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REVERSAL BY CYTIDINE OF CYCLOPENTENYL CYTOSINE-INDUCED TOXICITY IN MICE WITHOUT COMPROMISE OF ANTITUMOR ACTIVITY

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Abstract—Among nine compounds surveyed, cytidine was found to be the most effective in reversing the antiproliferative effects of cyclopentenyl cytosine (CPEC) on human T-lymphoblasts (MOLT-4) in culture. Cytidine, at concentrations of 1–25 μM, enabled cells to maintain normal logarithmic growth when added up to 12 hr after exposure to a 200 nM concentration of the oncolytic nucleoside, CPEC. The most abundant CPEC metabolite, CPEC-5′-triphosphate, is a potent [$K_i \approx 6$ μM] inhibitor of CTP synthetase (EC 6.3.4.2). Accumulation of this inhibitor resulted in a depletion of CTP levels to 17% of their original cellular concentration. Exogenous cytidine reversed CPEC-induced cellular cytotoxicity by suppressing the formation of CPEC-5′-triphosphate by 70%, and by partially replenishing intracellular CTP to at least 60–70% of its original concentration. *In vivo*, cytidine (500 mg/kg) administered intraperitoneally 4 hr after each daily dose of CPEC (LD_{10}–LD_{100}) for 9 days reduced the toxicity and abolished the lethality of CPEC to non-tumored mice. Of greater practical importance is the finding that, under these experimental conditions, cytidine did not curtail the antineoplastic properties of CPEC in L1210 tumor-bearing mice. Moreover, the concentration range over which CPEC exhibited antineoplastic activity was extended with cytidine administration.

Key words: cyclopentenyl cytosine; cytidine; MOLT-4 lymphoblasts; antidotes; L1210 leukemia; antimetabolite

Preclinical studies with CPEC‖ have shown that its spectrum of antitumor activity includes murine and human leukemias and certain solid human tumor xenografts (i.e. lung, colon and breast) [1–3]. The antineoplastic properties of this agent are attributable to its most abundant cellular metabolite, CPEC-TP¶, which inhibits CTP synthetase (EC 6.3.4.2) leading to a profound depletion of CTP and dCTP pools [2, 4]. Toxicological studies with CPEC have demonstrated that bone marrow and gastrointestinal epithelium are the principal sites of toxicity produced by its parenteral administration to beagle dogs [5]; a similar pattern of toxicity has been reported in the currently ongoing Phase I clinical trial with CPEC. Granulocytopenia and thrombocytopenia have been cited as the dose-limiting toxicities in human subjects given CPEC as a 24-hr infusion. Other toxicities included anemia, vomiting, mucositis, diarrhea and hypotension [6]. In previous studies, we and other investigators reported that certain physiologic nucleosides and their analogs were capable of reversing the *in vitro* cytotoxicity of CPEC [3, 4, 7]. The current study further examines the usefulness of these pyrimidines as reversing agents, and establishes the boundaries of their antidotal action against CPEC-induced toxicity to cultured MOLT-4 lymphoblasts. Additionally, utilizing the murine L1210 leukemia system, we have investigated the possibility that cytidine, the best of these reversing agents, could be used to moderate CPEC toxicity *in vivo* without compromising its antineoplastic activity. A preliminary account of these studies has appeared [8].

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‖ Abbreviations: ara-C, arabinofuranosyl cytosine; CPEC, cyclopentenyl cytosine; CPEU, cyclopentenyl uracil; CPEC-TP, CPEC-5′-triphosphate; dCTP, 2′-deoxy-CTP; 5-FU, 5-fluorouracil; ILS, increase in life span; LCK, $\log_{10}$ tumor cell kill; LD_{10}, dose lethal to 10% of animals; LD_{100}, dose lethal to 100% of animals; and NBMPR, nitrobenzylthioinosine.

¶ CPEC-5′-triphosphate (CPEC-TP) has become the accepted biological abbreviation for the triphosphorylated form of this nucleotide analog; however, according to chemical nomenclature this 5-carbon cyclopentenyl nucleoside analog would be designated CPEC-6′-triphosphate.

