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METABOLISM AND RNA INCORPORATION OF CYCLOPENTENYL CYTOSINE IN HUMAN COLORECTAL CANCER CELLS

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Abstract—We studied the cytotoxicity and metabolism of the investigational cytidine analogue cyclopentenyl cytosine (CPE-C) in three human colorectal cancer cell lines: HCT 116, SNU-C4, and NCI-H630. CPE-C potently inhibited cell growth and decreased clonogenic capacity at concentrations achieved in murine and primate pharmacologic studies. CPE-C produced a concentration-dependent depletion of CTP, accompanied by changes in the dCTP pools. CPE-C exposure was associated with an accumulation of cells in the S phase at 48 hr. [^{3}H]CPE-C was metabolized predominantly to the triphosphate (CPE-CTP) form. Saturation of phosphorylation to the monophosphate form occurred above 5–10 μM. Plateau CPE-CTP pools were of a magnitude similar to that of the physiologic ribonucleotide triphosphate pools. The intracellular half-life of CPE-CTP was 24 hr. After a 24-hr exposure to 0.5 μM CPE-C, CPE-CTP was detected for up to 96 hr post-drug removal, accompanied by persistent depletion of the CTP pools. Cesium sulfate density centrifugation of purified nucleic acids indicated that [^{3}H]CPE-C incorporated into RNA, but was not detected in DNA. Agarose-gel electrophoresis of RNA from [^{3}H]CPE-C-treated cells indicated that it localized predominantly in low molecular weight (4–8 S) RNA species. When CPE-C was administered concurrently with [^{3}H]adenosine (Ado), the proportion of [^{3}H]Ado migrating with low molecular weight RNA species increased. Concurrent exposure to 10 μM cytidine (Cyd), sufficient to replete CTP pools, provided essentially complete protection against lethality resulting from a 24-hr exposure to $\leq$ 0.5 μM CPE-C. While 10 μM Cyd substantially decreased CPE-CTP formation and CPE-C-RNA incorporation during the initial 3 hr of exposure compared to CPE-C alone, after 24 hr the levels were not significantly different. Cyd rescue did not affect the accumulation of [^{3}H]CPE-C or [^{3}H]Ado into low molecular weight RNA species after a 24-hr exposure to CPE-C. Our results indicate that depletion of CTP and dCTP pools is an important component of CPE-C cytotoxicity. While CPE-C incorporation into RNA may not be the critical cytotoxic event during a 24-hr exposure to CPE-C, it may play a role during prolonged exposure to CPE-C. CPE-C is a highly potent new agent and merits clinical evaluation in the treatment of colorectal cancer.

Cyclopentenyl cytosine (CPE-C,§ NSC 375575) is an investigational nucleoside with activity against murine leukemia and human solid tumor xenografts [1–4]. CPE-C is an analogue of cytosine with the substitution of a carbocyclic sugar for the ribose moiety. Kang *et al.* [4] documented in L1210 leukemia cells that CPE-C is metabolized to the monophosphate by Urd/Cyd kinase (EC 2.7.1.48). Using partially purified enzyme from P388 leukemia, the K_m was 196 μM, compared to a K_m of 67 μM for Cyd [4]. The triphosphate of CPE-C is a potent inhibitor of CTP synthetase (EC 6.3.4.2); using partially purified enzyme from bovine liver, the K_i with respect to UTP was 6 μM [4]. While CTP synthetase displays classical Michaelis–Menten kinetics with UTP as the variable substrate, CPE-CTP increases the apparent K_m for UTP and induces sigmoidal kinetic plots [4]. Dose-dependent depletion of CTP is evident within 2–4 hr in L1210 cells, HT29 human colon cancer cells and Molt-4 human leukemia cells [1, 2, 4, 5]. Polyacrylamide-agarose gel electrophoresis of RNA from HT29 cells following a 24-hr treatment with 0.25 μM CPE-C (which decreased colony formation by 60%) and [^{3}H]Ado showed equal inhibition of formation of 28 S and 18 S [^{3}H]-rRNA; no accumulation of [^{3}H]45 S rRNA was seen, suggesting interference with rRNA transcription rather than impairment of rRNA processing [1].

CPE-C will shortly be entering Phase I clinical evaluation as an anticancer agent. Given the paucity of useful agents for colorectal carcinoma. we have been interested in identifying new agents with potential activity. Based on these considerations and the activity of CPE-C against the HT29 colon cancer cell line, we wished to evaluate the cytotoxicity, biochemical effects and metabolism of CPE-C against three human colorectal carcinoma cell lines with different inherent sensitivities to 5-fluorouracil (FUra). We have reported previously that a biochemically active, but non-lethal, concentration of CPE-C increases the toxicity, metabolism and DNA incorporation of cytosine arabinoside [6]. We

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§ Abbreviations: CPE-C, cyclopentenyl cytosine; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PCA, perchloric acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid; SDS, sodium dodecyl sulfate; RNAse, ribonuclease; TCA, trichloroacetic acid; NaOH, sodium hydroxide; CPE-U, cyclopentenyl uridine and HCl, hydrochloric acid.

herein characterize the toxicity of CPE-C in both cell growth and clonogenic studies and its effect on cell cycle distribution, and correlate the biochemical effects with [^{3}H]CPE-C metabolite formation. The incorporation of CPE-C into RNA is quantitated, and its distribution into various RNA species by agarose gel electrophoresis is described, as is the effect of CPE-C exposure on distribution of [^{3}H]-Ado into RNA species. In addition, the effect of a protective concentration of Cyd on CPE-C pharmacodynamics is explored. Preliminary accounts of some of these studies have been presented previously [7, 8]. The cellular pharmacology of CPE-C in Molt-4 lymphoblast cells has been described recently by Ford *et al.* [5]; the differences between CPE-C metabolism in human colon carcinoma and leukemia cells will be highlighted.

