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Analytical and pharmacokinetic studies with 5-chloro-2′-deoxycytidine

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Abstract

5-Chloro-2′-deoxycytidine (NSC 371331, CDC) is in development as a possible radiosensitizing agent for cancer treatment. Previous studies have been done to demonstrate the *in vivo* efficacy of CDC with various modulators of its metabolism. This paper describes our preclinical studies to determine the pharmacokinetic properties of CDC and the disposition of the drug, both alone and in the presence of the metabolic modulator tetrahydrouridine (THU), a cytidine deaminase inhibitor. Detection of the drug in biological fluids was performed by HPLC analysis using a C-18 column, gradient elution with solvents composed of aqueous trifluoroacetic acid and acetonitrile, and ultraviolet absorbance at 290 nm. Samples were processed by treatment with ammonium sulfate prior to injection into the HPLC system. CDC was stable in aqueous solution and in mouse plasma. High doses of CDC (100 mg/kg) were given i.v. or i.p. to mice for the determination of CDC plasma half-life (10 min). CDC was not detectable in plasma after oral administration. It was converted rapidly to 5-chloro-2′-deoxyuridine (CDU) by cytidine deaminase, and CDU was readily discernable in plasma and urine samples collected after i.v. and i.p. administration of CDC. When CDC in doses ranging from 5 to 100 mg/kg was given with 100 mg/kg of THU, increased plasma levels of CDC were seen. CDC was eliminated through the kidneys, as well as by enzymatic deamination, and did not bind to plasma proteins. The initial steps of the CDC metabolic pathway were determined *in vitro* with isolated enzymes. Cytidine deaminase from mouse kidney converted CDC into CDU; thymidine phosphorylase converted CDU into 5-chlorouracil (5-CU). The conclusions of these studies are: (a) CDC is a drug with a short half-life and (b) it is excreted through the kidney, mainly in metabolite form. Administration of THU substantially increased the concentrations of CDC in mouse plasma, supporting proposals that the combination of THU with CDC should be evaluated in clinical trials.

Keywords: 5-Chloro-2′-deoxycytidine; Tetrahydrouridine; Pharmacokinetics; Cytidine deaminase; Radiation sensitization; Pyrimidine nucleoside metabolism

1. Introduction

CDC, like other 5-halogenated pyrimidine nucleosides, is known to sensitize cells to ultraviolet and ionizing radiation once it is incorporated into DNA. The related compounds IdU and BrdU, to be effective for radiosensitization, have to be infused continuously to overcome rapid catabolism by uridine and thymidine phosphorylases, as well as rapid dehalogenation. Another limitation of these two drugs is their severe toxicity to normal tissues, which includes bone marrow suppression and dermal phototoxicity [1,2]. CDC, on the other hand, is a pro-drug that is anabolized to the active form of CDU. Although CDU, like IdU and BrdU, is incorporated into DNA, CDC itself is not catabolized by uridine and thymidine phosphorylases nor is it dehalogenated by thymidylate synthetase like IdU and BrdU [2,3]. There is some evidence that CDC itself is phosphorylated and incorporated into DNA despite being a poor substrate for deoxycytidylate kinase [2], allowing for the drug to possibly exert some biological actions independent of those associated with the formation of CDU [4].

Studies done with human xenografts in mice have shown that CDC is tumor selective because many tumor cells have elevated amounts of deoxycytidylate deaminase, cytidine deaminase, and deoxycytidine kinase compared to normal cells [2]. Elevated enzyme levels allow more CDC to be converted to its active form in the tumor cell than in normal cells. Therefore, enzyme levels, rather than simple proliferative status of the tissue, determine drug incorporation; the CDU metabolite is formed by enzymatic deamination

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Abbreviations: CDC, 5-chloro-2′-deoxycytidine, cytochlor; CDU, 5-chloro-2′-deoxyuridine; THU, tetrahydrouridine; 5-CU, 5-chlorouracil; BrdU, 5-bromo-deoxyuridine; IdU, 5-iodo-deoxyuridine; ara-C, cytosine arabinoside; AUC, area under the curve.

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and subsequently phosphorylated and incorporated into DNA [5].

Since many tumors have elevated cytidine deaminase levels, THU, a cytidine deaminase inhibitor, can be used to prevent systemic deamination of CDC in the serum and liver with comparatively lesser inhibition of this pathway in the tumor cells. THU does not add any toxicity or itself enhance radiosensitization [5]. Even though THU limits the cytidine deaminase pathway, the deoxycytidylate deaminase pathway could still activate CDC after initial kinase-mediated phosphorylation. Because there are two metabolic pathways with two distinct kinases leading to active drug, it is unlikely for a cell to become resistant to the drug. Although the mechanism of radiosensitization produced by CDC treatment is not different from that produced by IdU and BrdU, CDC does appear to have a lower toxic potential in mice [2,6].

Because CDC is a non-hypoxic radiosensitizing prodrug when it is given to mice with THU, developmental research has been undertaken to assess its potential value as part of cancer treatment regimens. In these preclinical studies, we determined the pharmacokinetics of CDC alone and with THU. Plasma binding and mode of excretion also were determined. *In vitro* enzyme studies were conducted to establish that the principal metabolite observed in the *in vivo* studies was formed by the expected enzyme system.

