Metabolism and Effects of 5-(β-d-Ribofuranosyl)isocytosine in P815 Cells¹

Ting-Chao Chou, Joseph H. Burchenal, Jack J. Fox, Kyoichi A. Watanabe, Chung K. Chu, and Frederick S. Philips


ABSTRACT

5-(β-d-Ribofuranosyl)isocytosine (ψicCyd), a C-nucleoside, has been shown to be active against P815 leukemia in mice. In P815 cells treated with [2-14C]ψicCyd, we have detected radioactivity in nucleotide fractions and in RNA and DNA. Degradation to nucleosides of the labeled triphosphate nucleotide fraction and of RNA showed that the radioactivity present was chromatographically identical to ψicCy d. Half-saturation concentrations for the incorporation of [2-14C]ψicCyd into the triphosphate nucleotide fraction and into RNA and DNA were 370, 280, and 94 μg/ml, respectively, which were >100-fold higher than those for thtritated cytidine. The incorporation of ψicCyd was competitively inhibited by cytidine. Phosphorylation and incorporation of ψicCyd into nucleic acids of P815 cells and of a P815 subline resistant to 1-β-d-arabinofuranosylcytosine are about 2- to 20-fold higher than in P815 sublines resistant to ψicCyd or to both 5-azacytidine and 1-β-d-arabinofuranosylcytosine. These data suggest that the phosphorylation of ψicCyd and possibly its incorporation into nucleic acids are essential for therapeutic activity in P815 leukemias. In vitro metabolic studies also suggest that ψicCyd and 5-azacytidine are cross-resistant and that P815 cells resistant to ψicCyd are cross-resistant to 1-β-d-arabinofuranosylcytosine. These predictions were confirmed by therapeutic experiments carried out in mice bearing P815 leukemias.

INTRODUCTION

ψicCyd² is a C-nucleoside with the ribose moiety linked to the 5-position of the pyrimidine ring through a carbon-carbon bond (Chart 1). Burchenal et al. (3) have shown that ψicCyd is effective in inhibiting the growth of P815 and L1210 cells in vitro and in vivo. In addition, the effects of ψicCyd can be blocked by exogenous cytidine but not by dCyd or thymidine. Of particular interest is the finding that ψicCyd is effective against the P815 cell line resistant to ara-C, P815/ara-C (3).

In the present studies, [2-14C]ψicCyd has been synthesized and used for monitoring the metabolism of ψicCyd in P815 cells. Phosphorylation into triphosphate nucleotide and incorporation into nucleic acids have been compared in P815 cells and various sublines resistant to ψicCyd, ara-C, or azac-C. It is shown that the extents of phosphorylation of ψicCyd by different sublines of P815 cells in vitro closely correlate with the relative sensitivity of these cell lines toward ψicCyd in vivo. The preliminary accounts of this study have appeared earlier (6).

MATERIALS AND METHODS

Synthesis and Purity of [2-14C]ψicCyd. [2-14C]ψicCyd hydrochloride was synthesized by the following procedure. Metallic sodium (12 mg) was added to a solution of [14C]guanidine hydrochloride (5 mCi) (purchased from Moorek Biochemicals, City of Industry, Calif.) in ethanol (10 ml), and the mixture was stirred for 15 min at room temperature. Crystalline ethyl 3-methoxy-2-(2,3-O-isopropylidene-5-O-trityl-d-ribofuranosyl)acrylate (9, 10) (544 mg, 1 mmol) was added to the above solution, and the mixture was refluxed for 1.5 hr. A second batch of [2-14C]ψicCyd of lower radioactivity was obtained (prepared by dissolving 100 mg of metallic sodium in 3 ml of ethanol) and guanidine hydrochloride (170 mg, 1.9 mmol) was added to the above reaction mixture, and refluxing was continued for 1.5 days. TLC of the mixture on a Silica Gel GF₃₄ plate showed a single spot (Rₙ 0.6; benzene:methanol, 8:2). The mixture was concentrated to approximately 6 ml under reduced pressure, cooled in an ice bath, and carefully neutralized with N HCl (to pH 6.8 to 7). During the neutralization, a small amount of protected ψicCyd precipitated. Water (10 ml) was added to complete precipitation of 2',3'-O-isopropylidene-5-O-trityl-ψicCyd which was collected by filtration on a Büchner funnel and air-dried overnight. The protected product was dissolved in 10% methanolic hydrochloride (10 ml), and the mixture was stirred for 1.5 hr at room temperature after which [2-14C]ψicCyd hydrochloride precipitated. The precipitate was collected by filtration (the filtrate was not discarded), dissolved in water (4 ml), decolorized with charcoal, and then freeze-dried to a fluffy solid (195 mg, 11.3 μCi/mg; recovery of radioactivity, 44%). The specific radioactivity of the synthesized [2-14C]ψicCyd was measured by using [7-14C]benzoic acid as radioactivity standard.

