Biochemical, Pharmacological, and Phase I Clinical Evaluation of Pseudoisocytidine¹

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ABSTRACT

Pseudoisocytidine (ΨICyd) is a C-nucleoside with enhanced stability and resistance to enzymatic deamination when compared to 5-azacytidine and 1β-D-arabinofuranosylcytosine. Elimination kinetics in plasma using 14CΨICyd showed a β-phase t1/2 for 14C of 2 hr and a β-phase t1/2 of unchanged ΨICyd of 1.5 hr. Net recovery of radioactivity in urine over 24 hr varied between 40 and 80% of the administered dose; 50 to 90% was unchanged drug and the rest was pseudouridine. Human leukemic cells in vitro deaminated ΨICyd very slowly, formed appreciable quantities of pseudoisocytidine triphosphate, and incorporated small amounts into RNA and DNA. Clinical trials were done using a daily i.v. injection for 5 consecutive days. Hematological or intestinal toxicities were not seen, nor was depression of white blood cell count observed in leukemic patients. Hepatic toxicity proved to be dose limiting; this was characterized by an early phase with elevation of prothrombin time and aspartate aminotransferase. A later phase with cirrhosis was observed in two patients. Autopsy showed massive hepatic necrosis in patients dying of acute toxicity and micronodular cirrhosis in one patient dying with the chronic form.

INTRODUCTION

ara-C³ and aza-CR are among the most active agents in the management of acute leukemia; their activity and their limitations suggest that further nucleoside analogs should be synthesized and examined for antitumor activity. ara-C has an extremely short plasma half-life because of rapid enzymatic deamination (4, 18). This factor makes its optimal clinical use dependent upon either multiple daily injections or continuous infusion (9, 15, 24).

aza-CR is quite active against transplanted murine leukemias and possesses some activity against human leukemia and solid tumors (1, 11, 12, 21-23, 29-35). aza-CR also has a short plasma half-life because of chemical instability and rapid deamination by pyrimidine nucleoside deaminase (5, 17, 24-26). As part of a continuing synthetic program investigating pyrimidine analogs (7, 27), Chu et al. (6) have prepared ΨICyd, a C-nucleoside isomer of cytidine, in which ribose is linked to the pyrimidine ring by a carbon-carbon bond (Chart 1). It was demonstrated to have desirable chemical characteristics in that it is chemically stable and very slowly deaminated by pyrimidine nucleoside deaminase (2, 3, 6). Burchenal et al. (2) observed that ΨICyd was as active against transplanted murine tumors resistant to ara-C as was aza-CR. The cytotoxic effects of the drugs were blocked by cytidine and uridine but not by deoxycytidine or thymidine, indicating that ΨICyd acts as a ribonucleoside in a manner similar to that of aza-CR.

Further work on the metabolism of ΨICyd in P815 cells by Chou et al. (6) showed that phosphorylation of ΨICyd was essential for therapeutic activity; incorporation of ΨICyd into nucleic acids was observed predominantly into RNA. These studies also demonstrated that biological resistance to aza-CR conferred resistance to ΨICyd and vice versa. Zedeck has examined the nucleosides resulting from the hydrolysis of liver RNA and splenic DNA from mice that had been treated with labeled ΨICyd (37). Radioactivity was present in DNA as ΨICyd suggesting that, at the nucleotide level, ΨICyd is a substrate for ribonucleotide reductase since cleavage of the ribose-pyrimidine carbon-carbon bond is not thought to occur in vivo (13, 14).

Toxicological studies showed a lack of any acute lethal toxicity in any species in doses up to 40 mg/kg. The lethal effects observed on progressive toxicological studies included myelosuppression, intestinal damage, and hepatic toxicity. In most species, the hepatic toxicity occurred at dosages above those required to give myelosuppression; in cats, however, hepatic necrosis occurred with doses near those producing dose-limiting hematological toxicity.

We have undertaken clinical evaluation of ΨICyd because of its superior chemical and metabolic stability, its activity comparable to aza-CR in animal tumor screens, and the reported activity of aza-CR in human leukemias resistant to other agents.