2. Materials and methods

2.1. Chemicals

HPLC-grade trifluoroacetic acid, HPLC-grade water, HPLC-grade acetonitrile, and ammonium sulfate were purchased from Krackeler Scientific. CDU, 5-chlorouridine, cytidine, uridine, thymidine, thymidine phosphorylase from *Escherichia coli*, and anhydrous dibasic sodium phosphate were purchased from the Sigma Chemical Co. Potassium phosphate was purchased from Mallinckrodt, and dextrose from J.T. Baker. Mouse plasma used for generating standard curves and for the measurement of stability was purchased from Pel-freeze Biologicals. Cytidine deaminase was isolated and partially purified from mouse kidney homogenate as previously described [7]; mouse kidney acetone powder was purchased from Sigma. CDC and THU were provided by the National Cancer Institute (NCI).

2.2. Dosing

Normal male mice (CD_2F_1; weighing approximately 20–25 g) were provided by the NCI. Mice were given 5–100 mg/kg doses (bolus) of CDC in 0.2 mL volumes. THU was injected concomitantly at 100 mg/kg. Drug solutions were prepared in water containing 5% dextrose. Oral doses were administered with a feeding needle; i.v. doses were injected into the lateral tail vein and i.p. doses were injected laterally in the abdominal region.

2.3. Collection of samples

Blood was obtained by retro-orbital puncture and collected in heparinized capillary tubes. Samples were taken at various points over a time course of 5 min to 24 hr. Plasma was separated by centrifugation (6200 *g* for 5 min at room temperature). Urine was collected by clean catch during blood collection or through the use of a metabolism cage for the 24-hr samples. Mice were killed by cervical dislocation, organs were inspected for gross abnormalities, and all samples were frozen at -20° until analysis.

2.4. HPLC conditions

Chromatographic separation was achieved using an Econosphere C-18 reverse phase 250 mm × 4.6 mm column from Alltech. Hardware consisted of a 20-μL injection loop, a variable wavelength detector, gradient pump, dynamic mixer, and integrator from Spectra-Physics. The mobile phases consisted of 0.1% trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). CDC and metabolites were detected at 290 nm after gradient elution. The gradient consisted of holding the aqueous phase at 100% for 5 min, then a linear decrease to 85% aqueous over 5 min, and holding at 85% aqueous phase for 5 min before returning to initial conditions. The total run time was 25 min; the flow rate was 1 mL/min. The identity of CDC in the pertinent region of the chromatograms was confirmed by HPLC-MS, using a Finnigan MAT SSQ-710C LC-MS system in the electrospray mode. Characteristic peaks were noted at *m/z* 262.6 (protonated molecular ion) and at *m/z* 284.6 and 300.6 (sodium and potassium adducts, respectively). As expected, signals due to chlorine isotopes (^{37}Cl) were also noted. The identity of the material in the CDC peak was confirmed by Photo Diode Array spectroscopy using a Waters Millenium system.

2.5. Plasma assay

Plasma proteins were precipitated by mixing 50 μL of plasma with 250 μL of 0.5 mg/mL ammonium sulfate. After vortexing, the samples were centrifuged at 8000 *g* for 10 min at 4°. The supernatant solutions were then ready for HPLC analysis. Recovery of CDC from seeded samples typically was in the 90% range. Concentration–peak area relationships for CDC solutions in water and in mouse plasma proved linear (correlation coefficient 0.997) through the concentration range of 5×10^{-6} to 5×10^{-5} M. Same-day accuracy and within- and between-day precision were 3, 4, and 8%, respectively. External standards of CDC and CDU in water were used to determine the concentrations of the drug in the various samples by comparison of the peak areas. Estimates for derived pharmacokinetic parameters

were made using non-compartmental analysis where the AUC is calculated by the linear trapezoidal rule and λ_z, the terminal phase rate constant, is determined from the linear regression of time vs. log concentration (WinNonlin; Pharsight Corp.).

2.6. Organ assay

Selected organs (kidney and liver) were removed from mice post-mortem and frozen at −20° until processing. Approximately 0.2 g of each thawed organ was homogenized in 0.1 M potassium phosphate buffer (1 mL), and centrifuged at 8000 *g* for 10 min at room temperature; supernatant solutions were then analyzed as described above for plasma.

2.7. Stability

Solutions of CDC in water and in mouse plasma at a concentration of 20 μM were incubated in a 37° water bath. Samples (50 μL) were removed at various time points, processed, and analyzed as described above for plasma.

2.8. Plasma binding

Plasma binding was estimated by adding 200 μL plasma or water containing 20 μM CDC to Amicon centrifree assemblies (MW cutoff of 30,000). The assemblies were centrifuged in a Sorval centrifuge at 1000 *g* and 0° for 30 min. The ultrafiltrate volume was measured, and the sample was diluted 1:5 with 0.1 M potassium phosphate buffer (pH 7) prior to HPLC analysis.

2.9. Urine assay

Urine was diluted 1:40 with water prior to HPLC analysis. Solvents and the assay system were the same as described above except that no trifluoroacetic acid was used in solvent A, causing the CDC peak to elute after the CDU peak.

