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MECHANISM OF RESISTANCE TO CYCLOPENTENYL CYTOSINE (CPE-C) IN MOLT-4 LYMPHOBLASTS

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Abstract—Cyclopentenyl cytosine (CPE-C), a carbocyclic analogue of cytidine, has preclinical antineoplastic activity against ara-C resistant murine leukemias and a broad spectrum of human tumor xenografts. CPE-C is a prodrug and requires intracellular phosphorylation to cyclopentenyl cytosine triphosphate (CPE-CTP) which depletes endogenous CTP pools. The initial step in this activation process is catalyzed by uridine/cytidine kinase. We studied the mechanism of resistance to CPE-C in a Molt-4 T-cell leukemia line made resistant to CPE-C (Molt-4^R) by culturing it in the continuous presence of increasing concentrations of CPE-C. Using a tetrazolium based colorimetric assay to assess cytotoxicity, the IC_{90} for the parent Molt-4 cells (Molt-4^{WT}) was 0.5 μM after a 24 hr drug exposure. In contrast, cytotoxicity was not observed at concentrations as high as 1 mM in the Molt-4^R cells. Following a brief exposure to 1 μM CPE-C, parent drug could be detected intracellularly in the resistant and sensitive cell lines. However, CPE-CTP formation was reduced markedly in the resistant cell line. Measurement of the activity of anabolic and catabolic enzymes in the Molt-4^{WT} and Molt-4^R cells revealed equivalent activities of alkaline and acid phosphatases as well as cytidine and dCMP deaminase but there was a significant reduction in uridine/cytidine kinase activity in Molt-4^R cells. Endogenous ribonucleotide pools and CPE-CTP pools were measured in the absence and presence of CPE-C. CTP pools were reduced markedly in Molt-4^{WT} cells following exposure to CPE-C. However, CTP pools in Molt-4^R cells exposed to 100 μM CPE-C were two times greater than in the untreated Molt-4^{WT} cells. At high concentrations of CPE-C (10 and 100 μM), Molt-4^R cells were able to generate amounts of CPE-CTP equivalent to that seen in Molt-4^{WT} cells exposed to 1 μM CPE-C (a cytotoxic concentration of drug in Molt-4^{WT} cells), but no cytotoxic effect was seen in Molt-4^R cells. Therefore, in addition to decreased uridine/cytidine kinase activity, a second mechanism of resistance that is the result of alterations in CTP synthetase activity also appears to be operative. Elucidation of the mechanism of resistance *in vitro* may provide insight into the mechanism of action of the drug and potential mechanisms of resistance *in vivo*.

Cyclopentenyl cytosine (CPE-C[5]‖, NSC 375575) is a carbocyclic analogue of cytidine that has preclinical activity against cytarabine (ara-C) resistant murine leukemias and a broad spectrum of activity against human tumor xenografts. Phase I clinical trials with this agent have been initiated recently. CPE-C cytotoxicity correlates with the rapid depletion of intracellular endogenous CTP pools secondary to inhibition by cyclopentenyl cytosine triphosphate (CPE-CTP) of the conversion of UTP to CTP by CTP synthetase [1]. Like ara-C, CPE-C must be phosphorylated intracellularly to its triphosphate nucleotide form (CPE-CTP) to express its antitumor effect. However, the enzyme catalyzing the initial step to the monophosphate form differs for ara-C (deoxycytidine kinase) and CPE-C (uridine/cytidine kinase) [1–3]. Murine leukemia cell lines that develop resistance to ara-C as a result of a deoxycytidine kinase deficiency are actually collaterally sensitive to CPE-C, since these cells can still activate CPE-C, but have a reduced capacity to form the endogenous competing cytidine nucleotides [4, 5]. Other reported mechanisms of resistance to ara-C include: decreased ara-C transport [6], increased cytidine deaminase activity [7], and increased dCTP pools, resulting from either an increase in CTP synthetase activity or a deficiency of dCMP deaminase [8–10].

In the present study, the mechanism of acquired resistance to CPE-C was investigated in a Molt-4 human T-cell leukemia cell line made resistant to the drug by continuous exposure to increasing concentrations of CPE-C.

MATERIALS AND METHODS

Materials

CPE-C was obtained from the Pharmaceutical Resources Branch, National Cancer Institute,

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‖ Abbreviations: CPE-C, cyclopentenyl cytosine; ara-C, cytarabine; CPE-CTP, cyclopentenyl cytosine triphosphate; Molt-4^{WT}, wild type Molt-4 human T-cell leukemia cell line; Molt-4^R, CPE-C resistant Molt-4 human T-cell leukemia cell line; RPMI, Rosewell Park Memorial Institute; FBS, fetal bovine serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; CPE-U, cyclopentenyl uridine; CPE-UMP, cyclopentenyl uridine monophosphate; and PCA, perchloric acid.