Patients. Patients participating in the clinical trial had histologically confirmed cancers; they had exhausted conventional therapeutic measures and were entered in the study after giving informed consent. The patient population was characterized by a median performance status of 50 (range, 30 to 90), a male:female ratio of 10:11, and a median age of 31 years (range, 3 to 79). All patients had received prior therapy, 16 with chemotherapy alone, 5 with chemotherapy and radiation therapy. No patients were accepted with impairment of renal function as determined by a plasma creatinine of 1.5 mg/dl or greater. Patients with hepatic metastasis were not excluded providing that pretreatment bilirubins were less than 2 mg/dl. Pretreatment WBC and platelet counts exceeded 4000/μl and 100,000/μl respectively, except for patients with acute leukemia. No chemotherapy or radiation therapy was

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³ The abbreviations used are: ara-C, 1β-D-arabinofuranosylcytosine; aza-CR, 5-azacytidine; ΨICyd, pseudoisocytidine; ΨICyd, deoxypseudoisocytidine; ΨUrd, pseudouridine.

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given in the 4 weeks prior to entering the study. Table 1 gives the histology and primary site of the malignant neoplasm of the patients studied.

Blood counts (complete blood count, including hemoglobin concentration, WBC, and platelet count) were done daily during the first weeks of treatment. Screening profiles, urine analysis, chest X-rays, and electrocardiograms were done before initiation of treatment and were repeated approximately weekly for the first patients. Cardiograms and X-rays were repeated only as indicated while liver function tests including screening profiles, 5'-nucleotidase, prothrombin time, and partial thromboplastin time were repeated as often as every other day.

**MATERIALS AND METHODS**

Unlabeled and [2-14C]ΨCyd were synthesized by Dr. J. Fox. Specific activity was 12 μCi/mg, and radiochemical purity was greater than 97% by thin-layer chromatography (6, 8).

ΨCyd was prepared for clinical use by weighing 110% of the calculated dose into a sterile container, dissolving the hydrochloride salt of the drug with slightly less than the calculated amount of sterile 0.9% NaCl solution for injection (U.S.P.), and adjusting the pH to 7.2 with 0.1 N NaOH. The volume was adjusted with 0.9% NaCl solution and the solution was filtered through a 0.22-μm sterile filter (Swinnex-13 HS) into a sterile vial, and given within 4 hr by rapid i.v. injection. Radiolabeled drug was prepared in the same way by adding 200 μCi of labeled drug before neutralization and sterilization.

Blood samples were drawn into heparinized tubes; whole blood, plasma, and RBC fractions were separated and frozen at −5° until analysis. Urine samples were collected by asking the patients to void at specific times; volumes were then recorded and aliquots were frozen.

Blood and urine samples were evaluated for total 14C activity by oxidation of 0.1- to 0.3-ml aliquots in a Packard Model 306 sample oxidizer (Packard Instruments, Downers Grove, Ill.) and subsequent counting in a quench-correcting Packard Model 3380 liquid scintillation counter previously calibrated for the scintillation solutions used (Packard Instruments).

Respiratory gases were gathered by timed collections into a 100-liter gas bag using a nonbreathing valve. The 14CO2 in respiratory gases was recovered by bubbling through ethanolamine. The ethanolamine was evaluated for 14C by mixing with a scintillation solution and counting in a liquid scintillation counter. Quench correction was done by adding internal standards.

**Analysis of [2-14C]ΨCyd and Its Metabolites in Plasma and Urine.** Four ml of cold 5% perchloric acid were added to each ml of plasma or urine collected from patients and homogenized. After centrifugation, the supernatant was diluted with an equal volume of water, and 5 ml of this acid-soluble extract were applied onto an AG 50-X8 column, 0.7 x 10 cm (cation-exchange resin in hydrogen form purchased from Bio-Rad Laboratories, Richmond, Calif.). The column was further washed twice with 3.5 ml of water, and the pooled eluates were counted for radioactivity using a Packard Tri-Carb Model 3775 liquid scintillation spectrometer as described previously (6). The ΨCyd was eluted by the column and the deaminated product, ΨUrd, appeared in the aqueous eluate. Some urine samples were further analyzed with 2 additional chromatographic systems. The supernatants containing perchloric acid were neutralized with KOH, and the resulting neutralized extracts were concentrated by lyophilization and applied onto cellulose chromatogram sheets for thin-layer chromatography (solvent system, isobutyric acid:2.2 N NH4OH, 66:34, v/v). Two radioactive peaks were observed; one corresponded to ΨCyd (RF 0.75), and the other corresponded to ΨUrd (RF 0.54). The concentrated extracts were applied onto Whatman No. 3MM paper and were descending chromatographed (solvent system, isopropyl alcohol:concentrated HCI:H2O, 68:17:15, v/v/v). Two radioactive peaks were observed; one corresponded to ΨCyd (RF 0.36), and the other corresponded to ΨUrd (RF 0.46). The fractions of radioactivity in urine appearing as ΨCyd in the 3 chromatographic systems were in good agreement. The samples of plasma were analyzed with AG 50-X8 column chromatography only, since relatively low levels of radioactivity were present.