MATERIALS AND METHODS

Chemicals. CPEC (NSC 375575), CPEU (NSC

375574), zebularine* (NSC 309132) and tetrahydrouridine were obtained from Dr. V. Narayanan, Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program (DTP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI). [5-^{3}H]CPEC (sp. act. 15 Ci/mmol), synthesized under contract at the Research Triangle Institute (Research Triangle Park, NC), was provided by Dr. R. Haugwitz, Drug Synthesis and Chemistry Branch, DTP, DCT, NCI. Cytidine, uridine, 2′-deoxycytidine, 2′-deoxyuridine, NBMPR and orotidine were purchased from the Sigma Chemical Co. (St. Louis, MO).

In vitro *rescue studies.* MOLT-4 lymphoblasts in logarithmic growth were cultured at 37° in RPMI-1640 medium supplemented with 10% newborn calf serum and 50 μg/mL gentamycin sulfate in an atmosphere of air:CO_2 (95:5). MOLT-4 cells (1×10^5 cells/mL) were treated with a range of CPEC concentrations (200–1000 nM), and the putative antidote (or an equivalent aliquot of normal saline) was added at the concentration and times indicated. Cells were counted with a model ZB1 Coulter Counter, just prior to and 24 hr after the addition of CPEC. Cell doubling-time was determined using the exponential growth equation, $C_t = C_0 e^{kt}$ (where C_0 and C_t are the cell densities at times zero and t, respectively, and k is a rate constant).

In vivo *rescue studies.* Antitumor studies were conducted according to standard NCI L1210 protocols [9]. Female BALB/c × DBA/2 F_1 (hereafter called $CD2F_1$) mice and L1210 cells were obtained from Dr. J. Mayo, Biological Testing Branch, DTP, NCI). Mice weighing 18–21 g were inoculated subcutaneously in the axillary region with 1×10^5 L1210/0 cells. Compounds in normal saline were administered intraperitoneally in an injection volume of 0.5 mL. All treatments were administered based on the average body weight of a given dosage group (8–10 mice). Dosage and schedule are discussed in the Results. Mice were monitored for survival, toxicity (weight changes) and ILS relative to untreated controls on a daily basis. The ILS was calculated as previously described [9]. Calculations of net $\log_{10}$ units of tumor cells killed were made from the tumor-doubling time determined *in vivo* with a concomitantly run tumor titration [10].

Pharmacokinetic studies in mice. Normal male $CD2F_1$ mice, weighing 18–25 g, were fasted overnight and then inoculated intraperitoneally with a single dose of cytidine in normal saline (500 mg/kg) or normal saline at 1% body weight. At timed intervals, the mice were anesthetized with ether, and blood was drawn from the inferior vena cava into a heparinized syringe containing 10 μL of 5 mM tetrahydrouridine, a cytidine deaminase inhibitor [11]. Plasma was separated by prompt centrifugation; cytidine and uridine concentrations were then determined by reverse phase HPLC using a Vydac octadecylsilane column (250 × 4.6 mm, 10 μm particle size) with a mobile phase of 0.1 M ammonium formate, pH 5, pumped at a flow rate of 1 mL/min.

Determination of ribonucleotide pools. Ribonucleotide pools were determined in heat-inactivated 60% methanolic extracts of MOLT-4 cells by ion-exchange HPLC methods as described previously [4].

Statistical analysis. The Wilcoxon rank-sum test and a user-defined transform to compare two Kaplan–Meier survival curves were each used to compare the %ILS value for mice given CPEC 1.5 mg/kg (see Table 3), or 1.28 mg/kg (see Table 4) with the %ILS values for all combination groups of CPEC and cytidine.

* Zebularine (2-oxopyrimidine riboside), the "deamino" analog of cytidine (2-oxo-4-amino pyrimidine riboside), is a potent inhibitor of murine cytidine deaminase ($K_i = 2 \times 10^{-6}$ M).

Table 1. Effects of antidotes added simultaneously with CPEC on the doubling-time of MOLT-4 lymphoblasts

Treatment	Doubling-time (hr)
Saline	30 ± 1
CPEC	192 ± 37
CPEC + deoxycytidine	68 ± 4
CPEC + deoxyuridine	182 ± 29
CPEC + orotidine	199 ± 60
CPEC + zebularine	234 ± 56
CPEC + cytidine	31 ± 2
CPEC + uridine	30 ± 3
CPEC + CPEU	32*
CPEC + nitrobenzylthioinosine	33 ± 1
CPEC + dipyridamole	33 ± 2

MOLT-4 cells (1×10^5 cells/mL) were treated simultaneously with 200 nM CPEC and 25 μM antidote. Cell density was measured just prior to, and 24 hr after the addition of antidote. Values are the means ± SD of three experiments. Cell doubling-times determined in the presence of each counteragent alone did not vary more than 10% from those of the controls.