2.10. Isolated enzyme studies

All drug solutions (CDC, CDU, cytidine, uridine, and thymidine) were made in HPLC-grade water. To determine the effect of cytidine deaminase activity on CDC, 50 μL of cytidine deaminase solution was added to 0.05 M phosphate buffer containing CDC (100 μM), and the mixture (500 μL total volume) was incubated in a 37° water bath. The reaction mixture was analyzed by HPLC as described above for plasma. Inhibition of cytidine deaminase was evaluated by adding THU (100 μM) to the enzymatic reaction mixture. The K_m value for cytidine in the cytidine deaminase preparations from freshly isolated mouse kidney, determined by UV spectrometry and calculated using Lineweaver–Burk plots, was 9.2×10^{-5} M. This value is slightly higher than those reported in the literature ($(5-7) \times 10^{-5}$ M) [8]. This discrepancy may reflect the fact that the literature value is for mouse kidney acetone powder, rather than for freshly isolated enzyme.

The second step of the metabolic process was observed by spectroscopic analysis at 280 nm, using a Beckman DU-70 system in the kinetics mode. In these experiments, 1×10^{-4} M thymidine or CDU was incubated with 10 μL thymidine phosphorylase (1% dilution of the Sigma stock in 50 mM phosphate buffer) and 0.5–50 mM phosphate buffer (pH 7). The V_{max} and K_m of thymidine or CDU with thymidine phosphorylase were calculated using Lineweaver–Burk plots.

To study the deamination and phosphorolysis steps of the cytidine metabolic pathway in sequence, 10 μL cytidine deaminase and 10 μL thymidine phosphorylase (diluted as above) were mixed with 1×10^{-4} M CDC and 0.5–50 mM phosphate buffer to a total volume of 1 mL and incubated in a water bath at 37°. At various time points, 25-μL samples were removed and diluted 1:12 in 0.8 g/mL of ammonium sulfate. These fractions were centrifuged at 8000 *g* for 10 min at room temperature prior to HPLC analysis.

3. Results

The chromatographic conditions described for analyzing plasma resulted in the elution pattern seen in Fig. 1; in this representative chromatogram the peak for CDC was at 9.7 min and that for the deamination product, CDU, at 10.2 min. There were no interfering peaks in processed samples of control mouse plasma. THU does not absorb UV light at the analytical wavelength used. Concomitant administration of THU with CDC (Fig. 1C) resulted in an increase in the characteristic peak for CDC and a decrease in that for CDU relative to administration of CDC alone. CDC was stable in plasma for at least 6 hr at 37°, as judged by recovery of 95% of the drug after incubation at this temperature.

Mice appeared to tolerate high doses of CDC alone and of CDC with THU with no obvious adverse effects. The mice were alert and exhibited normal activity following drug administration. No gross abnormalities were observed in organs such as liver and kidney. The 10-min plasma concentration of CDC in mice administered either CDC alone or CDC with 100 mg/kg of THU increased with dose, as can be seen in Table 1. The pharmacokinetic profiles determined in these exploratory studies in mice are summarized in Table 2. CDC was eliminated rapidly from plasma after the i.v. administration of 100 mg/kg, with an estimated elimination half-life of 10 min. Concomitant administration of 100 mg/kg of THU, a cytidine deaminase inhibitor, increased the amount of CDC in the plasma at any given time whether the drugs were given i.v. or i.p. For both routes of administration, the averaged