MATERIALS AND METHODS

Materials

CPE-C and [^{3}H]CPE-C (15 Ci/mmol) were supplied by the Pharmaceutical Resources Branch, Developmental Therapeutics Program, Division of Cancer Treatment, NCI. CPE-CTP was a gift from Dr. Marquez of the Laboratory of Medicinal Chemistry, Developmental Therapeutics Program, NCI. The radiopurity of CPE-C was documented by injecting an aliquot onto a reversed-phase HPLC column (C8 μ-Bondapak, Waters Millipore, Milford, MA); water (at 2.0 mL/min) served as the mobile phase. One hundred percent of the tritium counts coeluted in a single peak with non-radiolabeled standard CPE-C at 6.0 min as determined by an on-line scintillation detector (Radiomatic, Tampa, FL). Moravek Biochemicals (Brea, CA) supplied the [5-^{3}H]Urd (20 Ci/mmol), [2,8-^{3}H]Ado (50 Ci/mmol) and [*methyl*-^{3}H]dThd (25 Ci/mmol). The radiopurity of the tritiated nucleosides was documented by HPLC as described above. Low-melting point agarose and the Horizon 11.14 horizontal gel electrophoresis apparatus were from Bethesda Research Laboratories (Gaithersburg, MD). Other chemicals were obtained from either NIH Stores (Bethesda, MD) or the Sigma Chemical Co. (St. Louis, MO).

Cell culture

The characteristics of HCT 116 cells, NCI-H630 and SNU-C4 cells have been reported previously [9, 10]. The cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, and 7% nondialyzed fetal bovine serum (FBS) (obtained from Biofluids Inc., Rockville, MD). Cell growth and colony formation assays were performed using previously described techniques [6, 11]. Colonies were defined as greater than 30 cells for NCI-H630 and SNU-C4 cells or greater than 50 cells for HCT 116 cells.

Assay for cytidine deaminase and deoxycytidylate deaminase

Cytidine deaminase (EC 3.5.3.5) activity was determined by the method of Chabner *et al.* [12]. Deoxycytidylate (dCMP) deaminase (EC 3.5.4.14) activity was determined by a modification of the method described by Drake *et al.* [13].

Determination of ribonucleotide pools

Cells were exposed either to phosphate-buffered saline (PBS), or to 0.05, 0.5 or 10 μM CPE-C for 24 hr. The cells were harvested by washing once with iced PBS, and then extracting with 0.5 N perchloric acid (PCA) for 20 min at 4°. The acid-soluble fraction was neutralized with 2 vol. of trichlorotrifluoroethane/tri-*n*-octylamine (3.4:1.5); after adjusting the pH to 7 with 0.5 N NaOH, the sample was frozen on dry ice, lyophilized, and stored at −70°. Immediately prior to analysis, the residue was resuspended in water, and analyzed according to a previously published anion-exchange HPLC method (termed HPLC method 1) [6] adapted from Plunkett *et al.* [14, 15]. The standard curve for peak area as a function of quantity of each ribonucleotide triphosphate, prepared by injecting a known amount of nucleotide triphosphate, was linear from 2 to 290 nmol. The retention times (min) were as follows: UTP, 13.7; CTP, 16.9; ATP 20.2; and GTP, 28.1.

Measurement of dCTP and dTTP pools

The enzymatic assay used for the measurement of dCTP and dTTP was a previously described modification [6, 11] of the method of Solter and Handschumacher [16] as described by Hunting and Henderson [17].

Analysis of CPE-C nucleotide formation and incorporation into methanol-precipitable material

Exponentially growing cells were exposed to 0.05 μM (sp. act. 3.8 μCi/nmol), 0.5 μM (sp. act. 0.24 to 0.64 μCi/nmol) or 10 μM (sp. act. 0.019 μCi/nmol) [^{3}H]CPE-C or PBS for the desired interval (3, 6 or 24 hr), then washed three times with iced PBS, and extracted with iced 60% methanol. The methanol-soluble fraction was separated and concentrated to dryness in a Savant Speed-Vac. In some experiments, the cells were extracted with 0.5 N PCA, neutralized and lyophilized as described above. The results with both extraction procedures were comparable.

Two different HPLC methodologies for analysis of CPE-C metabolites were employed. An aliquot of the methanol-soluble fraction was directly injected with nonradioactive standards into the HPLC system for analysis. Initially, a protracted gradient was used (HPLC method 2) to separate uridine and cytidine nucleotides. An SAX Radial-Pak column (Waters) was developed using two buffers as the mobile phase: buffer A was 0.001 M ammonium phosphate, pH 3.0, and buffer B was 0.75 M ammonium phosphate, pH 4.5. An isocratic gradient of 100% buffer A was run at 1.8 mL/min for 8 min, followed by a linear gradient at 2 mL/min to 94% buffer A/6% buffer B over 8 min. The following linear gradients were run in series (% buffer A/% buffer B): to 92%/8% over 44 min; 80%/20% over 6 min; to 70%/30% over 5 min, followed by an isocratic gradient (70% A/30% B) for 5 min; to 40%/60% over 30 min; then the column was washed with 100% buffer B for 4 min. The column was then allowed to equilibrate for 15 min at initial conditions prior to the next run.

The retention times (min) were as follows: CMP, 7.3; UMP, 18.3; CDP, 50.1; UDP, 56.5; UTP, 89.3; and CTP, 94.1.

After documenting that CPE-U nucleotide formation was negligible, an abbreviated method was thereafter used to quantitate CPE-C metabolites (HPLC method 3). A linear gradient was run from 100% Buffer A (0.007 M ammonium phosphate, pH 4.5) to 70% Buffer A/30% Buffer B (0.75 M ammonium phosphate, pH 4.5) over 6 min, followed by an isocratic gradient for the next 9 min. A linear gradient was then run over 15 min to 28% Buffer A/72% Buffer B; 100% Buffer B was run for an additional 4 min to wash the column. Four tritium peaks were apparent; the first and fourth peak coeluted with cold CPE-C (2.0 min) and CPE-CTP (27.2 ± 0.4 min), respectively. The second (6.7 ± 0.4 min) and third (11.6 ± 0.1 min) peaks were presumed to represent mono- and diphosphate metabolites, since this was the area in which cold CMP (6.8 min) and CDP (10.5 min) eluted; UTP eluted at 27.3 min.

Aliquots of the methanol-soluble fraction were concentrated to dryness in a Savant Speed-Vac, reconstituted in distilled water, and 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 centrifuged through a 0.22 μm Amicon Centrifree micropartition system (W. R. Grace & Co., Danvers, MA), and frozen until analyzed by reversed-phase HPLC (referred to as HPLC method 4) with 0.1 M ammonium formate, pH 5.0, as the mobile phase to distinguish CPE-C from CPE-U [18]. With this method, nucleotides elute with the void volume at 2 min; CPE-C and CPE-U elute at 11 and 14 min, respectively.

Incorporation into the methanol-precipitable fraction was determined by washing the pellet three times with iced 60% methanol; the pellet was dissolved in 0.5 N NaOH, neutralized with HCl, and then counted in a liquid scintillation counter.