A second batch of [2-14C]ψicCyd of lower radioactivity was obtained by treatment of the filtrate with 500 mg of "cold" ψicCyd hydrochloride and by stirring the mixture for 0.5 hr at room temperature. Crystals were collected by filtration, dissolved in water (5 ml), decolorized with charcoal, and then freeze-dried. [2-14C]ψicCyd hydrochloride (390 mg,

¹ This study was supported in part by American Cancer Society Grant CH 36 A, National Cancer Institute Grants 16534 and 00874, and by the Elsa u. Pardee Foundation.
² The abbreviations used are: ψicCyd, pseudoisocytosidine or 5-(β-d-ribofuranosyl)isocytosine; dCyd, deoxycytidine; ara-C, 1-β-d-arabinofuranosylcytosine; azac-C, 5-azacytidine; TLC, thin-layer chromatography; PCA, perchloric acid; ψrd, pseudoisouridine; HPLC, high-pressure liquid chromatography; TCA, trichloroacetic acid; ara-CTP, 1-β-d-arabinofuranosylcytosine 5′-triphosphate; 2′-deoxy-ψic, 2′-deoxy-pseudoisocytidine; ψCTP, pseudoisocytidine 5′-triphosphate.

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Metabolism of ψCyd

Chart 1. Chemical structure of ψCyd. *, position of the 2-¹⁴C labeling.

Chart 2. Paper chromatogram of synthesized [²-¹⁴C]ψCyd. Whatman No. 3MM paper was used for descending paper chromatography with the solvent system, isopropyl alcohol/concentrated HCl:H₂O, 68:17:15 (v/v); TLC cellulose plates with isopropyl alcohol:concentrated NH₄OH:0.1 M boric acid, 70:10:20 (v/v); and TLC cellulose with isobutyrlic acid:2.2 M NH₄OH, 66:34 (v/v). In each of the 3 systems, the UV absorption of the 2 batches of [²-¹⁴C]ψCyd (λₘₐₓ = 262 nm, ε = 7590; λₘᵲᵢₙ = 241 nm, ε = 4670 in 0.1 N HCl; and λₘₐₓ = 277 nm, ε = 7145, λₘᵲᵢₙ = 253 nm, ε = 2810 in 0.1 N NaOH) was identical with that of an authentic sample of ψCyd hydrochloride. The purity of both batches was checked further in 3 chromatographic systems: descending paper (Whatman No. 3MM) developed with isopropyl alcohol:concentrated HCl:H₂O, 68:17:15 (v/v); TLC cellulose plates with isopropyl alcohol:concentrated NH₄OH:0.1 M boric acid, 70:10:20 (v/v); and TLC cellulose with isobutyrlic acid:2.2 M NH₄OH, 66:34 (v/v). In each of the 3 systems, there was only a single UV-absorbing zone with Rₚ identical with that of authentic unlabeled ψCyd; the respective Rₚ's were 0.36, 0.33, and 0.71. Strips 0.5 to 1 cm in width were eluted with water, and the radioactivity of each eluate was measured. In each of the systems, radioactivity was recovered in a single peak with an Rₚ corresponding to that of authentic ψCyd; this is illustrated in Chart 2 for the first system.

Cell Lines. Leukemia P815 (i.e., P815/0) and its sublines resistant to ψCyd (P815/ψCyd), ara-C (P815/ara-C), and ara-C and aza-C (P815/ara-C/aza-C) were maintained by weekly transplantation in C57BL × DBA/2 F₁ (hereafter called BD2F₁) mice. The resistant cell lines had been derived by treating BD2F₁ mice bearing P815/0 leukemia i.p. every fourth day, starting on the second day after transplantation, with ψCyd (50 mg/kg) or ara-C (20 mg/kg). The drug treatment continued during weekly tumor transplantations for 5 weeks or longer to obtain sublines resistant to ψCyd or ara-C, respectively. Some mice bearing P815/ara-C leukemia were further treated with aza-C (2 mg/kg) to produce the P815/ara-C/aza-C line. Leukemic cells for metabolic studies were collected at 6 days after i.p. inoculation of 10⁶ cells in mice that did not receive drug treatment.

Incubation and Cell Extracts. For in vitro experiments, cell suspensions (5 to 19 × 10⁶/ml) along with radioactive precursor [²-¹⁴C]ψCyd, [⁵-³H]cytidine, [⁵-³H]dCyd, or [¹H]ara-C were incubated in Eagle’s basal medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal calf serum and 2 mM freshly added L-glutamine. The standard incubation (final volume, 1.5 ml) was carried out at 37° for 45 min in a capped tube containing a mixture of 5% carbon dioxide and 95% oxygen as described previously (5). Incubation was stopped by adding 3 ml of ice-cooled 10% PCA and homogenized with a Polytron (Brinkmann Instruments, Inc., Westbury, N. Y.) for 30 sec. The residue in the homogenizing tube was rinsed with 2 ml of the PCA solution and pooled. For in vivo experiments, BD2F₁ mice bearing P815/0 leukemia were given injections i.p. (Day 6 after i.p. inoculation of 10⁶ cells) with a specified dose of [²-¹⁴C]ψCyd (11.3 μCi/mg). At a specified time after injection, ascites were collected, pooled, and homogenized with cold 10% PCA. For both in vitro and in vivo experiments, PCA-soluble extracts were neutralized with KOH and chilled in ice; the resulting KClO₄ was removed by centrifugation.

Chromatographic Analyses of Acid-soluble Extracts from Leukemic Cells Exposed to [²-¹⁴C]ψCyd or [⁵-³H]Cytidine. For routine separation of radioactive metabolites of [²-¹⁴C]ψCyd and of [⁵-³H]cytidine, a simple anionic exchange chromatography procedure was used. Six ml of neutralized, acid-soluble extract were applied to a Chromaflex column containing 0.7 x 6 cm of AG 1-X₈ (anionic exchange resin in chloride form; Bio-Rad Laboratories, Richmond, Calif.). The nucleoside and mono-, di-, and triphosphate nucleotide fractions were obtained by stepwise elution with 12 ml each of H₂O and 0.15, 0.3, and 1 M NH₄HCO₃, respectively, as described previously for ara-C (or dCyd) and its nucleotides (5, 7). The procedure was also used herein for the fractionation of nucleotides formed from tritiated ara-C and dCyd.