* Average of two experiments.

RESULTS

Rescue studies in MOLT-4 lymphoblasts. In our previous study [4], we demonstrated that both cytidine and NBMPR, presumably acting through different mechanisms, could ameliorate the toxic effects of CPEC toward MOLT-4 cells. The present study was performed to include seven additional agents. For this study, MOLT-4 cells in logarithmic growth were incubated with 200 nM CPEC alone or simultaneously with a 25 μM concentration of the potential counteragents (Table 1). In the presence of CPEC alone, MOLT-4 lymphoblasts showed a 4-fold increase in doubling-time, the parameter used to indicate toxicity. However, cells exposed additionally to five of the potential counteragents—cytidine, uridine, CPEU, NBMPR and dipyridamole—proved capable of sustaining logarithmic growth over a 24-hr period at a rate that was indistinguishable from control samples. By contrast, 2′-deoxyuridine, zebularine and orotidine were ineffective as counteragents under these experimental conditions, while 2′-deoxycytidine was only moderately effective.

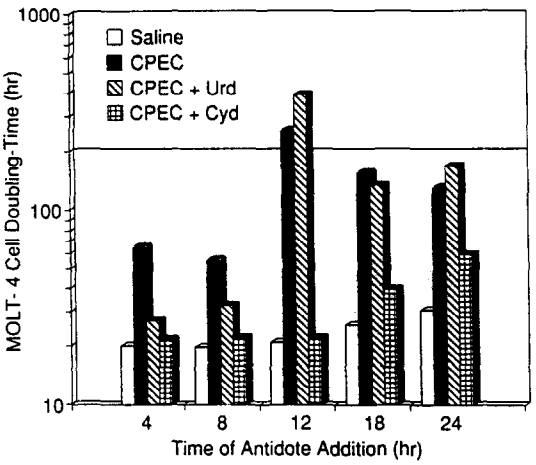

Fig. 1. Time-course of antidote rescue. MOLT-4 cells (5×10^5 cells/mL) in logarithmic growth at 37° were incubated with 200 nM CPEC. The addition of 25 μM reversing agents (cytidine, uridine, normal saline) was delayed 4, 8, 12, 18, or 24 hr after CPEC. Cellular doubling-times were determined just prior to the addition of CPEC, and 24 hr after each antidotal addition. Values shown are the averages of two experiments.