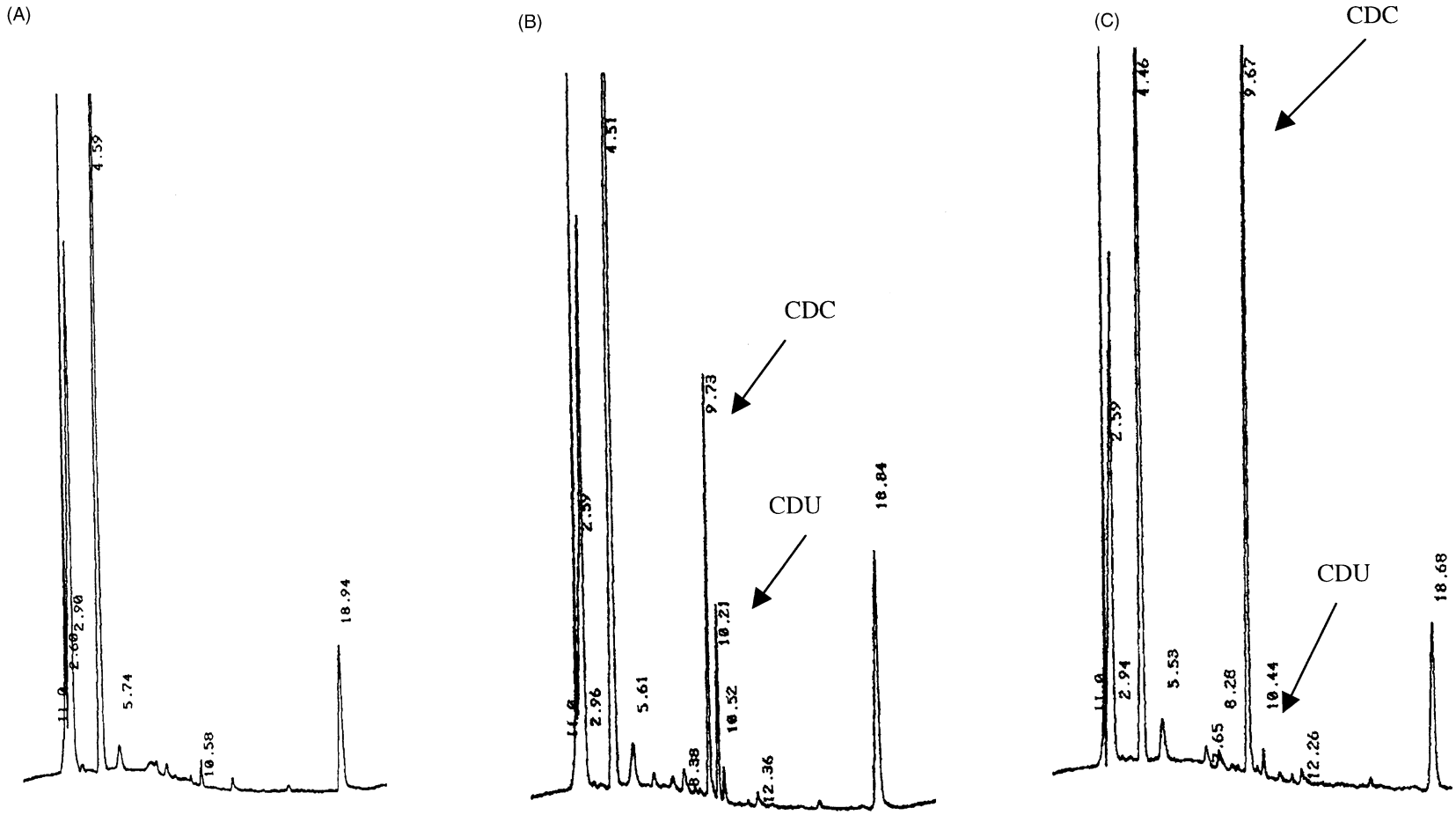

Fig. 1. HPLC analysis of mouse plasma samples. (A) Chromatogram of a blank plasma sample; (B) chromatogram 10 min after 20 mg/kg of CDC was given i.v. to normal mice; and (C) chromatogram 10 min after 20 mg/kg of CDC and 100 mg/kg of THU were given i.v. to normal mice.

Table 1
Plasma concentrations in mice of CDC administered with and without THU

Dose of CDC (mg/kg)	CDC alone (μM)	CDC + 100 mg/kg THU (μM)
5	4.8	20.5
10	14.09	N.A.
20	19.7	58.3
100	119.1 ± 17	214.3 ± 105

Plasma concentrations in mice 10 min after i.v. administration of CDC or CDC with 100 mg/kg of THU. Data for the 5, 10, and 20 mg/kg doses represent data from pilot studies, while the data for 100 mg/kg of CDC alone is the average ± SD of four studies and 100 mg/kg CDC with 100 mg/kg THU is the average ± SD of three studies. The study of 10 mg/kg of CDC with 100 mg/kg of THU is not available (N.A.). Five to nine mice were used in each study.

pharmacokinetic data show that, with THU, the area under the curve increased substantially, the half-life approximately doubled, volume of distribution decreased, and the rate of plasma clearance decreased. CDC appeared to be eliminated from plasma by first order kinetics as the half-lives of the drug given alone in doses of 5, 20, and 100 mg/kg were comparable (average 9.7 min; pilot studies). With 100 mg/kg of THU, the average half-life of these three doses was 28.6 min. Bioavailability after i.p. administration was approximately 80% when CDC was given alone. The one pilot study of CDC given with THU i.p. showed a significant increase in AUC. A pilot study in which 100 mg/kg of CDC was given i.v. 15 min after 100 mg/kg of THU given i.p. showed an even greater increase in the AUC (64,479 μM·min), compared to the i.v. studies. The half-life value found in the preincubation study (23.2 min) was approximately the same as when CDC and THU were given in the same dose. CDC was not detected in mouse plasma after oral administration.

THU inhibited CDC metabolism *in vivo*, as seen in Fig. 2. When a dose of 5 mg/kg of CDC was given alone, the plasma AUC was 113.4 μM·min; with 5 or 100 mg/kg of THU, the AUC values were 426.4 and 1179.7 μM·min, respectively. The half-life, volume of distribution, and clearance also increased with the increased dose of THU.

A pilot study was done in which 100 mg/kg of THU was given alone to determine if endogenous levels of cytidine were increased by treatment with this inhibitor. No substantial accumulations of cytidine were observed. Aliquots of plasma samples obtained in this study were incubated

Table 2
CDC pharmacokinetic data in mice

	AUC (μM·min)	$T_{1/2}$ (min)	Volume of distribution (mL)	Plasma clearance (mL/min)
CDC				
Intravenous[a]	3050 ± 265	10.3 ± 1.3	38.9 ± 7.2	2.4 ± 0.3
Intraperitoneal[b]	2450 ± 365	10.9 ± 1.6	44.1 ± 1.0	2.9 ± 0.3
CDC and THU				
Intravenous[c]	7880 ± 1920	21.6 ± 1.1	29.8 ± 6.4	0.9 ± 0.2
Intraperitoneal[d]	27,314	26.3	10.5	0.3

Pharmacokinetic data were obtained after the i.v. or i.p. administration of 100 mg/kg of CDC with and without 100 mg/kg of THU. Calculations were done using non-compartmental analysis (WinNonlin). There were 5–9 mice in each study.