To test the deamination of radioactive ψCyd, ara-C, cytidine, and dCyd in the nucleoside fractions of AG 1-X₈ anionic exchange column, an aliquot of each of these fractions was applied to a column containing 0.7 x 6 cm of AG 50-X₈ (cationic exchange resin in hydrogen form; Bio-Rad Laboratories). The radioactivity in the aqueous eluate of the AG 50-X₈ column represented the upper limit of deamination since ψrd, 1-β-D-arabinofuranosyluracil, uri-

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dine, and deoxyuridine appeared in aqueous eluates, whereas ψICyd, ara-C, cytidine, and dCyd, respectively, were retained by the AG 50-X8 column. For an analysis by HPLC of the distribution of radioactivity in total acid-soluble extracts, a Partisil SAX column procedure was used, similar to that reported previously for separating ara-C metabolites (16). To identify the radioactive substances present in nucleotide fractions, mice were given injections i.p. at 6 days after inoculation of P815/o leukemia with [2-14C]ψICyd (50 mg/kg, 21.5 μCi/mg). They were killed one hr later, and acid-soluble extracts of the ascites cells were prepared and chromatographed into nucleoside, mono-, di-, and triphosphate fractions as described above. The mono- and triphosphate fractions were flash evaporated at 80° under reduced pressure; water was added, and evaporation was repeated until NH4HCO3 was removed. The residue of the triphosphate fraction first was digested with 10% PCA in a boiling water bath for 10 min to convert triphosphate nucleotides to monophosphate nucleotides. After neutralization with KOH, KHCO3 was removed by centrifugation. The resulting supernatant and the residue of the monophosphate fraction were incubated with Escherichia coli alkaline phosphatase (5 units/ml) in 0.02 M Tris-HCl buffer (pH 8.2) at 37° for 18 hr to convert monophosphate nucleotides to nucleosides. The incubation mixtures were then deproteinized by shaking with equal volumes of chloroform. The aqueous layers were concentrated by lyophilization. They were streaked across Whatman No. 1 paper and chromatographed in the descending direction using Solvent System 1 (see Table 1). One-cm-wide vertical strips were cut from each channel for determination of the distribution of radioactivity. Single large zones of radioactivity were found (see "Results"), and the remaining portions of the chromatogram containing these zones were extracted with water. The eluates were concentrated by flash evaporation and then chromatographed along with reference spots of authentic ψICyd in 2 TLC systems (Table 1, Systems 2 and 3). Eastman TLC chromatogram sheets, cellulose type 6065 (Distillation Products Inc., Rochester, N. Y.) were used. Strips 0.5 to 1 cm in width were cut from each channel for elution and measurement of radioactivity.

**Incorporation of Labeled Precursors into Nucleic Acids.** Incorporation of [2-14C]ψICyd, [3H]ara-C, [5-3H]cytidine, and [5-3H]dCyd into RNA and DNA was measured routinely with the method previously described (7). After homogenization of radio-precursor-treated cells with 2 volumes of cold 10% PCA, the insoluble precipitates were washed twice with 5 ml cold 10% TCA, twice with 5 ml 100% ethanol and once with 5 ml diethyl ether. The RNA in the pellet was digested with 0.5 N NaOH at 37° for 17 hr and neutralized with 6 N HCl; 2 ml cold 10% TCA were then added and centrifuged. The precipitate was washed with another 2 ml of cold 10% TCA, and the supernatants were pooled for radioactivity counting. To the precipitate (containing labeled DNA), 2 ml of 10% TCA were added and then heated at 90° for 15 min to digest the DNA. The tubes were placed in cold water for 5 min and centrifuged, and the radioactivity was counted in the supernatant.

In a preliminary experiment carried out to identify the radioactive substances present in RNA, 2 mice were given injections i.p. of [2-14C]ψICyd (5 mg/kg, 11.3 μCi/mg) at 6 days after inoculation of P815/o. The animals were killed 90 min later, and the ascites cells were collected by centrifugation. The RNA of the cells was isolated by the method of Girard (13). To 1.1 mg of isolated RNA [dissolved in 0.1 M sodium acetate:EDTA buffer (pH 5.1)] were added 2.2 units of T2 RNase, 55 μg of T1 RNase, and 55 μg of pancreatic RNase (final volume, 1 ml). The mixture was incubated at 37° for 5 hr after which the pH was raised to 8.2; 20 units of E. coli alkaline phosphatase were added, and the mixture was incubated for an additional 16 hr. Following the incubation periods, the mixture was shaken with equal volumes of chloroform and then centrifuged. The deproteinized supernatant was concentrated and chromatographed, and the distribution of radioactivity was measured.

**Chemicals and Radiochemicals.** ara-C and 1-β-d-arabinofuranosyluracil were obtained from The Upjohn Co., Kalamazoo, Mich. [3H]ara-C (8.2 Ci/mmol) was obtained from Dr. R. R. Engle of the National Cancer Institute. [5-3H]Cytidine (28 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc., Irvine, Calif.; 1-β-d-arabinofuranosylcytosine 5'-phosphate, 1-β-d-arabinofuranosylcytosine 5'-diphosphate, and ara-CTP were purchased from Terra-Marine Bioresearch, La Jolla, Calif.; aza-C, cytidine, and dCyd were purchased from Calbiochem, San Diego, Calif.; ψICyd was a gift from Kyowa Hakko Co., Ltd., Tokyo, Japan.