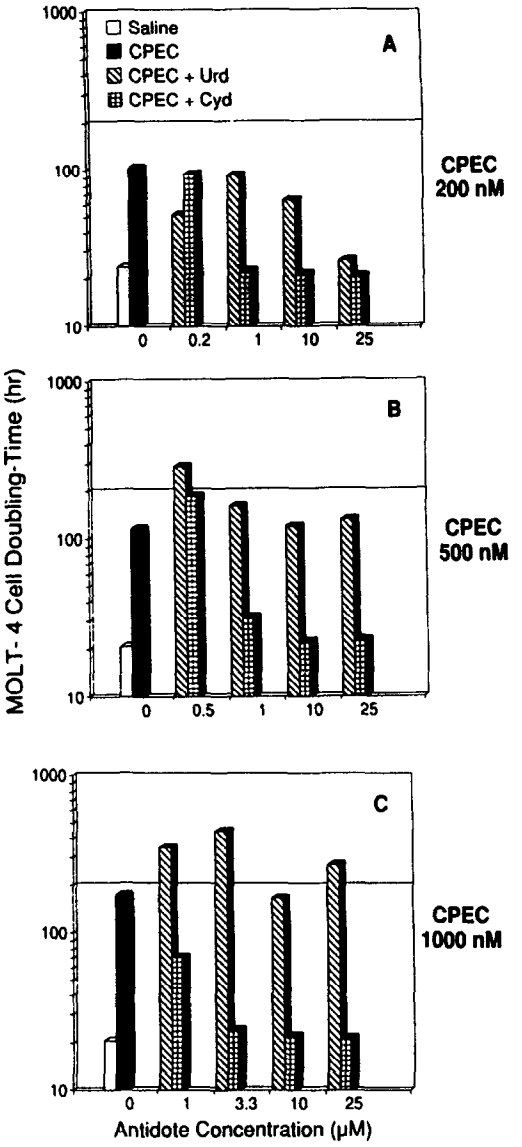

Fig. 2. Concentration–response to antidote rescue. Logarithmically growing MOLT-4 cells were exposed to (A) 200, (B) 500 or (C) 1000 nM CPEC for 4 hr at 37°. Cells were then counted, and counteragents were added at the indicated concentration. Cells were counted after 24 hr, and cell doubling-time was determined. Values shown are from a single experiment. A second experiment yielded essentially similar results.

Next examined was the question of whether the delayed addition of the effective antidotes was capable of preserving normal cell doubling-time after cellular exposure to CPEC. For this purpose, MOLT-4 cells were treated with 200 nM CPEC followed by the addition of the best reversing agents (cytidine, uridine, CPEU, NBMPR and dipyridamole) at 4, 8, 12, 18 and 24 hr. Cell densities were determined just prior to, and 24 hr after the addition of the potential antidote. Of the compounds tested, cytidine, uridine (Fig. 1) and CPEU (data not shown) were all effective when added 4 hr after CPEC, each permitting normal cell proliferation. Under these conditions, NBMPR and dipyridamole were markedly less effective and thus have not been studied further. When antidote addition was delayed for 8 hr after CPEC treatment, only cytidine permitted normal cellular doubling in the presence of 200 nM CPEC. When the addition of uridine was delayed for this same time period, the doubling-time was 33 hr (1.6 times longer than control samples). Cytidine, the most promising of these counteragents, permitted the maintenance of normal logarithmic growth when added as late as 12 hr after CPEC. However, this efficacious effect was diminished when addition of this nucleoside was delayed 18 hr, yielding an average doubling-time twice that of control samples.

Next, counteragents that exhibited some potential for rescue in delayed-addition experiments were tested to determine the concentration range over which their protective effect was expressed. To evaluate this point, MOLT-4 cells were incubated with 200, 500 and 1000 nM CPEC, and 4 hr later were exposed to concentrations of reversing agents ranging from 200 to 25,000 nM, as shown in Fig. 2. It was found that cytidine was protective over the broadest concentration range. Thus, at 200, 500 and 1000 nM CPEC, maintenance of logarithmic growth was achieved when the concentration of cytidine used was 1, 10, and 25 μM, respectively. By contrast the concentration of uridine and cyclopentenyl uracil (data not shown) required to restore a normal growth rate in cells exposed to 200 nM CPEC was 25 μM in both cases.

To better understand the influence of cytidine in restoring growth to cells exposed to CPEC, an additional experiment was performed where cell

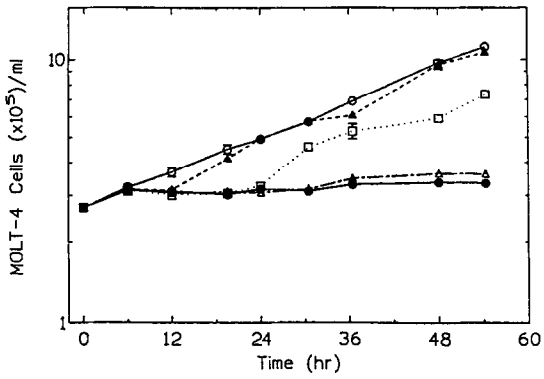

Fig. 3. Effect of cytidine on CPEC-induced cell growth inhibition. Logarithmically growing MOLT-4 cells (2.7×10^5 cells/mL, 50 mL) were exposed to 100 μL of physiologic saline (○) or 200 nM CPEC (●). To designated CPEC-containing vessels, cytidine (25 μM) was added at 6 (▲), 12 (□) or 20 hr (△). Cell counts were made at indicated intervals up to 54 hr after addition of CPEC. The data shown (means ± SD) are from triplicate determinations. MOLT-4 cells incubated with cytidine (25 μM) alone (data not shown) exhibited cell counts virtually identical to cells treated with saline above.