**Metabolism of [2-14C]ΨCyd by Human Leukemic Cells.** Blood or bone marrow samples from leukemic patients were incubated with [2-14C]ΨCyd (0.54 μg/ml) in Eagle’s basal medium containing freshly added L-glutamine (2 mm) and gassed with 95% O2 and 5% CO2.

The incubation was carried out at 37° for 45 min and was stopped by adding 2 volumes of cold 10% perchloric acid. Following homogenization and centrifugation, the acid-soluble extracts were neutralized with KOH and the resulting KClO4 was removed by centrifugation. An aliquot was applied to the AG 1-X8 column 0.7- x 6-cm (anionic exchange resin in chloride form purchased from Bio-Rad) for separating mono-, di-, and triphosphate nucleotides of [2-14C]ΨCyd as described previously (6). The incorporation of [2-14C]ΨCyd into RNA and DNA was also measured by a method described previously (6).

**RESULTS**

Pharmacological Studies in Vivo. Overall plasma radioactivity elimination kinetics observed in 3 patients receiving 30, 90, and [14C]ΨCyd (120 mg/sq m), respectively, is given in Chart 2. Each dose contained 200 μCi of [2-14C]ΨCyd. The kinetic
patterns are similar at all 3 dose levels with a β-phase \( t_{1/2} \) of 134 ± 22 (S.D.) min. Chart 3 shows the cumulative urinary recovery of radioactivity as a fraction of the administered dose. The wide variation in recovery does not appear to be dose related in these 3 patients, and it may be significant that the patient with the lowest urinary excretion developed severe hepatic damage.

The determination of plasma and urine levels of \([14C]ΨICyd\) when compared to \(^{14}C\) equivalents demonstrates that deamination of \(ΨICyd\) occurs. Overall, the β-phase \( t_{1/2} \) value of \(ΨICyd\) was 101 ± 16 (S.D.) min with little variation between the 3 dose levels as shown in Chart 4. Chart 5 gives the cumulative proportion of urinary \(^{14}C\) that was recovered as \([14C]ΨICyd\). As with the net \(^{14}C\) recovery, there is a large variation between 60 and 90% and no clear dose-effect relationship.

Chart 6 gives the simultaneous \(ΨICyd\) and \(ΨUrd\) levels in the plasma of the patient who received 90 mg/sq m. The reciprocal relationship is obscured by the semilogarithmic scale, but through 24 hr all of the plasma \(^{14}C\) can be accounted for as \(ΨICyd\) or \(ΨUrd\). At 5 hr after treatment, 50% of the plasma \(^{14}C\) is present as deaminated product. Only traces of \(^{14}CO_2\) could be detected in expired air. If extrapolated over 24 hr, the highest observed \(^{14}CO_2\) excretion rate could account for less than 0.1% of the administered radioactivity.

Pharmacological Studies in Leukemic Cells. Deamination of \([2-^{14}C]ΨICyd\) by human leukemic cells in vitro was measured by using AG 50-X8 cation exchange columns. Under standard incubation conditions, the deamination did not exceed 0.2% of the total \(ΨICyd\) present during the 2-hr incubation period. The large proportions of deaminated product of labeled \(ΨICyd\) (i.e., \(ΨUrd\)) in circulating blood and urine after giving \([2-^{14}C]ΨICyd\) to patients are probably due to high pyrimidine nucleoside deaminase activity in organs such as the liver (4, 35). \(ΨICyd\) appears to be a poor substrate for deamination by human leukemic cells since parallel studies using \([3H]ara-C\) indicated that 2 to 10% of ara-C was deaminated to \([3H]-1-β-D-arabinofuranosyluracil\).

In comparison, human leukemic cells anabolized more \(ΨICyd\) than did P815 cells that are resistant to \(ΨICyd\), but less \(ΨICyd\) than did P815 cells sensitive to \(ΨICyd\) under the same experimental conditions (Table 2). Acid and enzymatic degradation to nucleosides of the labeled triphosphate nucleotide fraction and of RNA (from P815 cells sensitive to \(ΨICyd\)) followed by chromatography indicated that the radioactivity present was incorporated into triphosphate nucleotides and RNA without

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**Chart 2.** Plasma radioactivity levels following \([2-^{14}C]ΨICyd\) (200 μCi i.v.) at 30 (Θ), 90 (Ο), and 120 ( Δ) mg/sq m.