Analysis of RNA incorporation

Isopycnic density centrifugation. Cells grown in replicate T175 cm^2 flasks were exposed to [^{3}H]CPE-C (0.05 μM, sp. act. 2.2 μCi/nmol; or 0.5 μM, sp. act. 0.24 to 0.64 μCi/nmol) for 6 or 24 hr. The medium was aspirated; the cells were washed three times with PBS, harvested by trypsinization, and then isolated by centrifugation. After overnight digestion in a lysis buffer [0.4 mg/ml Proteinase K (EC3.4.21.14), 10 mM Tris/10 mM EDTA (pH 7.4), 0.4 M NaCl, and 0.4% sodium dodecyl sulfate (SDS)] at 37°, the nucleic acids were purified by phenol/chloroform extractions and ethanol precipitation as previously described [6]. One of the duplicate samples for each cell line was treated with 50 μL DNAse-free RNAase (bovine pancreas, EC 3.1.27.5, 500 μg/mL; Boehringer Mannheim, Indianapolis, IN) for 60 min. Samples were then repurified by phenol/chloroform extractions and ethanol precipitation, and then were subjected to cesium sulfate density centrifugation as previously described [6, 19, 20]. Twenty-four 0.25-mL fractions were collected from the bottom to the top of the gradients, precipitated with 10% trichloroacetic acid (TCA), and collected onto Whatman type HA filters. The filters were washed three times with 5% TCA, once with 95% ethanol, and then counted. RNA (between density 1.62 and 1.68 g/mL) was precipitated in fractions 3 to 12, and the DNA (between density 1.42 and 1.48 g/mL) in fractions 18 to 24.

To document that the acid-precipitable radioactive material comigrating in the RNA fraction represented authentic CPE-C metabolites, purified nucleic acid was treated with DNAse-free RNAse. After 1 hr, ice-cold PCA was added to yield a final concentration of 0.5 N. The acid-soluble fraction was separated, neutralized and lyophilized. The residue was reconstituted in distilled water, and an aliquot was incubated with 5 U alkaline phosphatase at 37° for 90 min. The sample was filtered and analyzed by HPLC method 4.

Agarose gel electrophoresis. Cells in logarithmic growth phase were incubated for 24 hr with 0.5 μM [^{3}H]CPE-C (0.3 to 0.5 μCi/nmol), 20 μM [^{3}H]FUra (0.005 μCi/nmol) or 0.5 μM [^{3}H]Cyd (0.4 μCi/nmol); the medium was then removed and the cells were washed three times with ice-cold PBS. Total cellular RNA was then isolated by acid-guanidium thiocyanate phenol/chloroform extraction according to the method of Chomczynski and Sacchi [21] using a Stratagene RNA Isolation Kit (La Jolla, CA). To minimize [^{3}H] contamination from unincorporated nucleotide, the RNA was further purified on an RNAse-free Spehadex G-25 column (Select-D(RF)^{TM} G-25 column, 5 Prime-3 Prime, West Chester, PA). Purity of the RNA sample was documented by a 260 nm/280 nm O.D. ratio of 1.8 to 2.0. RNA integrity was documented by ethidium bromide staining of the major RNA species (28 S and 18 S rRNA) after electrophoresis on a 5 × 8 cm 1.1% low-melting point agarose/6.6% formaldehyde gel in 3-[*N*-morpholino]propanesulfonic acid (MOPS) buffer (Digene Diagnostics, Inc., Silver Spring, MD) for 2 hr at 50 V (room temperature).

To determine the distribution of [^{3}H]CPE-C among the RNA species, RNA samples were electrophoresed on a 11 × 14 cm gel (as above) for 18 hr at 20 V (room temperature). The gels were cut into 0.5-cm slices and melted; after addition of liquid scintillation fluid, the samples were counted.

RESULTS

Cytotoxicity of CPE-C

CPE-C inhibited the growth of each cell line over a 72-hr period in a concentration- and time-dependent fashion. The 50% inhibitory concentration (IC_{50}) of CPE-C ranged from 0.4 to 0.8 μM for an initial 3-hr drug exposure; approximately one log less CPE-C was required for 50% inhibition if the duration of drug exposure was extended to 72 hr: 0.01 to 0.06 μM (data not shown).

In clonogenic studies, the IC_{50} of CPE-C decreased as the duration of exposure increased; the IC_{50} values among the three cell lines varied by less than 2-fold for each duration of exposure (Table 1). In general, 5- to 9-fold more CPE-C was required to further decrease colonogenic capacity from 50% to 90%

Table 1. Lethality of CPE-C in colon carcinoma cell lines

	IC_{50} μM (mean ± SEM) or AUC [(μM·hr)] 3 hr	24 hr	168 hr
HCT 116	5.0 ± 1.5 [15.0]	0.19 ± 0.04 [4.6]	0.035 ± 0.005 [4.2]
SNU-C4	10.0 ± 4.4 [30.0]	0.37 ± 0.12 [8.9]	0.057 ± 0.011 [9.6]
NCI-H630	8.4 ± 2.5 [25.2]	0.75 ± 0.41 [18]	0.060 ± 0.011 [10.1]
	IC_{90} μM (mean ± SEM) or AUC [(μM·hr)] 3 hr	24 hr	168 hr
HCT 116	29 ± 14 [87]	1.0 ± 0.3 [24]	0.19 ± 0.09 [22.8]
SNU-C4	93 ± 74 [279]	14.1 ± 5.7 [338.4]	0.48 ± 0.04 [80.6]
NCI-H630	48 ± 21 [144]	2.3 ± 0.8 [55.2]	0.51 ± 0.12 [85.7]

Exponentially growing cells (300–500) were cloned in duplicate 6-well plates, and exposed to CPE-C at concentrations ranging from 0.01 to 50 μM for 3, 24 or 168 hr. The medium was gently aspirated following the 3- and 24-hr drug exposures, and the cells were washed once with PBS, following which fresh drug-free medium was replaced. The cells were stained following a 7-day incubation, and colonies were enumerated. For each experiment, the colony number as a percent of control was plotted against the concentration of CPE-C, and the IC_{50} value was extrapolated. The mean IC_{50} and IC_{90} values (± SEM) are from three (168 hr), five (3 hr) or six to ten (24 hr) separate experiments each done in duplicate. The area under the concentration time curve for each cell line was calculated by multiplying the IC_{50} or IC_{90} in μM for each duration of exposure by the hours of exposure. The cloning efficiency for each cell line was as follows (mean ± SEM): HCT 116, 41 ± 4% SNU-C4, 51 ± 5%; and NCI-H630, 37 ± 7%

(IC_{90}). In contrast, the uridine analog (CPE-U) was non-toxic at concentrations ≤ 1 mM for 144 hr.