Bethesda, MD. [^{3}H]CPE-C (sp. act. 15.0 Ci/mmol), labeled at position 5 of cytosine, was obtained from Research Triangle Institute (Research Triangle Park, NC). [5-^{3}H]FdUMP (sp. act. 22 Ci/mmol) and [6-^{3}H]uridine (sp. act. 20 Ci/mmol) were obtained from Moravek Biochemicals, Inc. (Brea, CA). EDTA was obtained from the Fisher Scientific Co. (Fair Lawn, NJ). FdUMP, uridine, ATP and all other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO).

Cell lines

The wild type Molt-4 human T-cell leukemia cell line (Molt-4^{WT}) was propagated continuously in a drug-free suspension culture consisting of Roswell Park Memorial Institute (RPMI) 1640 medium (Mediatech, Washington, DC) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biofluids Inc., Rockville, MD). Cells were passaged every 3–4 days and maintained in logarithmic growth phase.

A CPE-C resistant Molt-4 cell line (Molt-4^R) was developed by culturing cells in RPMI-1640/10% FBS containing gradually increasing concentrations of CPE-C. The starting concentration was 1×10^{-10} M which initially inhibited growth of this cell line. Once the growth rate approached that of Molt-4^{WT} cells in drug-free medium, the CPE-C concentration was increased 5-fold. This process was repeated over the ensuing 10 months until cells were growing in the presence of 500 μM CPE-C. The Molt-4^R cells were then propagated continuously in drug-free media throughout the course of these experiments.

Determination of drug sensitivity

A modified 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [11, 12] was used to determine the sensitivity of the Molt-4 lymphoblasts to CPE-C. Cells (135 μL of 1×10^5 cells/mL) were plated into 96-well microtiter plates and incubated at 37°. Twenty-four hours later, drug at specified concentrations was added to each well in replicates of 8. Cells were exposed to drug for 24 hr, at which time the number of surviving cells was quantitated using the previously described MTT assay of Adamson *et al.* [13].

HPLC methods

Several HPLC methods were used for qualitative and quantitative analysis of parent drug, metabolites, and endogenous ribonucleotide pools. The HPLC system was a Waters 600E multisolvent delivery system (Waters Millipore, Medford, MA) with a Waters 990 photodiode array detector set to monitor wavelengths from 250 to 270 nm and an online Flo-One βeta radioactive flow detector (Radiomatic Instruments, Tampa, FL). An NEC Powermate computer with Powerline/990 software (Waters Millipore) was used for keyboard control of the pump and 990 photodiode array detector, and for storage and analysis of chromatograms. For HPLC methods 1–4, a SAX Radial-Pak column (Waters) was eluted with a gradient made up of two buffers: buffer A was 0.007 M ammonium phosphate, pH 4.5, and buffer B was 0.75 M ammonium phosphate, pH 4.5. The total flow rate was 2.4 mL/min unless otherwise specified.

HPLC Method 1. Samples were analyzed for endogenous nucleotide content using a previously published anion-exchange HPLC method [14–16]. Buffer A (100%) was run for 6 min, followed by a linear gradient to 70% buffer A/30% buffer B over the next 30 min, followed by a linear gradient to 20% buffer A/80% buffer B for the next 1 min to wash the column. The column was allowed to equilibrate at initial conditions for 15 min between separations. The retention times and wavelengths monitored were as follows: UTP, 22 min at 260 nm; CTP, 25 min at 270 nm; ATP, 27 min at 260 nm; and GTP, 33 min at 250 nm. The intracellular content of nucleotide was quantitated by integration of the chromatographic peak at the wavelengths indicated above. The standard curves for each ribonucleotide were linear ($r^2 \geq 0.999$) over a range of 0.5 to 220 nmol for UTP, CTP, and GTP and 0.6 to 295 nmol for ATP.

HPLC Method 2. CTP and CPE-CTP pools were eluted with a gradient modified slightly from the above conditions: 100% buffer A was run for 20 min, followed by a linear gradient to 75% buffer A/25% buffer B over 10 min, followed by a linear gradient to 70% buffer A/30% buffer B for the next 32 min, followed by a linear gradient to 100% buffer A over the next 15 min. The column was allowed to equilibrate at initial conditions for 15 min between separations. CTP was monitored at 270 nm with a retention time of 45 min under these conditions. The tritium-labeled peak which co-eluted with cold CTP was presumed to be the triphosphate form of CPE-C.

HPLC Method 3. CPE-C nucleotides were eluted with a gradient modified slightly from the above conditions: 100% buffer A was run isocratically for 6 min, followed by a linear gradient to 70% buffer A/30% buffer B over the next 24 min, followed by a linear gradient to 100% buffer B over the next 4 min to wash the column. The column was equilibrated with 100% buffer A at initial conditions for 15 min between separations. Four tritium-labeled peaks were identified, the first (2.0 min) co-eluted with cold CPE-C, and the next three peaks which were presumed to be the mono-, di-, and triphosphate forms of CPE-C co-eluted with CMP (6.7 min), CDP (13.7 min) and CTP (30.3 min), respectively.

HPLC Method 4. A previously described protracted HPLC method of Yee *et al.* [17] was used to separate uridine and cytidine nucleotides. The nucleotide retention times (min) for this gradient were as follows: CMP, 7.3; UMP, 18.3; CDP, 50.1; UDP, 56.5; UTP, 89.3; and CTP, 94.1.