**Chart 3.** Cumulative urinary recovery of radioactivity following i.v. \([2-^{14}C]ΨICyd\) at 30 (Θ), 90 (Ο), and 120 ( Δ) mg/sq m.

**Chart 4.** Plasma \(ΨICyd\) levels following \([2-^{14}C]ΨICyd\) (200 μCi i.v.) at 30 (Θ), 90 (Ο), and 120 mg/sq m.

**Chart 5.** Cumulative urinary recovery of intact \([14C]ΨICyd\) expressed as percentage of total radioactivity excreted. \(ΨICyd\) doses: Θ, 30 mg/sq m; Ο, 90 mg/sq m; Δ, 120 mg/sq m.
alteration. The identification of radioactivity in triphosphates, RNA, and DNA fractions of human leukemic cells and of P815 cells resistant to $\Psi$Cyd was not analyzed due to relatively low radioactivity.

The results indicated that human leukemic blast cells phosphorylate $[2^{-14}C]$\PsiCyd into mono-, di-, and triphosphate nucleotides; the mono- and dinucleotide levels are low and not linear with time, suggesting that they are rapidly converted to triphosphate nucleotide. To a small extent, $[2^{-14}C]^ICyd$ was incorporated into RNA and DNA. There is no appreciable difference in metabolism of $\Psi$Cyd by acute myeloblastic leukemia and acute lymphocytic leukemia cells.

Clinical Evaluations. Therapy began by using a schedule of single daily injections for 5 consecutive days. Escalation of treatment to higher doses was done if toxicity was judged to be acceptable 2 weeks after completion of a course. The starting dose was 30 mg/kg; this was estimated as being one-third of the lowest toxic dose in the most sensitive species studied in preclinical testing. Doses were advanced to 60, 90, 120, and 180 mg/kg.

Retrospectively, even at the lowest doses, minor elevations in prothrombin time (2 to 4 sec) were noted in some patients. Because of the resolution of these abnormalities and the lack of other signs of biological effect, dose escalation was continued. After observation of variable hepatotoxicity in 12 patients without concomitant marrow or intestine toxicity, 4 patients were studied using a twice weekly schedule to see if this schedule change could alter the toxicity pattern. When hepatotoxic effects were noted in all 4 patients (Table 3), clinical trials were suspended.

Hematological toxicity was not detected, nor did the patients experience nausea, vomiting, stomatitis, alopecia, diarrhea, or cutaneous toxicity. Hepatotoxicity was noted in 12 of 21 patients; the frequency and severity of the hepatotoxicity increased as the dose was raised. No therapeutic responses were noted.

Elevations of the prothrombin time provided the earliest changes suggestive of hepatic damage. In patients treated on a 5-consecutive-day schedule, these changes could be observed on Days 4 to 8. There was no associated thrombocytopenia, hemolysis, or clinical bleeding; and return to pretreatment values could occur within 10 days of the last treatment. Twelve patients developed elevation of the aspartate aminotransferase during this same interval. Resolution of this phase occurred in 10 to 20 days; however, in 6 patients it was followed by progressive elevation of plasma bilirubin and alkaline phosphatase; 2 of these patients developed progressive hepatic insufficiency. Acute life-threatening hepatic toxicity occurred in 2 pediatric patients within 2 weeks of starting therapy at the highest dose level.

Chart 7 outlines the development of hepatotoxicity in a patient who had received $\Psi$Cyd (90 mg/sq m) for 5 consecutive days and illustrates the sequential biochemical abnormalities seen.

Pathological Findings. Autopsy material obtained from 2 patients manifesting severe liver toxicity illustrates the acute and chronic hepatotoxic changes seen. Fig. 1 illustrates changes in the liver of a 10-year-old boy who died with acute lymphoblastic leukemia and widespread hemorrhage. His liver...
Table 3
Liver function tests after ICyd treatment