Table 2. Effects of antidotes on cellular CTP and CPEC-TP pools in CPEC-treated MOLT-4 cells

Treatment	% of Control	
	CTP	CPEC-TP
Control*	100 ± 10	
Cytidine	149 ± 11	
Uridine	147 ± 13	
CPEC	17 ± 1	100 ± 14†
CPEC + cytidine at 0 hr	77 ± 13	28 ± 3
CPEC + cytidine at 4 hr	64 ± 11	23 ± 4
CPEC + uridine at 0 hr	51 ± 3	32 ± 3
CPEC + uridine at 4 hr	68 ± 19	29 ± 6

MOLT-4 cells (5×10^5 cells/mL) were treated with 200 nM [^{3}H]CPEC and 10 μM cytidine or uridine at the times indicated. Cells were incubated for 24 hr following the addition of CPEC after which cells were counted and intracellular contents extracted. Values are the means ± SD of three experiments. Nucleoside triphosphate pools were determined in 60% methanolic extracts of MOLT-4 cells by HPLC analysis using on-line UV–visible spectrophotometry and liquid scintillation counting.

* Endogenous levels of CTP and UTP in control samples were 0.41 ± 0.04 and 1.27 ± 0.05 nmol/10^6 cells, respectively. In all experiments with CPEC, UTP levels increased to 145–185% of control values.

† Cellular levels of CPEC-TP were 59.3 ± 7.9 pmol/10^6 cells.

growth was monitored periodically during a 54-hr exposure to saline, 200 nM CPEC or CPEC followed by cytidine (25 μM) at 6, 12, or 20 hr (Fig. 3). In control experiments, MOLT-4 cell growth was exponentially uniform (2.7×10^5 to 11.2×10^5 cells/mL, doubling-time 25.9 hr) showing a 315% increase in cell number over 54 hr. Exposure of these cells to 200 nM CPEC retarded growth substantially (after an ~ 6-hr normal growth period) allowing only a 23% increase in cell number (2.7×10^5 to 3.3×10^5 cells/mL, doubling-time 243 hr) in 54 hr. Cytidine added 6 hr after CPEC allowed logarithmic growth to resume—after an initial lag period—6 hr following the addition of the antidote (doubling-time 24.8 hr). Similarly, cytidine added to cultured cells previously exposed to CPEC for 12 hr restored cellular growth in an essentially logarithmic fashion 8–12 hr after the addition of the antidote, permitting a doubling-time of 30 hr. However, when cytidine was added 20 hr after CPEC, exponential growth was not restored to MOLT-4 cells so treated.

Metabolic effects of cytidine rescue. The effect of exogenous cytidine on CTP and CPEC-TP concentrations in MOLT-4 lymphoblasts was determined. As shown in Table 2, cells incubated for 24 hr with 200 nM CPEC exhibited an 83% depletion of intracellular CTP and a mean accumulation of CPEC-TP to 59.3 ± 7.9 pmol/10^6 cells (mean ± SD). Cytidine (10 μM), added simultaneously with 200 nM CPEC, restored the CTP level to 77% of the original value while reducing cellular CPEC-TP to 28% of the concentration reached when CPEC was used alone. When the addition of cytidine was delayed 4 hr, restoration of CTP was slightly less pronounced, increasing to only 64%; intracellular CPEC-TP reached 23% of the levels achieved when no antidote was present. Uridine showed a generally similar ability to slow CPEC-TP accumulation while partially restoring CTP levels to more than half their normal levels.

Rescue studies in mice. To determine if cytidine rescue was operative *in vivo*, non-tumored $CD2F_1$ mice, as well as mice with subcutaneously implanted L1210/0 ascites tumor cells, were treated once daily for 9 days with intraperitoneal injections of either CPEC alone or CPEC in combination with cytidine administered 4 hr after each CPEC treatment (Table 3).

In non-tumored animals, cytidine administered in this manner clearly abolished the lethality of CPEC over a range of doses that spanned the LD_{10} to the LD_{100} of the antimetabolite alone (Table 3). In addition, amelioration of CPEC-induced toxicity was demonstrated further as adjudged by weight loss. Mice given daily injections of CPEC alone at 2.08 mg/kg for 9 days showed a 7 g weight loss (33% of total body weight) on day 9. When cytidine (500 mg/kg) was given as adjunct therapy 4 hr after CPEC, mice exhibited only a 2 g weight loss during the same time period. By day 13, mice given this combination of CPEC and cytidine recovered their original body weight. However, seven of the eight mice given CPEC alone (2.08 mg/kg) expired by day 11 (data not shown).