HPLC Method 5. Samples were analyzed for CPE-U formation using a previously published reverse-phase HPLC method [18]. The mobile phase was 0.1 M ammonium formate, pH 5.0, run at 1.0 mL/min and the analytical column was a 5 μm particle size 25-cm × 4.6-mm Beckman Ultrasphere ODS (Beckman Instruments, San Ramon, CA). With this method, nucleotides were eluted with the void volume at 2 min; and the retention times for CPE-C and CPE-U were 11 and 14 min, respectively.

Determination of ribonucleotide pools

Exponentially growing Molt-4^{WT} and Molt-4^{R} cells (1×10^7) were incubated in the presence or absence of 1 μM CPE-C for 4 hr; then they were washed twice with iced PBS, and the cell pellets were extracted immediately or frozen at −70° until extraction. Perchloric acid (PCA, 0.5 N) was added and the mixture was incubated for 15 min at 4°. The supernatant was extracted with 2 vol. of trifluoroethane:tri-*n*-octylamine (2.3:1), and the aqueous phase was retained after adjusting the pH to 7.0 with 0.5 N NaOH. The acid soluble extract was frozen on dry ice, lyophilized to dryness, and stored at −70°. The lyophilized residue was resuspended in distilled water immediately before analysis and clarified by filtration prior to HPLC injection. Samples were analyzed for endogenous nucleotide content using HPLC Method 1.

Endogenous CTP pools in resistant and wild-type cells were measured in a similar fashion following incubation with 0 μM, 1 μM (0.13 μCi/nmol), 10 μM (0.013 μCi/nmol), or 100 μM (0.00134 μCi/nmol) [^{3}H]CPE-C. A separation of endogenous nucleotides was achieved using HPLC Method 2. CPE-CTP, which co-elutes with CTP, can be detected by UV absorption in Molt-4 cells following exposure to CPE-C concentrations ≥ 100 nM [19]. Therefore, [^{3}H]CPE-C was used for these experiments in an attempt to correct for this interference. CPE-CTP was quantitated by measuring tritium with the online Flo-One βeta radioactive flow detector. The amount of CPE-CTP was estimated using the specific activity of the radiolabel and then subtracted from the total area under the UV peak corresponding to CPE-CTP and CTP.

CPE-C uptake and metabolism

To estimate uptake of CPE-C into the Molt-4^{WT} and Molt-4^{R} cell lines, exponentially growing cells were incubated with 1.0 μM [^{3}H]CPE-C (0.2 μCi/nmol) for 15 min. Cells were washed once with 5 mL iced PBS and centrifuged for 10 min at 90 *g*. Two milliliters of iced methanol (60%) was added to the washed cell pellet. An aliquot of the methanol supernatant was placed in a scintillation vial and the sample was counted in a Packard 2500 TR liquid scintillation counter (Packard Instrument Co., Meriden, CT) following the addition of scintillation fluid.

CPE-C is catabolized to cyclopentenyl uridine (CPE-U) in non-human primates [18] and Ford *et al.* [19] detected CPE-U nucleotide metabolites in Molt-4 lymphoblasts exposed to CPE-C. We therefore attempted to determine if there were differences in the rate of intracellular deamination of CPE-C in exponentially growing Molt-4^{WT} and Molt-4^{R} cells exposed to 10.0 μM [^{3}H]CPE-C (0.013 μCi/nmol). Following extraction with PCA the resuspended lyophilized residues were analyzed for CPE-C, CPE-U and CPE-C nucleotides using HPLC Method 4. An aliquot of the resuspended lyophilized residue was also treated with alkaline phosphatase (EC 3.1.3.1) in alkaline phosphatase buffer (Promega, Inc., Madison, WI) for 60 min at 37°. The samples were clarified by centrifugation and analyzed for CPE-C and CPE-U by HPLC Method 5. After documenting that CPE-U nucleotide formation was negligible (< 5%), an abbreviated method of Yee *et al.* [17] was used thereafter to quantitate CPE-C metabolites (HPLC Method 3).

Enzyme assays

Uridine/cytidine kinase activity and alkaline and acid phosphatase activities were measured to determine if alterations in either phosphorylation of CPE-C or catabolism of CPE-CTP were present in