The data are also expressed as area under the concentration–time curve [μM·hr] (Table 1). At the IC_{50}, a 2.7- to 3.6-fold higher AUC was required for comparable cell kill with the 3-hr exposure compared to the 168-hr exposure; for the 24-hr exposure, a 1.5- to 3.4-fold higher AUC was required. The AUC associated with 90% lethality was similar for both 24-hr and 168-hr exposures in HCT 116 cells and NCI-H630 cells; for a 3-hr exposure, a 1.7- to 3.5-fold higher AUC was necessary. When the IC_{90}, expressed in μM, for a 3-hr versus a 168-hr exposure for each cell line was compared, the ratio ranged from 94 to 194. These results suggest that a comparatively narrow AUC range is associated with a given level of toxicity; as the duration of exposure increased from 3 to 24 hr, a lower total AUC was associated with 50% and 90% lethality.

Intracellular metabolism of CPE-C

The nucleoside transport inhibitor, dipyridamole, potently inhibited the uptake of [^{3}H]CPE-C into the acid-soluble fraction, and the IC_{50} for a 30-min uptake ranged from 0.002 to 0.005 μM for the three cell lines (data not shown). Our results agree with those of Ford *et al.* [5], and suggest that CPE-C enters human colon cancer cells via the facilitated nucleoside transport mechanism.

CPE-C is deaminated in primates [18]; further, CPE-U nucleotide metabolites were detected in Molt-4 lymphoblastic leukemia cells [5]. We therefore measured the activities of Cyd deaminase and dCMP deaminase in the three colon lines. Cyd deaminase activity ranged from 70 to 270 pmol/min/mg protein. The activity of dCMP deaminase was over two orders of magnitude greater, ranging from 18 to 40 nmol/min/mg protein.

A protracted (115 min) HPLC gradient was initially employed to distinguish CPE-C and any possible CPE-U nucleotide metabolites. Each cell line avidly phosphorylated CPE-C; the triphosphate, CPE-CTP, was the predominant nucleotide, followed in order of magnitude by CPE-CDP and CPE-CMP. CPE-UTP accounted for less than 1.5% of the total metabolites in all three cell lines; the mono- and diphosphate derivatives of CPE-U were not detected. To document that the tritiated nucleotides were indeed CPE-C metabolites, an aliquot of the cell extract was treated with alkaline phosphatase. When reanalyzed by the anion-exchange HPLC, tritium coeluting with the nucleotide fractions disappeared and all of the tritium counts coeluted with the solvent front at 2.0 min. When another aliquot of alkaline phosphatase-treated residue was analyzed by reversed-phase HPLC method 4 [18], virtually all of the tritium coeluted with cold CPE-C standard; [^{3}H]-CPE-U represented < 2% of the nucleosides in HCT 116 and SNU-C4 cells, but accounted for 5–9% of the [^{3}H]nucleosides in NCI-H630 cells.

Because the sensitivity of all three cell lines was similar, detailed studies were performed with the HCT 116 cell line. More limited studies were performed with the SNU-C4 and NCI-H630 cells to corroborate the results in HCT 116 cells. As the [^{3}H]nucleotide derivatives predominantly reflected CPE-C metabolites, thereafter a more expedient HPLC method was used to quantitate CPE-C metabolites. CPE-CTP formation in HCT 116 cells following a 3-hr exposure increased progressively with increasing concentrations of CPE-C from 0.05 to 5 μM (Fig. 1A), and then appeared to plateau. The peak CPE-CTP pool size following 5 μM CPE-C for 3 hr was 4370 ± 322 pmol/10^6 cells. Assuming that 10^6 cells have a 1 μL volume, the intracellular concentration of CPE-CTP is estimated to be 4.4 mM, which is of similar magnitude to the endogenous concentrations of UTP and CTP (Table 2). The percent of the total metabolites represented by CPE-CTP was 83–95% at concentrations at or below 10 μM, but decreased considerably with 25 and 100 μM CPE-C (Fig. 1B). The decrease in the percentage of CPE-CTP was accompanied by an increase in the proportion of metabolites represented by parent drug. The proportion of monophosphate (varying from 0 to 6.4% of the total) and diphosphate (varying from 4.5 to 13.5%), however, did not appear to be concentration dependent. CPE-CTP pools after a 24-hr exposure increased 5.3- and 1.7-

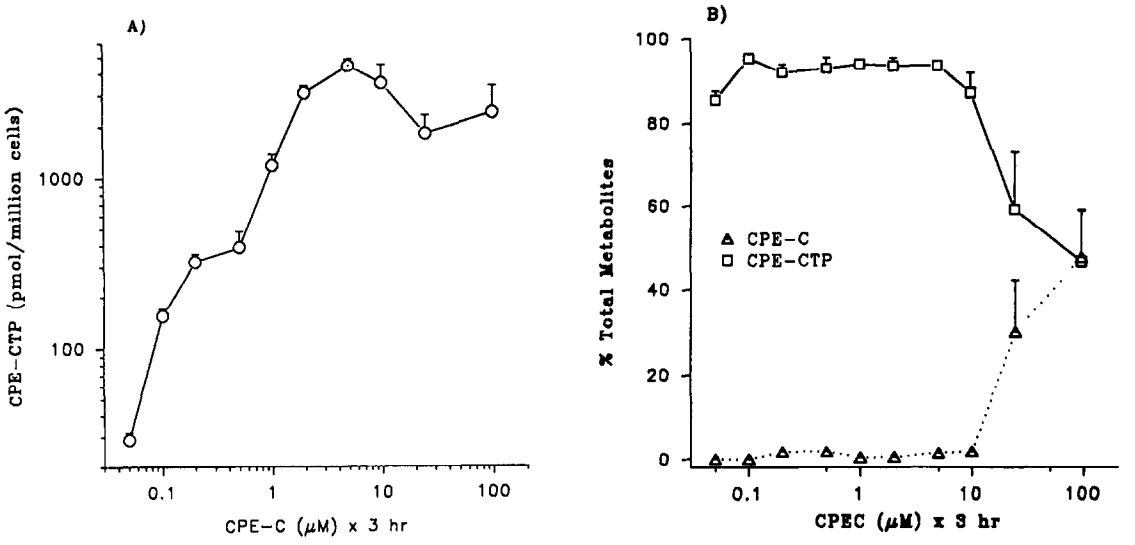

Fig. 1. CPE-CTP formation as a function of CPE-C concentration. Exponentially growing HCT 116 cells were exposed to various concentrations of [^{3}H]CPE-C ranging from 0.05 to 100 μM for 3 hr. Following aspiration of the medium, the cells were washed three times with iced PBS, and then extracted with 60% iced methanol. CPE-CTP formation was determined by HPLC method 3 as described in Materials and Methods. The data are presented as pmol/10^6 cells CPE-CTP as a function of CPE-C concentration (panel A). The data (mean ± range) for 0.05, 0.1, 0.2, 1, 2 and 5 μM CPE-C are from two determinations; the data for 0.5, 10, 25 and 100 μM CPE-C (mean ± SEM) are from four to seven determinations. The percentage of total CPE-C metabolites represented by parent drug and CPE-CTP is shown in panel B.