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**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Paper chromatographya</th>
<th>TLCb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solvent 1</td>
<td>Solvent 2</td>
</tr>
<tr>
<td>ψICyd</td>
<td>0.20</td>
<td>0.27</td>
</tr>
<tr>
<td>ψrd</td>
<td>0.24</td>
<td>0.33</td>
</tr>
<tr>
<td>2'-Deoxy-ψICyd</td>
<td>0.38</td>
<td>0.51</td>
</tr>
<tr>
<td>Isoctidine</td>
<td>0.28</td>
<td>0.43</td>
</tr>
<tr>
<td>Cytidine</td>
<td>0.26</td>
<td>0.35</td>
</tr>
<tr>
<td>Uridine</td>
<td>0.26</td>
<td>0.52</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0.32</td>
<td>0.26</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0.14</td>
<td>0.21</td>
</tr>
</tbody>
</table>

a Whatman No. 1 paper was used for descending chromatography. Solvent 1 consists of isopropanol:water:0.1 m boric acid:concentrated NH4OH, 70:20:10 (v/v). When Whatman No. 3MM paper was used (as indicated in text), the Rf's for ψICyd, ψrd, and 2'-deoxy-ψICyd were 0.26, 0.32, and 0.44, respectively.

b Cellulose TLC was used. Solvent 2 consists of isopropanol:concentrated HCl:H2O, 68:17:16 (v/v), and Solvent 3 consists of isobutyric acid:2.2 M NH4OH, 66:34 (v/v). When Solvent 2 was used for descending paper chromatography using Whatman No. 3MM paper (as indicated in text), the Rf's for ψICyd and ψrd were 0.36 and 0.46, respectively.

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2 Both [3H]ara-C and [5-3H]cytidine were purified with descending paper chromatography (Whatman No. 3MM) using Solvent System 2. The Rf's for [3H]ara-C and [5-3H]cytidine were 0.48 and 0.49, respectively. [7-14C]Benzoic acid (5.1 × 108 dpm/mg; used for radioactivity calibration) and [5-3H]dCyd
were prepared from ascites cells and fractionated by AG 1-X8 anionic exchange chromatography as described above. The distribution of the total radioactivity in the acid-soluble extract among the nucleoside mono-, di-, and triphosphate fractions was, respectively, 78, 8.5, 1.9, and 11.6%. Chromatography in Solvent System 1 of Table 1 of the triphosphate fraction, which had been hydrolyzed with acid, digested with alkaline phosphatase, and streaked on paper, revealed that more than 95% of the radioactivity was present in a single band with Rf corresponding to that of authentic \( \psi \text{ICyd} \) (Rf = 0.20). The eluate from the band was further analyzed by TLC using Solvent Systems 2 and 3 (Table 1). In both systems, >96% of the radioactivity was recovered as a single peak with Rf like that of known \( \psi \text{ICyd} \) (Rf = 0.27 and 0.75, respectively). These results provide evidence that \( \psi \text{IC} \) is incorporated unchanged into the triphosphate fraction as isolated by AG 1-X8 column chromatography.

The diphosphate fraction from the above experiment was not further analyzed because of its low radioactivity. When the monophosphate fraction, after digestion with alkaline phosphatase, was streaked on paper and chromatographed in Solvent System 1, a small peak of radioactivity, 1.4% of the total, was recovered in a band extending from Rf 0.18 to 0.23 that corresponded to the Rf of \( \psi \text{ICyd} \). A second small peak, 3.1% of the total, was present in a band from Rf 0.03 to 0.06. A third band with 91% of the total radioactivity was found in a zone from Rf 0.4 to 0.69. This band was eluted, concentrated, and rechromatographed by TLC in Solvent Systems 2 and 3. In System 2, 2 overlapping peaks at Rf 0.05 and 0.27 were present in a band, extending from the origin to Rf 0.40, that contained 93% of the radioactivity. There were also 2 overlapping peaks in System 3 at Rf 0.45 and 0.68 in a band of radioactivity extending from 0.35 to 0.78 and containing 98% of the total radioactivity. It would appear from these results that the radioactivity in the monophosphate fraction which is obtained by anionic exchange chromatography is present predominantly in one or more substances that are not pseudoisocytidine 5’-phosphate. The HPLC profile of acid-soluble extract (Chart 3) did not show double peaks at the monophosphate region.

This result suggests that the 2 overlapping peaks appearing in the TLC chromatograms may represent a partial digestion of an unknown metabolite by alkaline phosphatase used in digesting the monophosphate fraction.