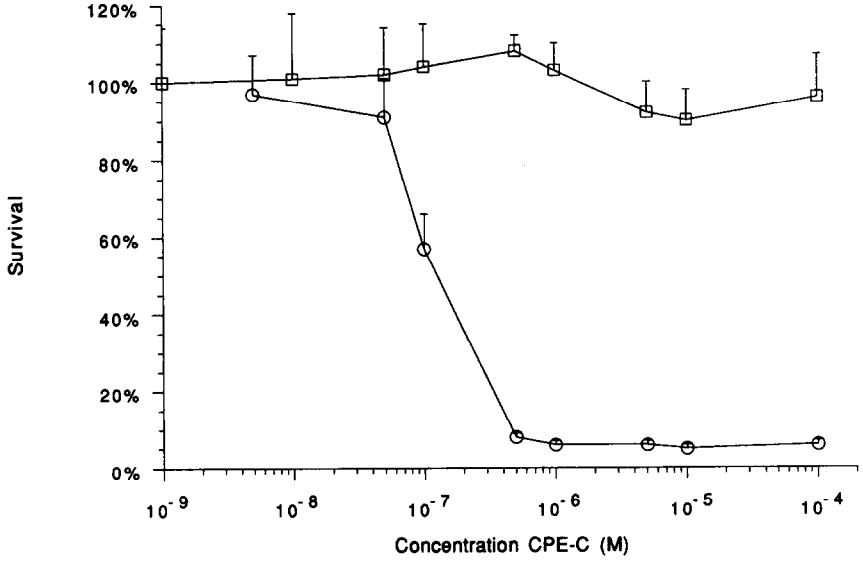

Fig. 1. Concentration–response curve for Molt-4^{WT} (○) and Molt-4^{R} (□) cells following a 24-hr continuous exposure to increasing concentrations of CPE-C. Values are means ± SD, N = 8. (Results of a representative experiment.)

Table 1. Effect of CPE-C on endogenous ribonucleotide triphosphate pools following a 4 hr exposure to 1 μM CPE-C

Cell line	CPE-C concentration (μm)	GTP	UTP	ATP	CTP
			(nmol/10^6 cells)		
Molt-4^{WT}		2.3 ± 0.5*	4.2 ± 1.0	13.4 ± 3.0	1.5 ± 0.3
	1.0	1.8 ± 0.5	6.0 ± 0.1	13.3 ± 0.2	0.5 ± 0.03
Molt-4^R		1.2 ± 0.03	1.4 ± 0.1	5.8 ± 0.2	2.6 ± 0.1
	1.0	1.5 ± 0.3	2.1 ± 0.5	7.8 ± 1.7	3.6 ± 0.9

* Mean ± SD of triplicate experiments.

the Molt-4^R cells. In addition, cytidine deaminase and dCMP deaminase activities, which have been reported previously to be extremely low in Molt-4^{WT} cells [20], were measured to confirm that there were no differences between the parent and resistant cell lines in the deamination of CPE-C. Cytidine deaminase catalyzes the conversion of the parent nucleoside, CPE-C, to its inactive uridine metabolite CPE-U whereas dCMP deaminase potentially converts CPE-CMP to cyclopentenyl uridine monophosphate (CPE-UMP).

Extracts of Molt-4^{WT} and Molt-4^R cells were prepared from freshly isolated cells or from cell pellets frozen at −70°. The cells were suspended in 100 μL of 50 mM Tris–HCl buffer containing 1 mM EDTA (pH 7.4) and lysed by sonication for three cycles of 3 sec at maximal output. The cell lysate was centrifuged at 8000 *g* for 5 min, and the supernatant was used as a crude enzyme source.

Uridine/cytidine kinase activity was measured according to the method of Peters *et al.* [21]. The reaction mixture contained 50 mM Tris–HCl (pH 7.4), 10 mM ATP, 10 mM $MgCl_2$, 1 mM [^{3}H]uridine (0.6 to 0.7 nCi/nmol) or 1 mM [^{3}H]CPE-C (0.7 to 0.9 nCi/nmol), and 85 μL of cell extract in a final volume of 150 μL. The mixture was incubated in a 37° shaking water bath for 30 min. After incubation, the reaction mixture was clarified by centrifugation at 3000 *g* for 30 sec. The supernatant was chilled on ice, and 50 μL was placed onto a Whatman DE-81 filter disc (Whatman Int. Ltd., Maidstone, U.K.) which retains the product of the reaction by adherence of the nucleotide to the DEAE disc [22]. The filter discs were airdried for 30 min, washed three times with distilled water, and placed into scintillation vials; 1 mL of 0.1 M KCl/0.1 M HCl solution was added to elute the nucleotide product from the disc. Ten minutes later, 10 mL of scintillation fluid was added and the samples were counted 12–14 hr later using a liquid scintillation counter. The activity of uridine/cytidine kinase is expressed as picomoles of CPE-C or uridine nucleotide formed per milligram of total protein per minute.

Acid and alkaline phosphatase activities were determined according to the method of Fernandes and Cranford [23] after cell extracts were prepared as above. The reaction mixture for the acid phosphatase contained 25 mM sodium acetate (pH 5.8), 1 mM [^{3}H]FdUMP (1.5 to 2.0 nCi/mmol) and 42 μL of cell extract in a final total volume of 80 μL. The reaction mixture for alkaline phosphatase activity was identical except that the buffer was 25 mM ammonium bicarbonate (pH 9.2). After incubation at 37° for 30 min, residual nucleotides were isolated and counted using the filter method described above. The activity of the phosphatases is expressed as picomoles of nucleoside formed per milligram of total protein per minute.

Cytidine deaminase (EC 3.5.3.5) activity was determined by the method of Chabner *et al.* [24]. dCMP deaminase (EC 3.5.4.14) activity was determined by a modification of the method described by Drake *et al.* [25]. The activity of cytidine deaminase and dCMP deaminase is expressed as nanomoles of CPE-U or CPE-UMP formed per milligram of total protein per minute, respectively.