In tumored mice, the antineoplastic properties of CPEC were neither diminished nor enhanced by cytidine treatment delayed 4 hr. Cytidine therapy, however, appeared to extend the therapeutic range of CPEC, in addition to increasing the number of 60-day survivors, especially at the lower CPEC doses (Table 3). Statistical analysis comparing the %ILS

(%ILS, calculated from dying mice only and not long-term survivors) from CPEC given alone (1.5 mg/kg) with the %ILS for all CPEC/cytidine combinations showed only one combination (2.44 mg/kg CPEC + 500 mg/kg cytidine) to be statistically different ($P = 0.026$, Wilcoxon rank-sum test; $P = 0.053$, user-defined transform to compare Kaplan–Meier survival curves).

A second experiment in tumored mice was performed, which further substantiated these conclusions (Table 4). Here again the number of long-term survivors was increased compared with mice not given cytidine. The modest increases observed in %ILS when CPEC was administered at doses of 1.76 to 2.44 mg/kg in combination with cytidine were, however, not significantly different compared with %ILS produced when CPEC was given at 1.28 mg/kg. The inverse trend seen in both experiments (Tables 3 and 4) where the ability of cytidine explicitly improved the number of survivors at lower CPEC doses, although not significant, proved to be reproducible. For Table 3, all animals that died between days 31 and 43 (no deaths after day 43) had gross evidence of disease. This was true of both the CPEC-treated and the CPEC/cytidine-treated groups. For Table 4, there were no deaths after day 27. Thus, such long-term survivors may have represented complete cures.

Plasma cytidine–uridine levels in mice. To determine the plasma levels of cytidine being reached in the *in vivo* experiments, and to permit comparison between these and the levels used in the cytidine-reversal experiments *in vitro*, plasma cytidine was measured following a single intraperitoneal dose of cytidine (500 mg/kg) in non-tumored mice. Cytidine administered in this manner resulted in a peak plasma cytidine concentration of 36.4 ± 18.2 μM at 10 min. Thereafter, the concentration of cytidine in plasma decreased rapidly ($T_{\frac{1}{2}} = 4.2$ min), reaching normal levels (2.6 ± 0.51 μM) after 60 min. Subsequently, plasma uridine levels increased 2-fold, peaking 15 min post-injection and thereafter decreasing more slowly than cytidine, with a return to normal levels by 130 min post-treatment.

DISCUSSION

Naturally occurring pyrimidine nucleosides have been used successfully to limit the toxicity and increase the therapeutic index of a number of antimetabolites. Patients treated with methotrexate, ara-C, or 5-FU in combination with thymidine show a modulation and often a decrease in drug-induced toxicities [12]. Clinical studies have shown that uridine, also, clearly reduces the toxic effects of 5-FU [13]. Similarly, 2′-deoxycytidine has been shown to limit host toxicity to lethal doses of ara-C [14] and to protect bone marrow progenitor cells against ara-C or gemcitabine (2′-difluorodeoxycytidine) induced toxicity *in vitro* [15]. Although we and other investigators have demonstrated the usefulness of cytidine for *in vitro* protection from CPEC-induced cytotoxicity [3, 4, 7], we have now been able to extend these observations to an *in vivo* system by showing that cytidine protects mice against otherwise lethal doses of CPEC and modulates its toxicity, without compromising the oncolytic properties of the drug.