fold over that achieved with a 3-hr exposure at concentrations ranging between 0.05 and 0.5 μM, respectively, but no further accumulation in CPE-CTP was seen with higher concentrations (a 0.9- and 1.2-fold increase for 1 and 10 μM CPE-C). Taken together, these observations suggest that saturation of phosphorylation to the monophosphate is the rate-limiting metabolic step.

Similar results were seen in the other two colon cancer cell lines. After a 3-hr exposure, the CPE-CTP pools were as follows (pmol/10^6 cells, mean ± SEM): SNU-C4: 0.5 μM, 161 ± 93 (N = 4); 10 μM, 1782 (N = 2); NCI-H630; 0.5 μM, 142 ± 77 (N = 4); 10 μM, 990 (N = 2). No further accumulation of CPE-CTP was evident in SNU-C4 cells after a 24-hr exposure; in NCI-H630 cells, however, CPE-CTP pools increased by 2.1- and 1.6-fold after a 24-hr exposure to 0.5 and 10 μM, respectively.

Destruction of ribonucleotides was carried out by incubating an aliquot of methanol-soluble extract with sodium periodate and methylamine [22]. [^{3}H]-CPE-C deoxyribonucleotide metabolites were not detected in any of the cell lines.

Effect of CPE-C on nucleotide triphosphate pools

The effect of CPE-C on ribonucleotide triphosphate pools was examined. The adequacy of the extraction procedure was confirmed by measuring the ratio of the area of ATP to ADP for each HPLC run; the ATP : ADP ratio for these experiments was 13.4 ± 0.6 (mean ± SEM). Depletion of CTP pools was evident within 3 hr of CPE-C exposure; following a 24-hr exposure, the CTP pools were decreased by 62–78% with 0.05 μM CPE-C, and by 82–90% from baseline with 0.5 μM CPE-C in the three cell lines (Table 2). No consistent changes were seen in the other ribonucleotide triphosphate pools. Figure 2 depicts depletion of CTP pools as a function of CPE-CTP formation in the HCT 116 cells. A 50% decrease in CTP pools occurred with 100–150 pmol/10^6 cells of CPE-CTP. CPE-CTP accumulation above 800 pmol/10^6 cells decreased CTP pools by more than 85% and was associated with over 90% lethality. The ratio of CPE-CTP to CTP increased from 2.6 to 23 with 0.5 μM and 10 μM CPE-C, respectively, after 24 hr.

A DNA polymerase (EC 2.7.7.7) assay was used for the determination of dCTP pools in HCT 116 cells. The baseline dCTP level was 27.8 ± 3.7 pmol/10^6 cells. A 3-hr exposure to 0.5 μM CPE-C reduced dCTP pools by 54% to 12.8 ± 3.0 pmol/10^6 cells (*t*-test, $P_2 = 0.006$). Extending the exposure to 24 hr further reduced dCTP pools by 72% to 7.7 ± 2.4 pmol/10^6 cells. Thus, CTP depletion was accompanied by parallel decrements in dCTP pools. In contrast to the effect on dCTP, CPE-C did not decrease dTTP pools (pmol/10^6 cells: control, 112 ± 21; 0.5 μM CPE-C for 3 hr, 132 ± 16).

Intracellular retention of CPE-CTP and duration of CTP depletion following drug removal

The stability of intracellular CPE-CTP after drug removal was determined in HCT 116 cells. After a 24-hr exposure to 0.05 μM CPE-C, CPE-CTP formation was 70 pmol/10^6 cells; 24 hr following washout, it decreased to 31 pmol/10^6 cells. Figure 3 illustrates initial CPE-CTP levels after a 24-hr exposure to 0.5 μM CPE-C and the residual metabolite over a 4-day period following washout. The CPE-CTP pool decreased by 49% in the first

Table 2. Effect of CPE-C on ribonucleotide triphosphate pools

Cell line	CPE-C (μM)	RTP pool (nmol/10^6 cells) UTP	CTP	ATP	GTP
HCT 116	None	6.0 ± 0.6	2.4 ± 0.3	21.6 ± 3.3	4.0 ± 0.4
	0.05	6.0 ± 0.5	0.8 ± 0.3*	25.4 ± 6.6	6.3 ± 2.2
	0.5	8.3 ± 1.8	0.43 ± 0.05*	20.9 ± 3.0	6.5 ± 1.6
SNU-C4	None	6.9 ± 0.5	0.8 ± 0.1	11.5 ± 3.9	3.2 ± 0.7
	0.05	3.8 ± 0.9	0.18 ± 0.08*	10.5 ± 2.4	2.1 ± 0.6
	0.5	6.6 ± 1.3	0.08 ± 0.03*	17.3 ± 3.1	3.2 ± 0.5
NCI-H630	None	9.1 ± 2.0	0.7 ± 0.1	10.4 ± 2.3	3.2 ± 0.7
	0.05	6.4 ± 1.0	0.22 ± 0.05*	9.8 ± 1.8	2.2 ± 0.4
	0.5	5.5 ± 1.3	0.12 ± 0.06*	13.9 ± 2.2	3.3 ± 0.04

Cells were exposed to PBS, or CPE-C at 0.05 or 0.5 μM for 24 hr, followed by PCA (0.5 N) extraction. The acid-soluble fraction was processed as described in Materials and Methods, and an aliquot was analyzed by anion-exchange HPLC method 1. The data, from three separate experiments each done in duplicate, are presented as the mean ± SEM.

* P_2 (Student's t-test) < 0.05.

24 hr post washout, but triphosphate was detected for up to 4 days. The persistence of CPE-CTP was accompanied by continued depletion of CTP pools below 0.5 nmol/10^6 cells up to 96 hr post washout (data not shown).