**Identification of \( \psi \text{ICyd} \) Moiety in RNA.** When P815/o cells were exposed to \( [2\text{-}^{14}\text{C}]\psi \text{ICyd in vivo } \) and RNA was isolated and then digested with RNase and alkaline phosphatase, the resulting radioactive nucleoside had a chromatographic behavior identical to authentic \( \psi \text{ICyd} \). The RNA from ascites cells that had been exposed to \( [2\text{-}^{14}\text{C}]\psi \text{ICyd in vivo } \) (See “Materials and Methods”) contained radioactivity equivalent to the incorporation of 38.7 pmol of \( \psi \text{ICyd} \) per absorbance of 1.0 at the \( \lambda_{\text{max}} \) of 259 nm. Chromatography, after enzyme digestion to nucleosides, in Solvent System 1 on paper (Whatman No. 3MM) revealed the presence of a single zone of radioactivity with 91% of the total between Rf 0.20 and 0.32 with a peak at 0.28. Authentic \( \psi \text{ICyd} \) in the same experiment also had an Rf of 0.28. The remainder of the radioactive zone was eluted, concentrated, and rechromatographed. In Solvent System...
2, using Whatman No. 3MM in the descending direction, 95% of the radioactivity was recovered in a single zone (Rf 0.31 to 0.41) with a peak at 0.36; the Rf of authentic ψICyd was 0.35. In Solvent System 2 by TLC, 91% of the radioactivity was recovered in a single zone (Rf 0.17 to 0.37) with a peak at 0.27, the same Rf as that of authentic ψICyd.

**Phosphorylation and Incorporation into Nucleic Acids of ψICyd and Cytidine in P815/o Cells in Vitro.** Because previous studies had shown that the inhibitory effects of ψICyd on cell growth of P815/o in vitro is blocked by exogenous cytidine but not by dCyd or thymidine (3), it was important to compare the metabolism of ψICyd and cytidine in the same leukemic cells. As shown in Chart 4, the radioactivity of [5-3H]cytidine appeared rapidly in the triphosphate nucleotide fraction and in RNA; lesser amounts appeared in DNA. By contrast, the phosphorylation of ψICyd as well as its incorporation into nucleic acid were one to 2 orders of magnitude less.

When P815/o cells were incubated in varied concentrations of ψICyd in the presence and absence of cytidine, the incorporation of the C-nucleoside into ψICTP, RNA, and DNA was found to be competitively inhibited by cytidine (i.e., marked inhibition at low concentration of ψICyd and no inhibition at very high concentration of ψICyd (Chart 5).

The double reciprocal plots of the saturation curves of ψICyd given in Chart 5 for intact cells are essentially straight lines. It is, therefore, reasonable to calculate the half-saturation concentrations and the estimated maximal rates of synthesis at saturating concentrations of ψICyd. As shown in Chart 5, the incorporation of ψICyd into DNA was easily saturable and shows much lower maximal rates than it does into triphosphate and RNA. The estimated maximal capability of ψICTP formation in the intact cells at high ψICyd concentration was greater than was the maximal capability of the incorporation into RNA or DNA during the 45-min incubation (Charts 4 and 5). The apparent inhibition index of cytidine (in ng/ml) for blocking the incorporation of ψICyd into triphosphate nucleotide and nucleic acids can be calculated from ψICyd saturation curves (see legend to Chart 5). The inhibition indices for cytidine are 140- to 700-fold lower than are the corresponding half-saturation concentrations for ψICyd. These marked differences between ψICyd half-saturation concentrations and inhibition indices of cytidine were consistent with the data given in Table 2 which show that ψICyd at 100-fold excess over cytidine (2.5 μg/ml) had little effect on cytidine anabolism (e.g., phosphorylation and incorporation into nucleic acids).

**Comparison of Metabolism of ψICyd and Some Nucleoside Analogs in P815 Sublines.** Phosphorylation of ψICyd, cytidine, dCyd, and ara-C into triphosphate nucleotide fractions of AG 1-X8 anionic exchange chromatography and their incorporation into RNA and DNA fractions in vitro were compared in P815 cells, sensitive and resistant to ψICyd, ara-C, or both ara-C and aza-C (Table 3). The selection of the concentrations of the antimetabolite substrates, ara-C (2.5 μg/ml) and ψICyd (25 μg/ml), was based on their therapeutically effective concentrations in vivo and in vitro, which were reported previously (1, 3, 14). The following observations were made: (a) the incorporation of ψICyd into triphosphate, RNA, and DNA is the following order of magnitude: P815/o > P815/ara-C > P815/ara-C/aza-C ≥ P815/ψICyd; (b) the order of incorporation into triphosphate, RNA, and DNA in 4 sublines is identical for ψICyd and cytidine but not for dCyd or ara-C; (c) P815/ψICyd cells convert less ψICyd into triphosphate and incorporate less into RNA and DNA than do P815/o cells, suggesting that phosphorylation of ψICyd may be a prerequisite for the therapeutic effect of ψICyd; (d) comparing data from 4 sublines with ψICyd as a substrate leads to the suggestion that P815 cells that develop resistance to aza-C are probably cross-resistant to ψICyd although P815/aza-C cells were not used in the present studies; (e) P815/ara-C cells synthesize considerable amounts of ψICTP from ψICyd and incorporate a considerable amount of radioactivity into RNA and DNA, but the metabolic data do not suggest that P815/ara-C should be more sensitive to ψICyd than are P815/o cells; (f) the data are consistent with the earlier finding (19) that P815/ara-C cells are lacking in dCyd kinase. The results also indicate that P815/ara-C cells are not lacking in cytidine kinase but do show that P815/ψICyd cells are lacking in cytidine kinase and not in dCyd kinase; (g) there is a particularly low incorporation of ara-C into DNA and RNA in P815/ara-C and in P815/ara-C/aza-C cells. Compared with other nucleosides, the relatively low incorporation of ara-C into DNA in all 4 sublines is probably due to self-inhibition of DNA polymerase through the ara-CTP effect (12); (h) P815/ψICyd cells synthesize more ara-CTP (or deoxycytidine 5'-triphosphate) from ara-C (or dCyd) than do P815/o cells, suggesting that P815/ψICyd cells could be more sensitive to ara-C than are P815/o cells (i.e., they could have collateral sensitivity to ara-C).
Table 2

Effects of ψlCyd on incorporation of [5-3H]cytidine in P815/o cells

The incubation mixture (1.5 ml) contained 1.9 × 10⁶ cells and [5-3H]cytidine (2.5 μg, 2.1 μCi/ml). The incubation was carried out at 37°C for 45 min.