The mechanism mediating the protective effect of cytidine is likely multifactorial. Commencing at cellular entry, cytidine can compete with, and thus interfere with, the access of CPEC to what is likely a shared facilitated nucleoside transport mechanism, which is susceptible to potent inhibition by both NMBPR [4] and dipyridamole [7]. Cytidine can also interfere with the initial CPEC phosphorylation step, a reaction catalyzed by uridine–cytidine kinase (EC 2.7.1.48) [2]. This enzyme converts cytidine, uridine, and CPEC to their respective 5′-monophosphates, and has an apparent binding constant (K_m), which favors cytidine three times over CPEC, but can be competitively inhibited by the latter (K_i 144 μM) [2, 16]. With exposure to high exogenous cytidine concentrations, competition in this initial phosphorylation step (and probably successive steps) is likely to favor the natural substrate over the cytosine analog, and ultimately leads to partial replenishment of CTP levels at the expense of CPEC-TP. Thus, direct competition for transport and/or phosphorylation may likely predominate and result in a *prevention* of CPEC-induced cytotoxicity by reducing formation of CPEC-TP when exposure of the antimetabolite and cytidine is concurrent.

However, with delayed addition of cytidine a *rescue* mechanism may predominate. Several factors should be considered. First, we have demonstrated, herein, that cell growth is arrested 6 hr after a single exposure to 200 nM CPEC and cells are maintained in essentially a cytostatic state for at least 54 hr. Second, a rapid and sustained CPEC-induced depletion of intracellular CTP levels—75–80% within 4 hr ($T_{\frac{1}{2}}$ 1.4 hr)—has been described previously [4, 17]. Third, intracellular CPEC-TP formation is rapid and plateaus within 4–12 hr in MOLT-4 or L1210 depending upon dose and cell type [4, 18]. Fourth, facilitative transport inhibitors (NMBPR and dipyridamole) adequate to protect cells when simultaneously given with CPEC, offer markedly less protection when administration is delayed just 4 hr. Similarly, described herein and elsewhere [18], CPEU can prevent cytotoxicity only if addition is made within 4 hr after CPEC, after which antidotal properties are rapidly attenuated. The limited rescue properties of CPEU have been attributed to inhibition of uridine–cytidine kinase with decreased formation of CPEC-5′-monophosphate [18]. Thus, CPEC exerts its cytotoxicity within hours through formation of sufficient and persistent amounts of the active metabolite (CPEC-TP, *vide infra*), which results in a significant inhibition of CTP synthetase and dramatic reduction of CTP. Since this CPEC-induced cytotoxicity can be reversed *in vitro* up to 12–18 hr with cytidine, cytidine delayed-rescue is likely attributable to relieving enzymatic inhibition (*vide infra*) with production of the natural nucleotide, CTP.

Additionally, cytidine deaminase (EC 3.5.4.5) and 2′-deoxycytidylate deaminase (EC 3.5.4.12) provide additional rescue pathways by which cytidine or 2′-deoxycytidine-5′-monophosphate (after deamination) can generate uridine nucleotides (and ultimately UTP) which, in turn, can directly relieve

Table 3. Effects of cytidine on the L1210 antitumor activity and toxicity of CPEC in mice

Compound	Dose (mg/kg/injection)	Tumored mice* (antitumor activity) %ILS‡	LCK§	60-Day survivors per 10 mice	Non-tumored mice† (toxicity) Weight change‖ (g)	30-Day survivors per 8 mice
CPEC	2.44	−10	Toxic	0	−5	0
	2.08	−10	Toxic	0	−7	1
	1.76	+45	Toxic	0	−5	2
	1.50	+90	+1.0 (+5.1)	1	−6	4
	1.28	+90	+1.0 (+5.1)	1	−3	7
Cytidine	500	0	−3.1	0	0	8
CPEC + 500 mg/kg cytidine	3.38	+100	+1.5	0	−3	8
	2.87	+109	+2.0 (+5.1)	1	−3	8
	2.44	+118	+2.5 (+5.1)	1	−3	8
	2.08	+113	+2.3 (+5.3)	2	−2	8
	1.76	+109	+2.0 (+5.4)	3	−2	8
	1.50	+63	−0.5 (+5.5)	4	−2	8

* Single drug treatment: groups of 10 mice were inoculated (s.c.) with 10^5 L1210/0 ascites cells on day 0. An undivided i.p. dose of CPEC or cytidine was administered beginning on day 1 for nine consecutive days (QD 1–9). Drug combination treatment: CPEC was administered as described in the single drug treatment. Cytidine was administered as an undivided i.p. dose 4 hr after each CPEC dose.

† Toxicity test conditions were the same as described for tumor tests under the previous footnote without L1210 inoculation.

‡ Median percent increase in life span relative to untreated controls based on median day of death (dying mice only, 60-day survivors excluded).