Total protein concentration in the cell extracts was measured with the Bio-Rad Assay method (Bio-Rad Laboratories, Richmond, CA) [26].

UTP and CTP synthesis after exposure to [^{3}H]*uridine*

CPE-CTP inhibits the conversion of UTP to CTP in a reaction catalyzed by CTP synthetase [19]. To assess indirectly the activity of CTP synthetase, 5×10^6 cells in exponential growth phase were incubated for 4 hr with 1 μM (0.20 to 0.24 μCi/nmol) [^{3}H]uridine in the presence or absence of 1 μM CPE-C. The amounts of [^{3}H]UTP and [^{3}H]CTP that were formed following the addition of the exogenous [^{3}H]uridine were measured using HPLC Method 2.

RESULTS

Cytotoxicity of CPE-C in Molt-4^{WT} and Molt-4^R cells

Cell survival curves for Molt-4^{WT} and Molt-4^R cells exposed to a range of CPE-C concentrations for 24 hr are shown in Fig. 1. The IC_{90} of CPE-C for Molt-4^{WT} cells was 0.5 μM, but there was no evidence of cytotoxicity in Molt-4^R cells at concentrations of up to 1.0 mM. Molt-4^R cells have maintained resistance to the cytotoxic effects of CPE-C after propagation in drug free medium for greater than 1 year.

Ribonucleotide pools

Changes in endogenous ribonucleotide pools following a 4-hr exposure to 1 μM CPE-C are summarized in Table 1. Endogenous CTP pools in Molt-4^{WT} cells decreased by 66% following exposure

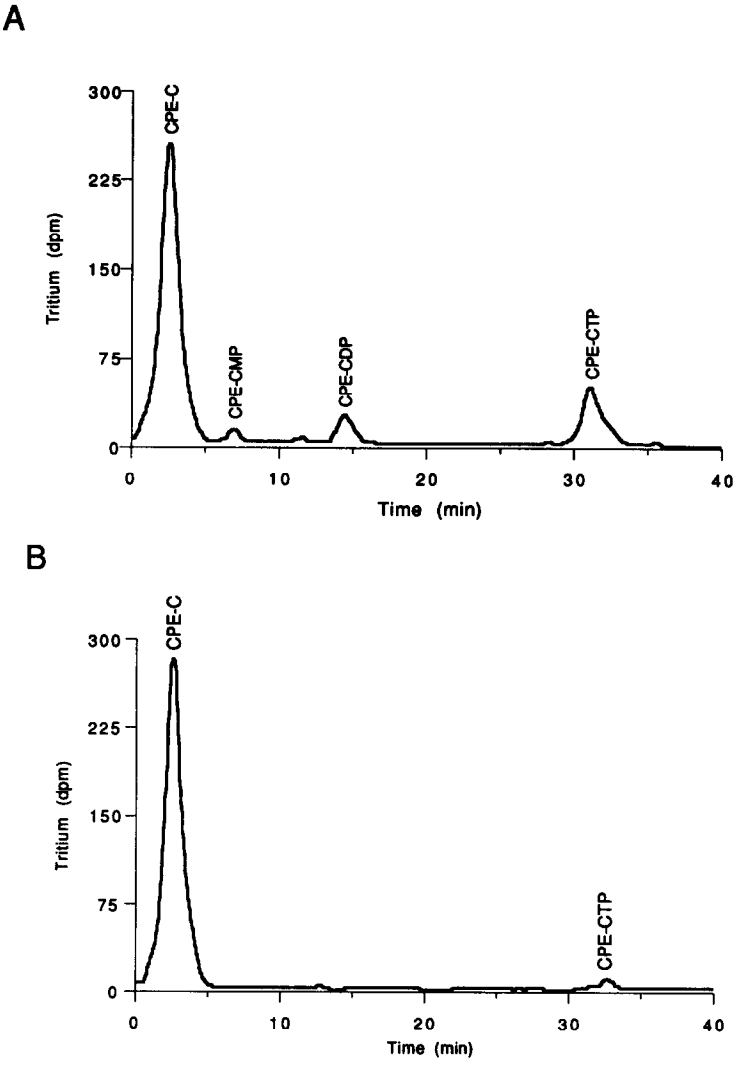

Fig. 2. Representative chromatograms (HPLC Method 3) illustrating CPE-C uptake and CPE-C nucleotide formation in Molt-4^{WT} (A) and Molt-4^{R} (B) cells following a 15-min exposure to 1 μM [^{3}H]CPE-C.

to 1 μM CPE-C, whereas CTP pools were unchanged or increased in the Molt-4^{R} cells. There were no significant changes in the other endogenous ribonucleotide pool sizes following exposure to CPE-C in either the parent or resistant cell line. The relatively smaller endogenous GTP, UTP and ATP pools in both the treated and untreated Molt-4^{R} cells compared to the Molt-4^{WT} cells may reflect alterations in either enzymatic regulation or absolute enzyme levels in the resistant cell line.