Inhibition of RNA and DNA synthesis and effect on cell cycle distribution

The effect of various concentrations of CPE-C on RNA and DNA synthesis was estimated by measuring incorporation of nucleoside precursors into acid-precipitable material in HCT 116 cells (Fig. 4). After a 24-hr drug exposure, the cells were washed to remove CPE-C from the extracellular medium; then radiolabeled precursor was added. Inhibition of [^{3}H]-uridine (Urd) incorporation into acid-precipitable material by CPE-C was not a reliable measure of RNA synthetic inhibition because the uptake and phosphorylation of Urd was inhibited by 75% in HCT 116 cells pretreated with 10 μM CPE-C compared to control (data not shown). Therefore, [^{3}H]Ado incorporation was used to estimate RNA synthesis because CPE-C did not affect Ado uptake into the acid-soluble cell fraction. CPE-C inhibited

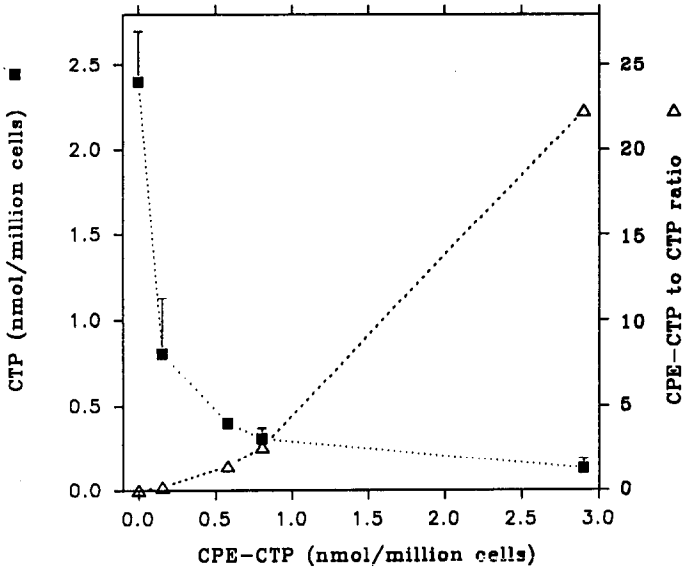

Fig. 2. CTP depletion as a function of CPE-CTP formation. After a 24-hr exposure to 0.05, 0.2, 0.5 and 10 μM CPE-C, HCT 116 cells were extracted with PCA as described in Materials and Methods. CTP levels were determined by anion exchange HPLC method 1. In separate experiments, cells were exposed to [^{3}H]CPE-C for 24 hr at the above concentrations, and CPE-CTP levels were measured by HPLC method 3. The CTP pool size (left axis) is plotted against the mean CPE-CTP level (■); mean CTP level ± range is presented for 0.05 and 0.2 μM; mean CTP level ± SEM are shown for 0.5 μM (N = 7) and 10 μM CPE-C (N = 4). The ratio of CPE-CTP to CTP pools is shown on the right axis (△).

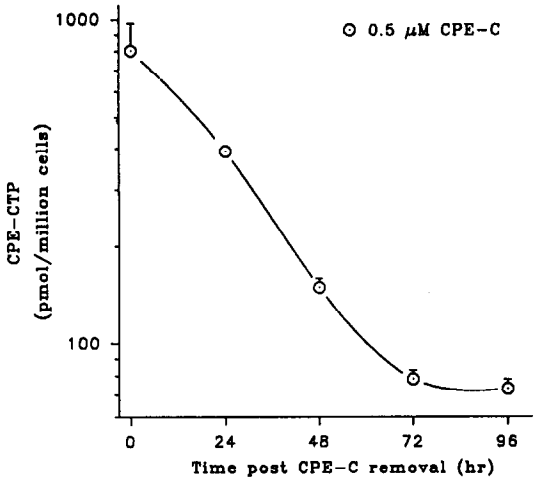

Fig. 3. CPE-CTP retention after drug removal in HCT 116 cells. After a 24-hr exposure to 0.5 μM [^{3}H]CPE-C, the medium was aspirated and the cells were washed twice; then drug-free medium was replaced. At various intervals after drug exposure, the cells were extracted and CPE-CTP levels were determined by HPLC method 3. The data, presented as the mean ± range, are from two to three separate experiments.

Ado incorporation into RNA in a concentration-dependent fashion, but even at the highest concentration tested, 10 μM, inhibition of Ado incorporation was incomplete (53% of control).

[^{3}H]dThd incorporation was used as an index of DNA synthesis. Inhibition of DNA synthesis by

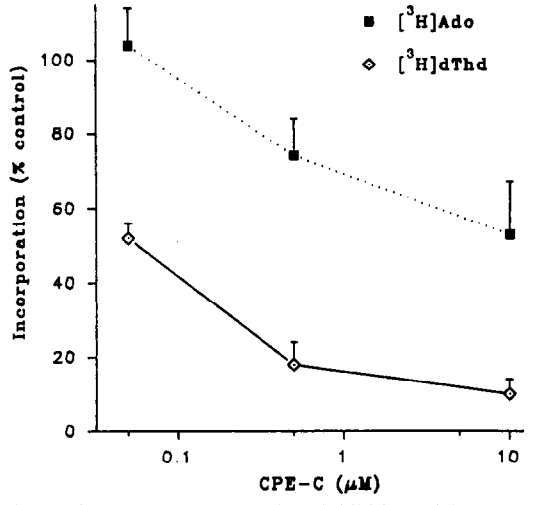

Fig. 4. Concentration-dependent inhibition of RNA and DNA synthesis by CPE-C. HCT 116 cells were exposed to the desired concentration of CPE-C for 24 hr; then they were washed three times with PBS and drug-free medium was added. The cells were pulse-labeled with 1.0 μCi [^{3}H]-Ado or [^{3}H]Thd for 60 min, and then acid-precipitable incorporation was determined. The data, presented as the percent of control incorporation (mean ± SEM), are from three (dThd) or four (Ado) separate experiments each done in duplicate. [^{3}H]dThd incorporation for control cells was 2.2 ± 0.3 pmol/10^6 cells; [^{3}H]Ado incorporation for control cells was 61.0 ± 15.3 fmol/10^6 cells.