<table>
<thead>
<tr>
<th>Dose</th>
<th>Evaluable courses</th>
<th>Bilirubin</th>
<th>Aspartate aminotransferase</th>
<th>Prothrombin time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median peak</td>
<td>Peak day</td>
<td>Median peak</td>
</tr>
<tr>
<td>30 mg/sq m, qd x 5</td>
<td>4</td>
<td>0.85 (0.6–11.8)</td>
<td>7 (5–23)</td>
<td>35 (19–110)</td>
</tr>
<tr>
<td>60 mg/sq m, qd x 5</td>
<td>4</td>
<td>0.60 (0.6–1.0)</td>
<td>3 (1–27)</td>
<td>24 (19–26)</td>
</tr>
<tr>
<td>90 mg/sq m, qd x 5</td>
<td>7</td>
<td>0.80 (0.6–2.68)</td>
<td>35 (22–50)</td>
<td>100 (29–544)</td>
</tr>
<tr>
<td>120 mg/sq m, qd x 5</td>
<td>4</td>
<td>1.55 (0.8–2.5)</td>
<td>7 (3–8)</td>
<td>220 (41–1140)</td>
</tr>
<tr>
<td>180 mg/sq m, qd x 3 or 4</td>
<td>2</td>
<td>5.5 (1.3–9.8)</td>
<td>5 (3–7)</td>
<td>1040 (575–1500)</td>
</tr>
<tr>
<td>90 mg/sq m, biw x 5</td>
<td>4</td>
<td>1.1 (0.3–1.7)</td>
<td>15 (4–21)</td>
<td>7 (10–105)</td>
</tr>
</tbody>
</table>

* qd x 5, single daily injections for 5 consecutive days; biw x 5, twice weekly injections for 5 doses; qd x 3 or 4, single daily injections for 3 or 4 days.
* Numbers in parentheses, range.
* Planned 5-day courses terminated due to acute hepatotoxicity.

showed severe acute hepatocyte necrosis and hemorrhage involving more than one-half of each lobule. The process was principally periportal with some sparing of the central zones. He died 17 days after the first dose of ICyd.

By comparison, Fig. 2 demonstrates comparable changes in the liver of a lethally intoxicated monkey receiving 40-mg/kg 5-consecutive-day doses of ICyd.

Fig. 3 illustrates the changes present in the liver of a 63-year-old man who died with metastases from an epidermoid carcinoma of the skin 90 days after the first dose of ICyd. His liver showed marked bile duct proliferation with fibrosis and bridging zones. In addition, there was severe cholestasis, especially marked in the portal regions. A few polymorphonuclear leukocytes and lymphocytes were present in the portal regions. Only a rare necrotic hepatocyte was noted at the periphery of the lobule.

**DISCUSSION**

These studies demonstrate that ICyd fulfilled the concepts guiding its synthesis; it is chemically stable, only slowly deaminated enzymatically in vivo, and is incorporated into human leukemic cell nucleic acids. Its deaminated metabolite, Urd, is without any known biological effect.

If Phase II studies were to be undertaken, 120 mg/sq m given on 5 consecutive days would be a reasonable schedule. This dose caused mild hepatotoxicity, with median bilirubin peak of 1.6, median aspartate aminotransferase peak of 220, and median prothrombin time peak of 20.2. The next highest dose gave unacceptable hepatotoxicity.

The hepatotoxicity correlated well with the vigorous incorporation of ICyd into hepatic cell RNA observed in rats. The acute cellular necrosis observed clinically resembles both that produced in monkeys by near lethal doses of ICyd and that reported to occur in some patients receiving aza-CR (31).

Other clinical observations of aza-CR toxicity including symptomatic hypophosphatemia and frank renal tubular acidosis (10, 16) were not seen with ICyd therapy. As adminis-
analog may be of interest. (a) there are specific inhibitors of nucleoside transport that might be used to alter the relative distribution of the drug and thereby avoid toxicity; (b) schedule dependency of the hepatotoxicity can be further explored to avoid hepatotoxicity; (c) the unexpected recovery of $\mathrm{IdCyd}$ from mouse of the hepatotoxicity can be further explored to avoid hepatoxicity; (a) there are $\mathrm{IdCyd}$ or the arabinosyl analog may be of interest.

REFERENCES

Fig. 1. Liver from patient with extensive leukemic infiltrate (small dark round cells). There is hemorrhagic necrosis (dark areas) surrounding residual focally fatty liver tissue. From patient who died 17 days after starting therapy with ICyd. H & E x100.

Fig. 2. Liver from rhesus monkey showing severe, acute hepatocyte necrosis. Many of the nuclei are karyorrhectic, the cytoplasm is disrupted and cells boundaries and liver plates are distorted or lost. Monkey killed while moribund at 5 days after starting ICyd (40 mg/kg daily for 5 days). H & E x400.

Fig. 3. Liver from 63 year old man with epidermoid carcinoma. There is extensive bile ductular proliferation and chronic inflammatory cell infiltrate with fibrosis in portal zones. There is also cholestasis. Autopsy 90 days after ICyd (90 mg/sq m daily for 5 days). H & E x300.
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