CPE-C uptake and metabolism

After a 15 min exposure to 1 μM [^{3}H]CPE-C, the amount of parent drug measured intracellularly in Molt-4^{WT} and Molt-4^{R} cells was 10 and 20.5 pmol/10^6 cells, respectively. However, CPE-CTP formation was diminished markedly in Molt-4^{R} cells (Fig. 2). CPE-CTP was detectable but below the limits of quantitation in Molt-4^{R} cells, while 3.7 pmol/10^6 cells of CPE-CTP was measured in the Molt-4^{WT} cells. Even with prolonged exposure to 1 μM [^{3}H]-CPE-C, only minimal CPE-CTP formation was observed in Molt-4^{R} cells (Fig. 3), whereas in Molt-4^{WT} cells CPE-CTP levels increased to 60 pmol/10^6 cells by 2 hr and then appeared to plateau at that level. CPE-CTP pool sizes were also measured following a fixed duration of exposure (4 hr) to 1, 10, and 100 μM [^{3}H]CPE-C in both cell lines (Fig. 4). Although CPE-CTP was formed in the Molt-4^{R} cells after exposure to higher concentrations of CPE-C, the CPE-CTP pool sizes in the resistant cell line were consistently an order of magnitude lower than in the parent cell line.

As shown in Fig. 4 at high concentrations of CPE-C (10 and 100 μM), Molt-4^{R} cells were able to generate amounts of CPE-CTP equivalent to that

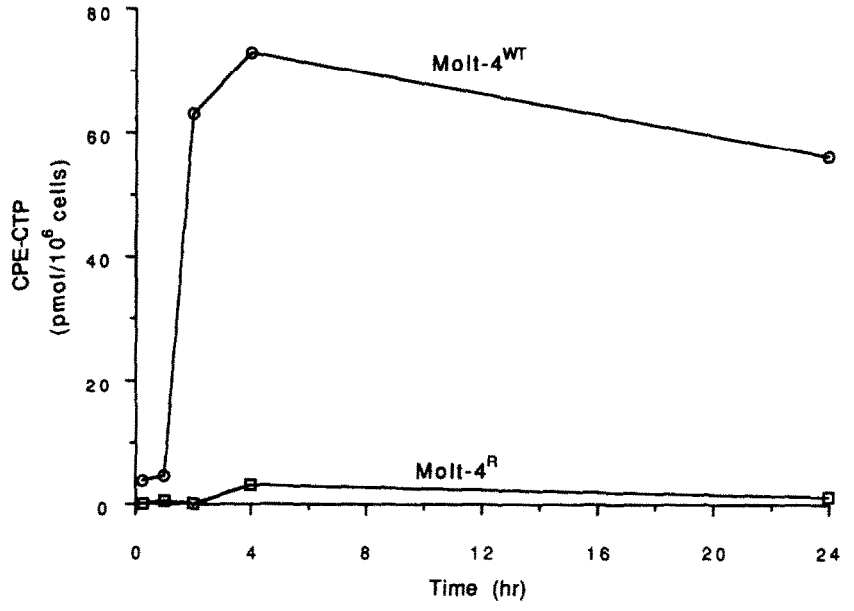

Fig. 3. Intracellular CPE-CTP concentrations in Molt-4^{WT} and Molt-4^{R} cells following increasing durations of exposure to 1 μM [^{3}H]CPE-C. Values are means of duplicate experiments.

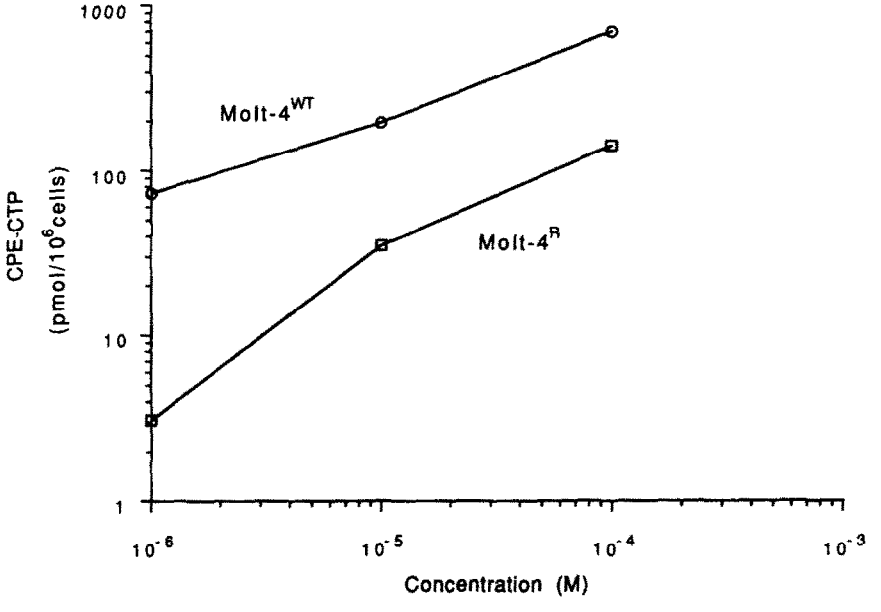

Fig. 4. Intracellular CPE-CTP concentrations in Molt-4^{WT} and Molt-4^{R} cells following a 4-hr exposure to increasing concentrations of [^{3}H]CPE-C. Values are means of duplicate experiments.