Table 3. Effect of CPE-C on cell cycle distribution

	Fraction of cells in DNA cycle phases (%)		
CPE-C (μM)	G_1	S	G_2 + M
0	50	26	24
0.1	6	59	35
1.0	4	70	26

Exponentially growing NCI-H630 cells were exposed to no drug or to CPE-C at the indicated concentrations. After 48 hr, viability of adherent control cells and drug-treated cells was assessed by trypan blue exclusion. Single cell suspensions were prepared from duplicate flasks. The cells were fixed in 50% ethanol, treated with RNAse A (EC 3.1.26.2), stained with propidium iodide, and analyzed on a Becton-Dickinson FACStar flow cytometer to determine the fraction of cells in each DNA cycle phase. The data are from a representative experiment; similar results were seen in other experiments.

CPE-C was concentration-dependent for 3- and 24-hr exposures in all three cell lines. For SNU-C4 and NCI-H630 cells, 86–91% inhibition of [^{3}H]dThd incorporation following a 24-hr exposure was observed with 0.05 μM CPE-C. In HCT 116 cells, greater than 80% inhibition of DNA synthesis required 0.5 μM CPE-C for 24 hr (Fig. 4). Inhibition of DNA synthesis appeared to be directly related to depletion of CTP and consequently dCTP pools by CPE-C.

After a 24-hr exposure to 0.1 and 1.0 μM CPE-C, a shift in cell cycle distribution was apparent with an accumulation of cells in S phase. After 48 hr, S phase accumulation was more pronounced, accompanied by a decrease in the proportion of cells in G_1 phase (Table 3). Similar results were obtained at 48 hr when cells were treated with CPE-C for only the initial 24 hr, consistent with persistent biochemical effects after drug removal.

Nucleic acid incorporation of CPE-C

[^{3}H]CPE-C incorporation into methanol-precipitable material after a 3-hr exposure in HCT 116 cells increased as the drug concentration increased from 0.05 to 5 μM (0.1 ± 0.04 to 23.8 ± 0.4 pmol/10^6 cells), but no further incorporation was evident at 10, 25 or 100 μM, consistent with the plateau in CPE-CTP formation. Similar results were obtained in NCI-H630 and SNU-C4 cells.

To further clarify whether CPE-C incorporated into nucleic acids, cells were exposed for 6 hr to 0.5 μM [^{3}H]CPE-C; nucleic acids were then purified and separated by cesium sulfate density centrifugation. When total nucleic acids were analyzed (without RNAse treatment), all the acid-precipitable counts localized in the RNA fractions; DNA incorporation was not detected. When the purified nucleic acids were treated with RNAse prior to density centrifugation, no tritium counts were recovered in the RNA fractions.

To document the identity of the tritium counts appearing in the RNA fraction, purified nucleic acids were enzymatically digested to nucleosides, and

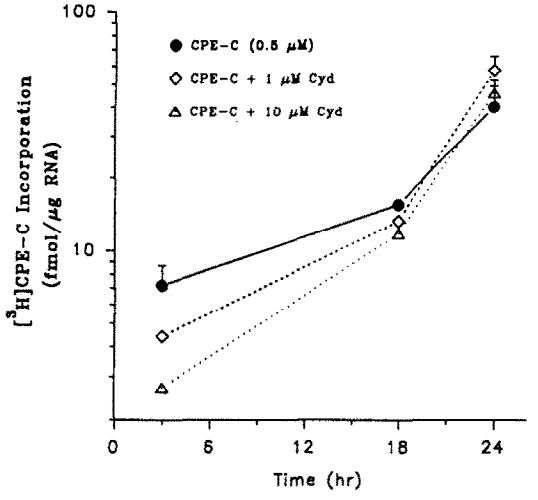

Fig. 5. Time-dependent incorporation of CPE-C into RNA. Exponentially growing HCT 116 cells were exposed to 0.5 μM [^{3}H]CPE-C in the presence and absence of Cyd (1 or 10 μM) for 3, 18 or 24 hr. The RNA was extracted and purified as described in Materials and Methods. For CPE-C alone, the data are shown as the mean ± SEM (3 and 18 hr, N = 4; 24 hr, N = 17). For CPE-C plus Cyd, the data for 3 and 18 hr are from a single experiment; the 24-hr data are shown as the mean ± SEM (1 μM Cyd, N = 14; 10 μM Cyd, N = 10).

analyzed by reversed-phase HPLC. Tritium co-eluted with cold CPE-C standard, but not with CPE-U or with other pyrimidine nucleosides, indicating that the tritium incorporated into nucleic acids represented genuine CPE-C metabolites.

RNA incorporation was quantified using an alternate purification method: acid guanidinium thiocyanate phenol/chloroform extraction [21]. The purity and integrity of the RNA were assessed spectrally and by agarose gel electrophoresis. The data for [^{3}H]RNA was considered reliable if the RNA appeared to be non-degraded as indicated by ethidium bromide staining of intact 28 S and 18 S rRNA bands. The amount of [^{3}H]CPE-C incorporated into RNA at a fixed concentration, 0.5 μM, increased 5.6-fold from 3 to 24 hr (Fig. 5); the effect of concurrent exposure to Cyd on CPE-C incorporation will be discussed in the following section. With a fixed duration of exposure, 24 hr, CPE-C RNA incorporation increased 21-fold as the concentration increased from 0.01 μM (2.7 fmol/μg) to 1.0 μM (Table 4). To demonstrate that the [^{3}H] counts reflected CPE-C-RNA rather than unincorporated [^{3}H]CPE-C-nucleotides, the RNA was further purified with a Select D(RF)^{TM} (Sephadex G-25) column. [^{3}H]CPE-C incorporation into RNA after a 24-hr exposure to 0.5 μM post-column purification (30.4 ± 3.8 fmol/μg RNA) was not significantly different from that determined prior to the Select-D column (P = 0.42). Digestion of purified RNA to the nucleoside level followed by HPLC analysis indicated that the tritium comigrated with cold CPE-C standard; [^{3}H]CPE-U was not detected in the RNA digests.

At equal concentrations, [^{3}H]Cyd incorporation into cellular RNA was 250-fold (1.0 μM) and 354-fold (0.1 μM) greater than CPE-C incorporation (Table 4). Following a 24-hr exposure to 20 μM FUra (IC_{70}), the magnitude of [^{3}H]FUra-RNA was 113-fold higher than CPE-C-RNA incorporation after 0.5 μM CPE-C (IC_{90}) (Table 4).