<table>
<thead>
<tr>
<th>Total nucleotides</th>
<th>RNA (ng equivalents/10⁶ cells/45 min)</th>
<th>DNA (ng equivalents/10⁶ cells/45 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[5-3H]Cytidine</td>
<td>134.3</td>
<td>24.1</td>
</tr>
<tr>
<td>[5-3H]Cytidine + ψlCyd</td>
<td>141.9</td>
<td>22.1</td>
</tr>
</tbody>
</table>

Since cytidine, dCyd, ara-C, and aza-C are substrates of pyrimidine nucleoside deaminase (4, 14, 21), it was important to determine whether significant deamination had taken place in the experiments of Table 3. Chromatographic studies with P815/o cells of their sublines that were incubated under standard conditions for 45 min indicated that the radioactivity in the nucleoside fractions eluted from the AG 1-X8 column contained mainly unchanged ψlCyd (>99%). There was, however, a small fraction of radioactivity in the nucleoside fraction (<1%) which appeared to have lost the cationic group of ψlCyd since it was no longer retained by the AG 50-X8 cationic exchange column when it was eluted with water. This small amount of radioactivity contained an unknown metabolite(s) (68%) which has an Rf of 0.22 in Solvent System 2 using Whatman No. 3MM paper. Studies with P815/o using labeled ara-C, cytidine, and dCyd also showed a small proportion (0.2 to 1.5%) of radioactivity in the nucleoside fractions that were eluted from AG 50-X8 column by water. (In other studies, we have found that ψlCyd is virtually resistant to deamination by the pyrimidine nucleoside deaminase present in cell-free extracts of BD2F1 mouse kidney, although the same extracts extensively deaminate cytidine and ara-C.)

Metabolism of ψlCyd in P815 Sublines in Vivo. [2-14C]ψlCyd (2.5 mg, 28.3 μCi/kg) was injected s.c. into BD2F1 mice bearing P815/o, P815/ψlCyd, P815/ara-C, or P815/ara-C/aza-C cells. The animals were killed 90 min later, and the radioactive metabolites were analyzed. The in vivo experiments (Table 4) gave results qualitatively similar to the in vitro experiments shown in Table 3 and, thus, conclusions similar to those drawn from Table 3 can be made (see above).

Therapeutic Effectiveness of ψlCyd, ara-C, 2,2'-Anhydro-1-β-D-arabinofuranosyl-5-fluorocytosine, and aza-C in Mice Bearing Leukemias of P815 Sublines. Therapeutic treatment of tumor-bearing mice started at Day 1 after i.p.
Comparison of metabolism of $\psi$Cyd, cytidine, dCyd, and ara-C in P815 subcell lines

The standard incubation mixture containing 5 x 10^6 to 19 x 10^6 cells/ml was incubated at 37° for 45 min. The labeled substrate concentrations used and the number of experiments were: [2-$^{14}$C]$\psi$Cyd, 25 $\mu$g, 0.54 $\mu$Ci/ml, n = 6 to 13; [5-$^{3}$H]cytidine, 25 $\mu$g, 1.5 $\mu$Ci/ml, n = 3 to 7; [5-$^{3}$H]dCyd, 25 $\mu$g, 1.5 $\mu$Ci/ml, n = 2 to 4, and [3H]ara-C, 2.5 $\mu$g, 1.5 $\mu$Ci/ml, n = 6 to 10.

<table>
<thead>
<tr>
<th>Leukemic cells</th>
<th>$\psi$Cyd</th>
<th>Cytidine</th>
<th>dCyd</th>
<th>ara-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP P815/o</td>
<td>9.130 ± 0.710</td>
<td>139.01 ± 13.11</td>
<td>10.57 ± 0.45</td>
<td>28.85 ± 2.02</td>
</tr>
<tr>
<td>P815/$\psi$Cyd</td>
<td>0.840 ± 0.300</td>
<td>12.33 ± 1.52</td>
<td>18.70 ± 2.10</td>
<td>41.80 ± 6.46</td>
</tr>
<tr>
<td>P815/ara-C</td>
<td>3.330 ± 1.400</td>
<td>51.75 ± 32.32</td>
<td>0.95 ± 0.28</td>
<td>0.58 ± 0.40</td>
</tr>
<tr>
<td>P815/ara-C/aza-C</td>
<td>1.920 ± 1.080</td>
<td>18.00 ± 7.91</td>
<td>2.05 ± 1.85</td>
<td>0.28 ± 0.18</td>
</tr>
<tr>
<td>RNA P815/o</td>
<td>1.040 ± 0.340</td>
<td>22.44 ± 9.36</td>
<td>1.28 ± 0.19</td>
<td>0.031 ± 0.003</td>
</tr>
<tr>
<td>P815/$\psi$Cyd</td>
<td>0.194 ± 0.055</td>
<td>2.35 ± 1.88</td>
<td>0.60 ± 0.07</td>
<td>0.027 ± 0.003</td>
</tr>
<tr>
<td>P815/ara-C</td>
<td>0.688 ± 0.447</td>
<td>4.75 ± 2.86</td>
<td>0.12 ± 0.02</td>
<td>0.004 ± 0.001</td>
</tr>
<tr>
<td>P815/ara-C/aza-C</td>
<td>0.200 ± 0.053</td>
<td>2.50 ± 1.76</td>
<td>0.13 ± 0.02</td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td>DNA P815/o</td>
<td>0.136 ± 0.028</td>
<td>3.36 ± 1.02</td>
<td>4.53 ± 0.51</td>
<td>0.016 ± 0.009</td>
</tr>
<tr>
<td>P815/$\psi$Cyd</td>
<td>0.006 ± 0.003</td>
<td>0.50 ± 0.11</td>
<td>2.40 ± 0.56</td>
<td>0.008 ± 0.002</td>
</tr>
<tr>
<td>P815/ara-C</td>
<td>0.032 ± 0.042</td>
<td>1.24 ± 0.52</td>
<td>1.11 ± 0.03</td>
<td>0.003 ± 0.002</td>
</tr>
<tr>
<td>P815/ara-C/aza-C</td>
<td>0.032 ± 0.011</td>
<td>1.06 ± 0.42</td>
<td>0.13 ± 0.05</td>
<td>0.001 ± 0.001</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.E.