seen in Molt-4^{WT} cells exposed to 1 μM CPE-C (a cytotoxic concentration of drug in Molt-4^{WT} cells), but no cytotoxic effect was seen in Molt-4^{R} cells. This suggests the presence of a second mechanism of resistance in addition to decreased activation of CPE-C by uridine/cytidine kinase. The higher endogenous CTP pools in the resistant cell line could account for the lack of effect of high CPE-C concentrations and may be the result of increased CTP synthetase activity in the resistant cell line. To investigate this, the amounts of radiolabeled UTP and CTP formed following exposure to 1 μM [^{3}H] uridine in the presence and absence of 1 μM CPE-C were determined to assess indirectly the activity

Table 2. Formation of [^{3}H]UTP and [^{3}H]CTP from [^{3}H]uridine in the presence or absence of 1 μM CPE-C for 4 hr

Cell type	[^{3}H]UTP (pmol/10^6 cells)	[^{3}H]CTP	Ratio CTP/UTP
Molt-4^{WT}			
[^{3}H]Uridine	56.6*	13.2	0.23
[^{3}H]Uridine + CPE-C	199.5	2.9	0.015
Molt-4^R			
[^{3}H]Uridine	17.8	11.5	0.65
[^{3}H]Uridine + CPE-C	16.9	7.4	0.44

* Mean of two experiments.

of CTP synthetase (Table 2). As can be seen, the total amount of nucleotide formed (UTP + CTP) was diminished considerably in the Molt-4^R cells. This can be attributed to a decrease in the activity of uridine/cytidine kinase in the resistant cells (see next section of Results). Despite this overall reduction of uridine phosphorylation in the resistant line, the formation of CTP approximated that formed in the wild-type cells in the absence of CPE-C. After exposure to CPE-C, CTP formation in the resistant line was actually greater than that in the Molt-4^{WT} cells. The ratio of CTP to UTP formed was also higher in the resistant line and unlike the parent line was relatively unaffected by exposure to CPE-C. This suggests that the resistant cells are more efficient at synthesizing CTP and that CTP synthetase in the Molt-4^R line is not inhibited by CPE-CTP.

CTP pools were subsequently measured in both the wild type and resistant cell lines after exposure to 1, 10, and 100 μM [^{3}H]CPE-C. As shown in Fig. 5, CTP pools were almost two times greater in the in the Molt-4^R cells exposed to 100 μM CPE-C than in the untreated Molt-4^{WT} cells, and CTP pools declined in the Molt-4^{WT} cells exposed to CPE-C. The slight increase in CTP levels in Molt-4^{WT} cells exposed to high concentrations of CPE-C probably represents CPE-CTP interference with the UV measurement of CTP that was not totally corrected for by the use of [^{3}H]CPE-C.

Uridine/cytidine kinase activity

Uridine/cytidine kinase activity was significantly lower in the Molt-4^R cell line when either uridine or CPE-C was used as a substrate (Table 3), suggesting that uridine/cytidine kinase deficiency may be a

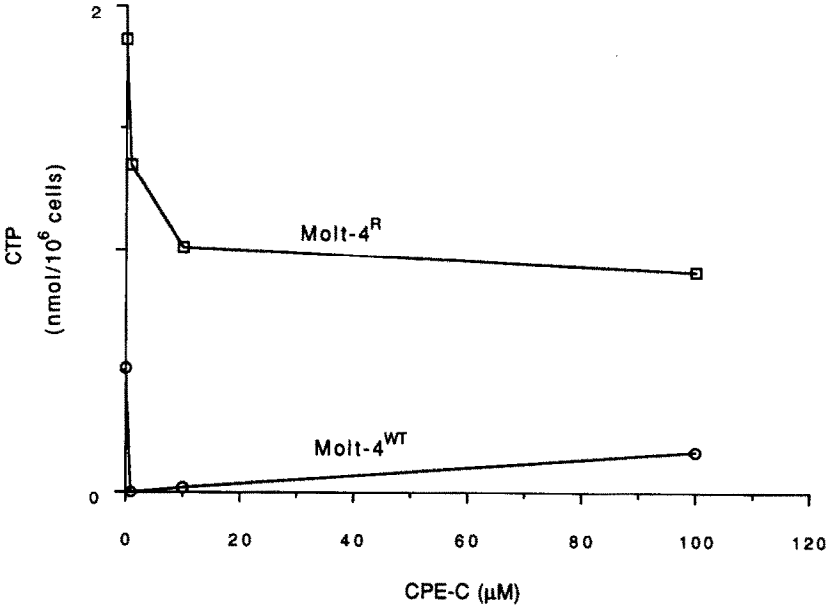

Fig. 5. Intracellular CTP concentrations in Molt-4^{WT} and Molt-4^R cells following a 4-hr exposure to increasing concentrations of [^{3}H]CPE-C. The apparent increase in the CTP concentration in the Molt-4^{WT} cells probably represents interference in the measurement of CTP by CPE-CTP (see text). Values are means of duplicate experiments.