§ Net $\log_{10}$ tumor cell kill for dying mice. Figures in parentheses include data from 60-day survivors. A value of +3 means a 99.9% decrease in tumor cell burden at the end of therapy.

‖ Difference in mean weights between day 9 and day 1.

Table 4. Effects of cytidine on the L1210 antitumor activity and toxicity of CPEC in mice: experiment 2*

Compound	Dose (mg/kg/injection)	Tumored mice* (antitumor activity) %ILS	LCK	60-Day survivors per 10 mice
CPEC	2.44	−5	Toxic	0
	2.08	0	Toxic	0
	1.76	+9	Toxic	0
	1.50	+63	Toxic	0
	1.28	+72	0 (+5.1)	1
Cytidine	500	0	−3.1	0
CPEC + 500 mg/kg cytidine	3.38	+72	0 (+5.1)	1
	2.87	+86	+0.7	0
	2.44	+72	0 (+5.1)	1
	2.08	+72	0 (+5.1)	1
	1.76	+63	−0.5 (+5.3)	2
	1.50	+63	−0.5 (+5.4)	3

* See footnotes to Table 3.

the competitive inhibition of CTP synthetase engendered by CPEC-TP [2].

Of the nine compounds tested in the present study as rescue agents, cytidine showed the best potential as a delayed-rescue agent. Yet, its rescue potential was lost when treatment with cytidine was deferred longer than 18 hr *in vitro*. The disappearance of the cytidine rescue phenomenon in MOLT-4 cells may

reflect an accumulation of intracellular CPEC-TP (and/or a build-up of aberrant RNA with incorporated CPEC), which is refractory to reversal by cytidine. CPEC is capable of massive intracellular accumulation as the 5′-triphosphate—to hundreds of times its extracellular concentration [2, 4]. This feature, coupled with the long intracellular half-life of CPEC-TP (9–14 hr) [4], may cooperate to produce a sustained toxic insult to cells and may explain why cytidine rescue is limited to 18 hr. Indeed, Yee *et al.* [7] have shown *in vitro* that about 50% of the intracellular CPEC-TP persists 24 hr after CPEC is removed from the medium and that the triphosphate persists in detectable concentrations up to 4 days after washout. These same investigators have also shown that CPEC induces preferential accumulation of CPEC-containing low molecular weight RNA in colon cancer cells. Once formed, these RNA species are little influenced by the administration of cytidine 3 hr after CPEC exposure [7].

The cytotoxic effects of another, less potent, CTP synthetase inhibitor, 3-deazauridine, can also be reversed by cytidine and to a lesser extent by uridine. As is the case with CPEC-5′-triphosphate, 3-dezauridine-5′-triphosphate rapidly depletes intracellular CTP and dCTP levels and interferes with DNA and RNA synthesis [19].

Studies evaluating the safety of human cytidine administration, at concentrations likely to achieve protection are lacking, although European investigators have shown that low intravenous doses of cytidine (450–750 mg/day) can be administered safely to patients [20, 21]. However, Phase I studies of two likely metabolites of cytidine have been performed. Both single (10–12 g/m^2) and multiple-dose (5 g/m^2 every 6 hr for 3 days) oral uridine studies achieved plasma uridine concentrations as high as 60–80 μM and 50 μM, respectively, with diarrhea being the dose-limiting toxicity [22]. 2′-Deoxycytidine given as a 120-hr infusion at 22.5 g/m^2/day achieved steady-state plasma levels to 100 μM with a concomitant elevation of 2′-deoxyuridine plasma levels to 114 μM with no apparent toxicity [23].

In summary, we have shown that cytidine can act as a rescue agent for CPEC-induced toxicity in an animal model. Additional experiments should be performed to define the dose and schedule that optimize cytidine rescue *in vivo*. In light of the amount of exogenous cytidine used in our *in vivo* rescue studies, we agree with Dahut *et al.* [24] that physiologic levels of cytidine found in mice (1.5 to 2.5 μM) or humans (0.5 μM) would probably be insufficient to abrogate CPEC toxicity. Higher levels than these, however, whether due to dietary or pharmacologic factors, could materially modulate the toxicity of CPEC, with the distinct likelihood that oncolytic activity will not, at the same time, be abrogated.

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