Table 4

Metabolism of [2-$^{14}$C]$\psi$Cyd in P815 subcell lines in BD2F1 mice in vivo

[2-$^{14}$C]$\psi$Cyd (28.3 $\mu$Ci, 25 mg/kg) was injected s.c. into BD2F1 mice bearing different subcell lines of P815 leukemia. Animals were used at 5 days after i.p. inoculation of 1 x 10^6 leukemic cells. At 90 min after [2-$^{14}$C]$\psi$Cyd was injected, animals were killed, and ascites cells were collected and counted. The radioactive $\psi$ICTP in PCA-soluble extract of the cells and the incorporation of radioactivity into RNA and DNA were assayed as for in vitro experiments (see "Materials and Methods"). Data given are means of duplicated experiments in which the average deviation did not exceed 20% of each mean.

<table>
<thead>
<tr>
<th>Leukemic sublines</th>
<th>TP</th>
<th>RNA</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>P815/o</td>
<td>154.2</td>
<td>171.7</td>
<td>14.0</td>
</tr>
<tr>
<td>P815/$\psi$Cyd</td>
<td>42.4</td>
<td>53.0</td>
<td>2.4</td>
</tr>
<tr>
<td>P815/ara-C</td>
<td>74.3</td>
<td>102.9</td>
<td>5.3</td>
</tr>
<tr>
<td>P815/ara-C/aza-C</td>
<td>28.8</td>
<td>45.8</td>
<td>3.4</td>
</tr>
</tbody>
</table>

DISCUSSION

The above studies have shown that P815 cells convert $\psi$Cyd into substances retained by anionic exchange columns. These substances can be eluted stepwise in fractions containing, respectively, the mono-, di-, and triphosphates of natural pyrimidine nucleosides. When the triphosphate fraction, obtained from P815 cells that had been exposed to [2-$^{14}$C]$\psi$Cyd, was degraded to the nucleoside state, most of the radioactivity was found to be present in a moiety which was chromatographically identical to authentic $\psi$Cyd in 3 different solvent systems (Table 1). These systems effectively distinguish $\psi$Cyd from the following substances: 2′-deoxy-$\psi$Cyd and adenosine (Solvent 1); 2′-deoxy-$\psi$Cyd, isocytidine, and uridine (Solvent 2); and $\psi$rd and guanosine (Solvent 3). Thus, there was no evidence suggesting extensive breakdown of $\psi$Cyd and reutilization of $^{14}$C for de novo synthesis of natural nucleosides, nor was there evidence for deamination to $\psi$rd and conversion of the latter to the triphosphate state or for conversion of $\psi$Cyd to isocytidine. The resistance of $\psi$Cyd to catabolic reactions is not unexpected since the carbon-carbon linkage between pyrimidine base and ribose is resistant to nucleoside phosphorylases of mammalian tissues (11, 20). Finally, 2′-deoxy-$\psi$Cyd was not detected as a distinct peak in the chromatography of the degraded triphosphate fraction; <2% of radioactivity was recovered in the zone corresponding to the deoxynucleoside.

Degradation of the monophosphate fraction, obtained from anionic exchange chromatography of acid solubles from treated P815 cells, showed the presence of unknown metabolites of $\psi$Cyd. The pharmacological significance of these substances is unknown. However, it seems reasonable to suggest for future study the possibility that they are complex substances like pseudoisocytidine 5′-diphosphate-choline or -ethanolamine. Preliminary studies in this laboratory have shown CDP-choline, when used as a reference
ethanolamine-containing nucleotides are formed from AG 1-X8 anionic exchange columns. Moreover, the unnat
compound, appears in the monophosphate fraction from that the radioactive nucleoside in the labeled nucleic acid
ural nucleoside, ara-C, is converted to 1-3-D-arabinofu
DNA fractions from P815 cells treated with [2-14C]i/ilCyd
ble to expect that it might also be incorporated into RNA. In
fact, in the present study, RNA that had been isolated from
in a variety of mammalian cells (17, 18). If choline- and
ranosylcytosine 5'-diphosphate-choline and -ethanolamine
appeared as 2'-deoxy-@j,ICyd after degradation of the tn
deoxy-@lCyd. The low rates of incorporation seem consist
by analogy with the natural pathway that utilizes CTP (2).
(Tables 3 and 4; Chart 4). For this reason, labeled DNA was
showed that the incorporation of [2-'4C]@ICyd into the DNA
ent with the fact that relatively little, if any, radioactivity
not isolated and degraded to detect the presence of 2'-
reductase (15).