[a] Average ± SEM of four studies.
[b] Average ± range of two studies.
[c] Average ± SEM of three studies.
[d] Pilot study.

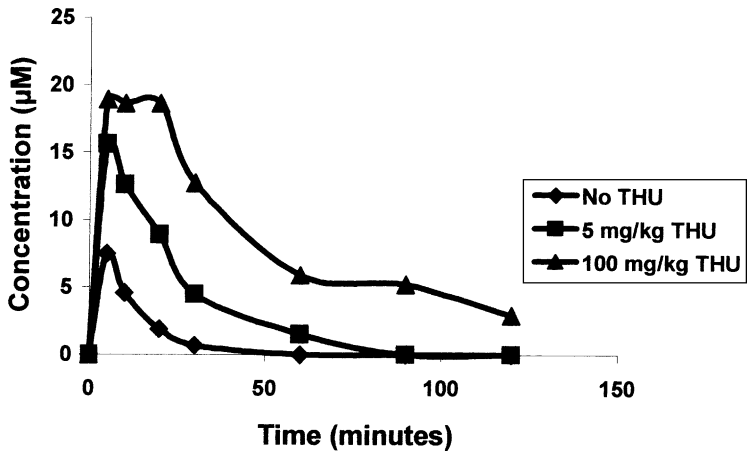

Fig. 2. Concentration–time relationship for 5 mg/kg of CDC following i.v. administration with and without THU. Representative data from one study; each point is indicative of one mouse.

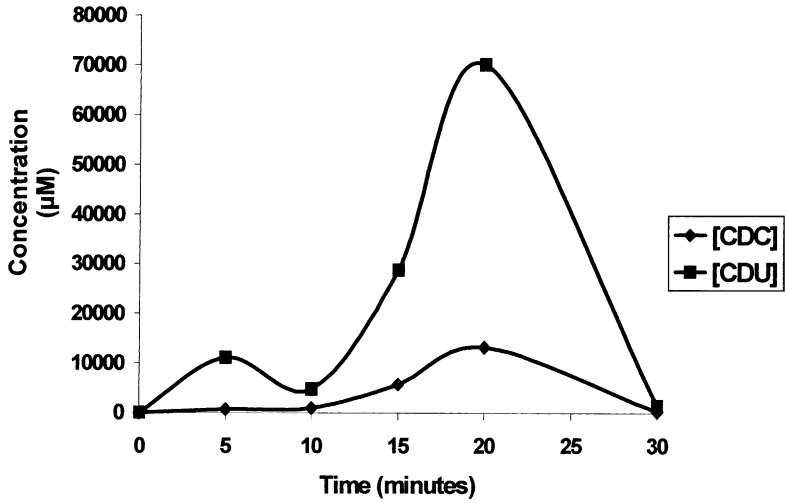

Fig. 3. Excretion of CDC and its primary metabolite, CDU, after 100 mg/kg i.v. administration in normal male mice. Representative data from one study; each point is indicative of one mouse.

with CDC and cytidine deaminase to ascertain (via HPLC analysis) the effective duration of the THU exposure. There was no CDU formation until the 1-hr plasma sample when the THU concentration was low enough to permit 0.6 μM CDU to be formed per minute compared with 6.8 μM/min in control plasma, as analyzed by HPLC.

CDC was detectable in urine samples collected after administration of CDC to mice; the most prominent species detected in urine samples was the deaminated metabolite CDU (Fig. 3). Peak urinary concentrations of parent drug and metabolite were observed around 20 min after administration, and both could be detected in urine for at least 4 hr in three studies in which 100 mg/kg of CDC was administered. When 5 mg/kg of THU was administered concurrently with 5 mg/kg of CDC, the expected preservation of CDC was noted, as exemplified by observed concentrations of 870 μM CDC in urine at 120 min as compared with no detectable drug at 90 min after injection of 5 mg/kg of CDC alone (Fig. 4).

CDU was found in the kidneys at a concentration of 156 μg/g 5 min after 100 mg/kg of CDC was given i.v., and the peak due to CDU gradually decreased until it was indistinguishable from small peaks due to endogenous substances. CDC could not be detected in processed kidney homogenates because of interference from endogenous substances. We were unable to detect CDC or CDU in liver samples, because of the presence of endogenous peaks in processed liver in the chromatographic areas of interest.

Plasma protein binding was determined by comparing the concentration of free drug in plasma after ultrafiltration to an aqueous standard containing the drug at the same concentration as the plasma. CDC binding to plasma proteins was very low in triplicate determinations, with virtually all the drug (>99%) in the free form.

