE OF CA TO INHIBIT THE SYNTHESIS OF RNA

Compound added	RNA Total counts/min × 10^4	DNA Total counts/min × 10^4
None	210	1·5
CA	200	0·3
CA + dCR	200	0·6

After extraction of the acid-soluble portion of the cells (see Table 2), the RNA was obtained from the residue by extracting twice with cold perchloric acid (1 N) for a period of 24 hr and twice with hot sodium hydroxide (0·2 N). The DNA-fraction was isolated by hydrolysis of the residue with 1 N perchloric acid at 80 °C for 30 min. Radioactivity was determined by liquid phosphor-counting, corrected to gas-flow counts. Each value given represents the average of two experiments.

presence of CA and CdR, when compared with that of the DNA from inhibited cells, is in agreement with the evidence previously presented (Table 1), which suggests that, under the conditions of whole cell-incubation, the synthesis of DNA continues, although the CA-mediated blockade of the synthesis of dCDP is maintained. The lower activity of the DNA in the presence of CA and CdR, when compared with the uninhibited controls, may result from the following circumstances: (1) CdR after phosphorylation presumably dilutes the ^{3}H-dCMP derived from ^{3}H-UR, and (2) CdR (or a phosphorylated derivative of it), may be deaminated to yield eventually 2′-deoxyuridylic acid (dUMP), thereby diluting the ^{3}H-dUMP derived from ^{3}H-UR. Delamore and Prusoff have shown[12] that CdR increases the uptake of formate into the acid-soluble phosphorylated derivatives of thymidine and into DNA in L5178Y cells. Such a dilution of ^{3}H-dUMP by non-radioactive dUMP would be expected to decrease the specific activity of the DNA.

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