### Table 5

**Therapeutic effectiveness of ψICyd, ara-C, 2,2'-anhydro-1-β-D-arabinofuranosyl-5-fluorocytosine, and aza-C on P815 subcell lines**

Therapeutic treatment of tumor-bearing BD2F, mice started at the end of Day 1 after i.p. inoculation of 10⁶ cells. Drugs in mg/kg were administered i.p. with one of the following schedules: q4d x 3, q4d x 4, qd x 5, or qd x 10, as indicated.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Typical schedule</th>
<th>P815/o</th>
<th>P815/ara-C</th>
<th>P815/ψICyd</th>
<th>P815/aza-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>ψICyd</td>
<td>50</td>
<td>qd x 10</td>
<td>44.0</td>
<td>104.0 ± 3.0</td>
<td>-12</td>
<td>-12</td>
</tr>
<tr>
<td></td>
<td>67-75</td>
<td>qd x 4</td>
<td>92.1 ± 20.0</td>
<td>116.6 ± 19.5</td>
<td>-5.5 ± 2.5</td>
<td>-9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>qd x 4</td>
<td>48.7</td>
<td>86.7 ± 16.5</td>
<td>-10.9</td>
<td>-4.2 ± 8.2</td>
</tr>
<tr>
<td></td>
<td>200-300</td>
<td>qd x 4</td>
<td>66.2 ± 20.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ara-C</td>
<td>20-30</td>
<td>qd x 10</td>
<td>184.9 ± 48.8</td>
<td>1.1</td>
<td>95.5 ± 58.0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>200-400</td>
<td>qd x 4</td>
<td>102</td>
<td></td>
<td>256.9 ± 82.7</td>
<td>8.2</td>
</tr>
</tbody>
</table>
| 2,2'-Anhy-
|dro-1-β-D-
|arabinofu-
|ranosyl-5-
|fluorocyto-
|sine      | 400          | qd x 4           | -1.4 ± 2.4 | 290.7 ± 42.0| -7.0 ± 8.0  |
| aza-C      | 1.5-3        | qd x 5           | 109.8 ± 49.0| 3.8         | -11.7 ± 4.7 | -6.2        |
|            | 4.5-6.7      | qd x 3           | 44 ± 0     | 7.9         |             |             |
|            | 10           | qd x 3           | 14.6 ± 7.5   | -2.0        | -7.4 ± 4    |             |

* a q4d, every four days; qd, every day.
* b Number following multiplication sign, number of cycles per drug treatment.
* c Percentage increase of life span over the untreated control (n = 2 to 6 experiments). Each experiment consists of 9 to 10 mice. Single entries are data from single experiments. The life span of tumor-bearing mice without any chemotherapy were: P815/o, 9.8 ± 0.3 (n = 6); P815/ara-C, 10.6 ± 1.4 (n = 6); P815/ψICyd, 10.2 ± 0.1 (n = 4); and P815/ara-C/aza-C, 9.9 ± 0.1 (n = 3).
* d Mean ± S.E.
* e Toxic manifestations to the host as seen by losses of body weight and death at early stage of treatment.

Comparative metabolic studies of ψICyd, ara-C, cytidine, and dCyd in P815/o, P815/ψICyd, P815/ara-C, and P815/ara-C/aza-C cells (Tables 3 and 4) provide evidence which suggests that phosphorylation and possibly incorporation into nucleic acids may be required for the therapeutic effect of ψICyd. The metabolic studies also predict that ψICyd is more effective against P815/o and P815/ara-C and less effective (or not effective) against P815/ψICyd and P815/ara-C/aza-C cells, that there is collateral sensitivity to ara-C in P815/ψICyd cells, and that P815/ψICyd and P815/ara-C/aza-C cells may be cross-resistant to ψICyd. These predictions have been confirmed by therapeutic experiments in mice (Table 5). The in vitro studies also suggest that the development of resistance toward ψICyd in P815/ψICyd cells is due to a lack in cytidine kinase activity, but not to a lack in dCyd kinase.

ψICyd is a relatively poor substrate when compared with cytidine or ara-C, for phosphorylation. Nevertheless, the phosphorylated ψICyd can be incorporated into RNA and, to a lesser extent, into DNA. The relatively high half-saturation concentrations of ψICyd for conversion into triphosphate and nucleic acids (Chart 5) are consistent with the fact that its incorporation can be readily inhibited by the natural nucleoside, cytidine, which has much lower half-saturation concentrations in the same metabolic processes. Results in Table 2 and preliminary studies in vitro (6) and in vivo (22) give no indication that ψICyd is a potent metabolic inhibitor. These negative findings lead to the suggestion.
that phosphorylation and incorporation into nucleic acids might be the primary mode of pharmacological action rather than inhibition of a natural pathway of pyrimidine metabolism.

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Metabolism and Effects of 5-\((\beta\text{-d-Ribofuranosyl})\text{isocytosine in P815 Cells}

Ting-Chao Chou, Joseph H. Burchenal, Jack J. Fox, et al.


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