Table 3. Uridine/cytidine kinase activity in wild-type and CPE-C resistant Molt-4 cells

Cell line	Substrate CPE-C* (pmol/mg/min)	Uridine* (pmol/mg/min)
Molt-4^{WT}	221 ± 53†	771 ± 235
Molt-4^{R}	66 ± 16	253 ± 139
	P = 0.009‡	P = 0.0017

* Activity is expressed as pmol of CPE-C or uridine nucleotide formed per mg of total protein per min.
† Mean ± SD of two experiments performed in duplicate.
‡ Mann–Whitney U test.

basis for the decreased production of CPE-CTP and resistance to CPE-C in the Molt-4^{R} cells.

Alkaline/acid phosphatase activity

No significant differences in either alkaline or acid phosphatase activity were observed between the parent and resistant cell lines. The acid phosphatase activity for the Molt-4^{WT} cells was 610 ± 143 pmol/mg/min versus 695 ± 223 pmol/mg/min in the Molt-4^{R} cells. The alkaline phosphatase activity was 419 ± 153 pmol/mg/min in the Molt-4^{WT} cells versus 382 ± 232 pmol/mg/min in the Molt-4^{R} cells.

Cytidine and dCMP deaminase activity

Cytidine deaminase activity was not detected in either the Molt-4^{WT} or the Molt-4^{R} cell lines and dCMP deaminase activity was similar in both cell lines. The average dCMP deaminase activity in Molt-4^{WT} cells was 10.3 nmol/mg/min (range 8.5 to 12. 1) and in Molt-4^{R} cells was 9.5 nmol/mg/min (range 7.1 to 11.9). These findings are consistent with the absence of measurable CPE-U formation in both cell lines following exposure to [^{3}H]CPE-C.

DISCUSSION

Drug resistance to CPE-C, a new cytidine analogue, was investigated *in vitro* in a human leukemia cell line made resistant to CPE-C through continuous exposure to increasing concentrations of this agent. A high level of drug resistance was achieved (>2000-fold) and the Molt-4^{R} cells retained this resistant phenotype when propagated in drug free medium. Studies to evaluate the mechanism of resistance demonstrated that approximately equivalent amounts of parent drug could be detected intracellularly in the resistant and sensitive cell lines following a brief exposure to 1 μM CPE-C, suggesting that resistance is not the result of a defect in drug uptake. In contrast, formation of the presumed cytotoxic intracellular metabolite of CPE-C, CPE-CTP, was reduced markedly in the resistant cell line. Measurement of the activity of anabolic and catabolic enzymes in both the Molt-4^{WT} and Molt-4^{R} cell lines revealed equivalent activities of alkaline and acid phosphatases but a reduction of uridine/cytidine kinase activity in the Molt-4^{R} line. Thus, decreased uridine/cytidine kinase activity, resulting in decreased formation of CPE-CTP, appears to be a primary mechanism of acquired resistance to CPE-C. However, uridine/cytidine kinase activity was not entirely absent in the resistant line and CPE-CTP was formed in Molt-4^{R} cells exposed to higher yet non-cytotoxic concentrations (10 and 100 μM) of CPE-C. In fact, the amount of CPE-CTP formed at these higher CPE-C concentrations approached or exceeded that known to be cytotoxic in the sensitive cell line exposed to 1 μM CPE-C, suggesting that other factors in addition to the absolute intracellular concentration of CPE-CTP are critical to determining the degree of cytotoxicity.

One factor may be the level of intracellular CTP, the endogenous cytidine nucleotide that competes with CPE-CTP [19]. After exposure to CPE-C, CTP pools in the Molt-4^{WT} cells were reduced markedly, whereas in the Molt-4^{R} cells CTP pools remained essentially unchanged or slightly increased. This expansion of CTP pools in the Molt-4^{R} cells may be the result of either increased or altered CTP synthetase activity. Indirect evidence to support this hypothesis includes the following observations: (1) CTP pools in Molt-4^{R} cells exposed to high concentrations of CPE-C were almost 2-fold higher than CTP pools in untreated Molt-4^{WT} cells and (2) there was more CTP formed in the Molt-4^{R} cells after simultaneous exposure to [^{3}H]uridine and CPE-C, and the efficiency of conversion of UTP to CTP appeared to be unaffected.

In summary, decreased uridine/cytidine kinase activity appears to be the primary mechanism of acquired resistance to CPE-C in this Molt-4 leukemia cell line. However, a second mechanism of resistance that is most likely the result of either increased or altered CTP synthetase activity also appears to be operative. Similar mechanisms of resistance involving deoxycytidine kinase and CTP synthetase have been described in ara-C resistant leukemia cell lines. Elucidation of the mechanism of resistance to CPE-C *in vitro* is important since this may provide some insight into the mechanisms of action of the drug and potential mechanisms of resistance *in vivo* and ultimately to therapeutic strategies to prevent or circumvent this resistance.

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