In vitro studies were done for comparison with the *in vivo* studies to confirm that the metabolic pathway, seen in Fig. 5, was mediated by the expected enzymes. The conversion of CDC to CDU mediated by cytidine deaminase from fresh mouse kidney progressed at a rate of approximately 8 μM/min. This reaction was inhibited by THU, resulting in a deamination rate of approximately 3.5 μM/min. Preincubation with THU was not tested, as it is not reported to increase the inhibition of mouse kidney cytidine deaminase [9]. These studies were also performed with the enzyme preparation from mouse kidney acetone

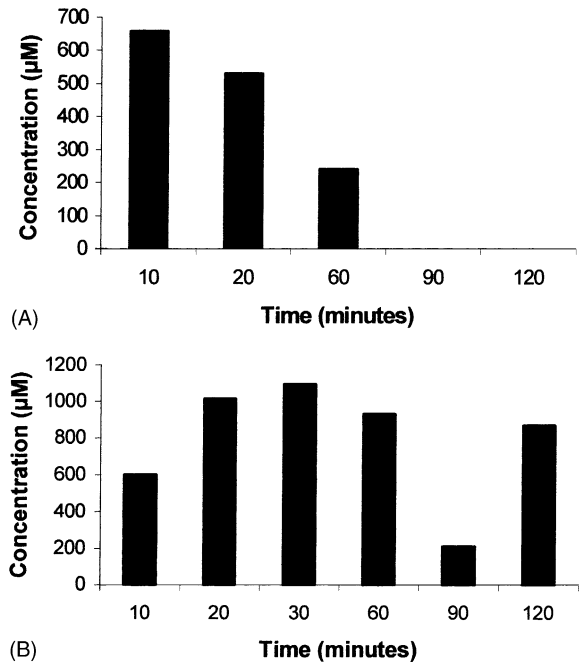

Fig. 4. Comparison of the preservation of CDC, administered with and without THU, in the urine. (A) Urine data after the i.v. administration of 5 mg/kg of CDC. (B) Urine data after the i.v. administration of 5 mg/kg of CDC and 5 mg/kg of THU. Urine samples were "clean-catch." Each bar represents an individual mouse.

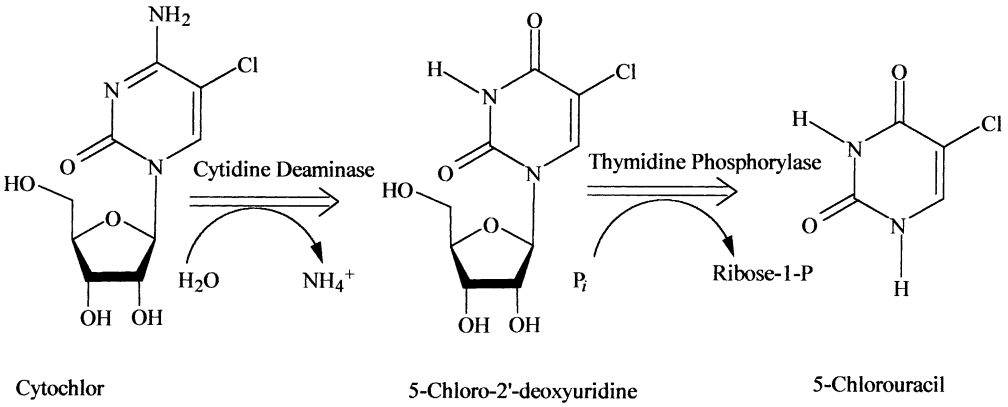

Fig. 5. First two steps of the CDC metabolism pathway.

powder. Similar results were seen with THU inhibiting the production of CDU, but the enzyme functioned at a slower rate than it did in the fresh preparation. Thymidine phosphorylase converts CDU to 5-CU by phosphorolytic removal of the sugar substituent. The K_m and V_{max} for thymidine and CDU are reported in Table 3 at three different concentrations of phosphate buffer, pH 7. The complete progression of the conversion of CDC to 5-CU over time can be seen in Fig. 6, while the rates for CDC disappearance and CDU or 5-CU appearances in each concentration of phosphate buffer are summarized in Table 4.

Table 3
Rates for thymidine phosphorylase

Sample	K_m (mM)	V_{max} (mM/min)
Thymidine in 50 mM PO_4	0.317 ± 0.01	166 ± 15
Thymidine in 5 mM PO_4	0.255 ± 0.01	127 ± 4
Thymidine in 0.5 mM PO_4	0.728 ± 0.4	131 ± 56
CDU in 50 mM PO_4	0.110 ± 0.01	143 ± 19
CDU in 5 mM PO_4	0.140 ± 0.03	225 ± 79
CDU in 0.5 mM PO_4	0.079 ± 0.01	68.5 ± 0.09

K_m and V_{max} values and for thymidine and CDU with thymidine phosphorylase when incubated at 37° 5–50 mM phosphate buffer. Data are average ± SEM of three experiments.

Table 4
Rate of CDC disappearance

PO_4 (mM)	CDC (μM/mm)	CDU (μM/min)	5-CU (μM/min)
50	−3.90	0.0016	3.17
5	−3.95	0.37	2.07
0.5	−5.16	0.61	1.44

CDC (1×10^{-4} M) was incubated with cytidine deaminase and thymidine phosphorylase in 0.5–50 mM phosphate buffer, and the rates of drug disappearance and metabolite formation were measured. Data are from one experiment at each concentration.

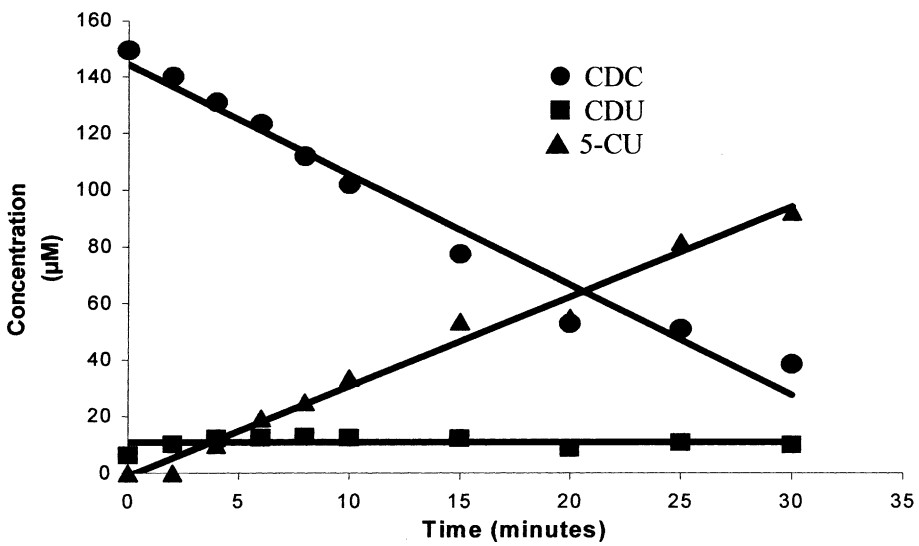

Fig. 6. Kinetics of CDC degradation and metabolite formation after 1×10^{-4} M CDC was incubated at 37° with cytidine deaminase and thymidine phosphorylase in 50 mM phosphate buffer. Representative data from one study.

4. Discussion

CDC and its primary metabolite, CDU, have similar UV spectroscopic properties, which is advantageous in that each is readily detected at the analytical wavelength of 290 nm. CDC and CDU are similar chemically, but resolution of HPLC peaks was generally good even though retention times were quite close. Although we evaluated a large number of compounds, pyrimidine and purine nucleosides as well as other compounds, we were unable to identify an appropriate internal standard and, therefore, used external standards of CDC and CDU for estimation of drug concentrations in the processed samples. CDC did not bind to a significant extent to plasma proteins. The lack of significant binding of CDC to plasma proteins parallels observations with the nucleoside gemcitabine (2′,2′-difluorodeoxycytidine), which also has negligible plasma binding to mouse, rat, and dog proteins [10].

We chose to use 100 mg/kg of CDC and THU for the majority of our *in vivo* studies. Pharmacodynamic studies in the literature have reported using 25 mg/kg of THU to inhibit CDC catabolism, but these studies also used higher doses of CDC (167–444 mg/kg) along with other metabolic modulators [2,6,11]. One study used an infusion of CDC at 628.08 mg/kg with and without THU at 1256.16 mg/kg over the course of 3 days [5]. It is hypothesized that low doses of THU may be more effective in enhancing CDC efficacy since higher doses may also inhibit the tumor enzymes [12]. CDC has good bioavailability when given to mice i.p., as seen by the similar AUC and half-life values for i.v. and i.p. administration of the drug given alone. The estimated elimination half-life of 9.7 min is comparable to the alpha phase half-life of 12 min reported for the analogous compound, ara-C, in humans [13]. Gemcitabine also has a similar plasma half-life of 17 min in humans and mice [10,14]. THU appears to have a greater effect on inhibiting metabolic processes with i.p. administration than with i.v. administration. The half-life of the combination was 30% longer and clearance was reduced in the i.p. dosing when compared with the i.v. administration. The volume of distribution remained approximately the same between the two modes of combined drug administration. Although preincubation of THU with cytidine deaminase does not enhance its inhibitory effects *in vitro* [9], our 15-min ‘‘preincubation’’ *in vivo*, with THU administered i.p. prior to i.v. administration of CDC, showed significant effects on the AUC, while the half-life remained the same compared to concomitant administration. A study of the interaction of ara-C and THU in humans involved administration of a portion of the THU dose prior to administration of ara-C, because the onset of inhibition is slow, reversible, and time-dependent even though the compound binds tightly to hepatic cytidine deaminase [15]. It is possible that the significant differences seen in the AUC when THU was given i.p. as a preincubation step, relative to the i.v. combination, are related to the onset of inhibition of CDC metabolism and/or altered distribution. It is interesting to note, in this regard, that Kreis and his colleagues [16] have reported that THU affects not only the deamination of ara-C but also its distribution. The half-life does not change in this case due to higher initial plasma concentrations of CDC related to the altered distribution of the drug, while the elimination process remains the same. The net effect is that THU significantly increases the effective plasma concentration of CDC. THU has a comparable effect in humans after i.v. administration to patients receiving ara-C, reflected in substantial increases in the plasma concentration of ara-C compared to those observed with ara-C alone [16].

CDC was not detected in plasma after oral administration. The drug is stable in dilute hydrochloric acid, pH 1, at 37° for 5 hr, suggesting that failure to detect the drug in plasma after oral administration is due to first pass metabolism and not to degradation in the stomach. Analysis of urine 60 min after oral administration of CDC showed the presence of CDU, in the 40 μM range, but no detectable CDC, supporting the idea that the drug is absorbed from the gastrointestinal tract, but is metabolized extensively in the first pass through the liver. This conclusion is consistent with observations that ara-C also has poor oral bioavailability due to rapid hepatic deamination [17]. Another study involving ara-C and ara-U looked at the activity of cytidine deaminase in liver and kidney. This study found that, although cytidine deaminase activity is higher in mouse kidney (639.5 IU/g of wet tissue), it is also significant in the liver (35.2 IU/g of wet tissue) [18]. Because THU is reported to have only 10% oral bioavailability, we did not give CDC orally with THU, although it may have been helpful, as there are bacterial gut flora having significant cytidine deaminase activity that may also decrease absorption [16]. It is possible that if the drug is given with zebularine, an orally active cytidine deaminase inhibitor [19], CDC might be detectable in plasma after oral administration.

Comparatively low doses of THU have substantial effects on the metabolism of CDC as evidenced by the similar effects observed after administration of THU at doses of 5 and 100 mg/kg. Administration of THU alone did not show a detectable increase in endogenous cytidine levels in plasma up to 1 hr after administration. Plasma from mice treated with THU inhibited CDC deamination by cytidine deaminase *in vitro*, and THU remained in the mouse plasma at concentrations sufficient to completely inhibit production of CDU for at least 1 hr. Even in the 1-hr plasma sample, there was still enough THU present to only allow a small amount of CDC to be metabolized. The elimination half-life of THU was reported to be 40 min in mice after 50 mg/kg was injected i.p. [20]. A previous study showed that 12.5–100 mg/kg of THU could completely inhibit cytidine deaminase in rhesus monkeys for up to 2 hr [16].

It is not surprising that the majority of the drug excreted through the kidneys was the deaminated metabolite. The

kidneys contain large amounts of cytidine deaminase, which is why kidneys were used to isolate the enzyme for *in vitro* work. As THU is also excreted by the kidneys (roughly 90–100% within 24 hr) [16,20], it can inhibit enzyme found in the kidney as well as cytidine deaminase in other tissues, giving rise to the increased concentration of parent drug in the urine observed when CDC and THU are given together. Gemcitabine is excreted via the kidney mainly in the uracil metabolite form in the mouse and dog, but the parent drug is the main compound found in rat urine [10]. Similarly, ara-C is excreted mainly in the ara-U form in mice, monkeys, and humans [20].

Tissue distribution studies of ara-C and its water-soluble relative, cyclocytidine (2,2′-*O*-cyclocytidine), gave results comparable to observations in our pilot tissue study. After a dose of 34 mg/kg, cyclocytidine was found at the highest concentrations in the mouse kidney 10 min after injection, with a concentration of 150 μg/g [21]. The concentration of ara-C found in mouse kidney depends upon the breed. Swiss mice have high kidney pyrimidine nucleoside deaminase activity, and the parent compound is not detected. BDF1 and AKD2F mice are presumed to have lower amounts of deaminase, as ara-C could be detected at low levels. All three types of mice have higher levels of the metabolite ara-U (compared with ara-C) in the kidneys than in other tissues; these levels were lower when ara-C was given with THU [20].

It is possible that some of the parent and metabolite compounds in the tissues converted to their nucleotides. A study of the pharmacokinetics of ara-C demonstrated that small amounts of nucleotides were located in AKD2F mouse livers and kidneys after 2 hr, and there were appreciable concentrations of nucleotides in the livers of Swiss mice after 30 min [20]. In each case, however, the concentration of ara-C or ara-U, depending on the presence of THU, was much greater than the concentration of nucleotides [20].

HPLC methodology was required for the *in vitro* enzyme work with CDC and its metabolite, CDU, because both compounds have similar ultraviolet absorption properties, thus obviating the use of conventional spectroscopic analysis. In the presence of THU, the rate of enzymatic deamination of CDC was decreased by nearly 50% for both the fresh kidney and the acetone powder enzyme preparations. These observations parallel those made *in vivo* where equal amounts of CDC and THU nearly doubled the half-life of CDC.

Thymidine phosphorylase is reported to have its maximal activity at a pH of 6.0 *in vitro*. Thymidine phosphorylase from *Lactobacillus casei* in 300 mM sodium phosphate buffer, pH 6.0, gave a K_m value of 1.32 mM for thymidine [22]. Thymidine phosphorylase from horse liver with thymidine is reported to have a K_m value of 0.11 mM in 0.1 M potassium phosphate buffer at pH 7.0 [23]. Despite the fact that we used a pH of 7.0, which is closer to the body's pH, and lower concentrations of phosphate buffer, our data appear to be comparable to published results. CDU is reported to have a K_m of 0.186 mM when thymidine phosphorylase is in 0.1 M potassium phosphate at pH 6.0 [24], which is similar to our results. Three different concentrations of phosphate buffer were used because the related enzyme, uridine phosphorylase, shows complex product inhibition in the presence of phosphate buffer at concentrations greater than 3 mM [25]. However, product inhibition was not observed with thymidine phosphorylase using either thymidine or CDU in any of the three concentrations of phosphate buffer.

Both cytidine deaminase and thymidine phosphorylase were incubated with CDC so that both steps of the metabolic progression could be seen at once. Although some variation was observed in the rates of enzymatic activity associated with the different concentrations of phosphate, the metabolic process seen in the mouse studies was effectively modeled *in vitro*.

In conclusion, CDC has potential as a radiosensitizing drug. It has a short plasma elimination half-life and is excreted by the kidney, mainly in metabolite form. THU substantially increased plasma levels of CDC in the mice, supporting proposals that the combination should be evaluated in clinical trials of CDC as a radiosensitizing agent.

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