The distribution of [^{3}H]CPE-C into the various RNA species was determined by counting the radioactivity in 0.5-cm gel slices after electrophoresis. CPE-C predominantly localized in low molecular weight RNA (4–8 S) species after both brief and 24-hr exposures; the ratio of [^{3}H]CPE-C in low molecular weight species to 28 S + 18 S rRNA was 1.9 and 6.4 after a 24-hr exposure to 0.5 and 10 μM, respectively (Table 4). In contrast, when cells were labeled with [^{3}H]Cyd under control conditions, 10% or less of the total tritium counts were identified in the low molecular weight RNA species. These results suggested several possibilities: preferential incorporation of CPE-C into low molecular weight species, chain termination, enhanced degradation of RNA containing CPE-C, and enhanced synthesis or decreased turnover of low molecular weight species. Of note, the ratio of [^{3}H]FUra in low molecular

Table 4. RNA incorporation of CPE-C, FUra and Cyd

Treatment (μM)	Incorporation after 24 hr (fmol/μg RNA)	Distribution into RNA species (% total)		
		28 S	18 S	Low mol. wt species
[^{3}H]CPE-C 0.5	40.2 ± 9.2	14 ± 4	21 ± 6	65 ± 9
[^{3}H]CPE-C 0.5 + Cyd 1	57.6 ± 8.4	3	2	95
[^{3}H]CPE-C 0.5 + Cyd 10	46.0 ± 6.3	13	18	69
[^{3}H]CPE-C 1.0	58.0 ± 7.8	7 ± 1	6 ± 2	83 ± 3
[^{3}H]FUra 20	4,541 ± 710	30	32	28
[^{3}H]Cyd 0.1	1,273	60	15	7
[^{3}H]Cyd 1.0	14,500	83	7	10

HCT 116 cells were exposed to [^{3}H]CPE-C (alone or with Cyd), [^{3}H]Cyd, or [^{3}H]FUra for 24 hr. The RNA was extracted and purified as described in Materials and Methods. The CPE-C data, presented as means ± SEM, are either from six to seventeen determinations (for total CPE-C incorporation) or from four experiments (RNA distribution). The data for total [^{3}H]FUra incorporation (means ± SEM) are from ten experiments; the FUra-RNA distribution data are averages of two separate determinations. The data for [^{3}H]Cyd are from a single experiment.

Table 5. Effects of CPE-C on adenosine incorporation and distribution into RNA

Duration of exposure	CPE-C (0.5 μM)	fmol/μg RNA (% Ado alone)	Distribution into RNA species: % total (fmol/μg) 28 S	18 S	Low mol. wt species
6 hr	No	3710	56% (2078)	16% (594)	20% (742)
	Yes	1940 (52%)	42% (815)	14% (272)	30% (582)
12 hr	No	3920 ± 523	36 ± 6% (1411)	23 ± 6% (902)	24 ± 4% (941)
	Yes	2364 ± 372 (60%)	25 ± 7% (591)	14 ± 2% (331)	46 ± 3% (1087)
24 hr	No	4750 ± 564	55 ± 3% (2612)	24 ± 3% (1140)	12 ± 2% (570)
	Yes	3829 ± 417 (81%)	46 ± 4% (1761)	27 ± 2% (1034)	22 ± 3% (842)
	Yes (+ 10 μM Cyd)	5293 ± 504 (111%)	43 ± 3% (2276)	27 ± 2% (1429)	22 ± 2% (1164)

HCT 116 cells were exposed to [^{3}H]Ado alone or with 0.5 μM CPE-C. The RNA was purified and analyzed by agarose gel electrophoresis as described in Materials and Methods. The data are presented as total RNA incorporation (6 hr, mean fmol/μg; 12 hr and 24 hr, mean ± SEM). The number of experiments for each duration of exposure is as follows: 6 hr, N = 2; 12 hr, N = 5; 24 hr, N = 18–24. The distribution into three RNA species, 28 S, 18 S and 4–8 S, is presented as the percent of the total counts in all gel slices (6 hr, mean, N = 2; 12 hr, mean ± SEM, N = 4; 24 hr, mean ± SEM, N = 12–18).

weight RNA species to 28 S plus 18 S rRNA after a 24-hr exposure, 0.45, was also higher than that seen with [^{3}H]Cyd (0.1).

In an attempt to distinguish among these possibilities, the effect of CPE-C exposure on [^{3}H]-Ado distribution into RNA species was investigated (Table 5). Concurrent exposure to 0.5 μM CPE-C during the initial 6–12 hr of [^{3}H]Ado treatment decreased total Ado incorporation into RNA by 52 and 60%, respectively. At the 6-hr time point, CPE-C decreased the absolute amount of Ado incorporated into 28 S, 18 S, and low molecular weight RNA, consistent with global inhibition of RNA transcription. After a 12-hr exposure, CPE-C treatment was associated with a 58–63% decrease in the absolute amount of Ado incorporated into 28 S and 18 S RNA; a shift in the distribution of Ado in the various RNA species was evident, however, with a significant increase in the percentage of [^{3}H]Ado-labeled low molecular weight RNA ($P_2 = 0.04$). After a 24-hr exposure to CPE-C, a significant increase in Ado incorporation into low molecular weight RNA species was again evident ($P_2 = 0.022$). These data suggest, therefore, that CPE-C exposure is accompanied by an accumulation of low molecular weight RNA species, rather than preferential incorporation of CPE-C into low molecular weight species.

Mechanism of cytidine protection from CPE-C cytotoxicity

In an effort to clarify the relative importance of the biochemical effects of CPE-C versus the RNA incorporation on toxicity, rescue experiments with Cyd were performed. When cells received concurrent CPE-C and 1 μM Cyd, protection was noted only against 0.1 μM CPE-C (Fig. 6). When 1 μM Cyd was administered continuously during and following CPE-C exposure, however, protection was seen against all three CPE-C concentrations. In contrast, a concurrent 24-hr exposure to 10 μM Cyd was sufficient to fully protect HCT 116 cells against CPE-C concentrations at or below 1.0 μM. If the administration of 10 μM Cyd were delayed until after the 24-hr exposure to CPE-C, only partial rescue was achieved. A physiological concentration of Urd, 5 μM, given continuously during and after CPE-C exposure only protected cells against 0.1 μM CPE-C (data not shown).

The ability of Cyd to protect HCT 116 cells during continuous exposure to CPE-C rather than 24-hr pulse exposures was also examined. No colonies were evident following a 7-day exposure to 0.1 and 0.5 μM CPE-C, while 0.05 μM CPE-C reduced colony formation (mean ± SEM) to 30 ± 13% of control. Concomitant exposure to 1 μM Cyd increased the colony number (mean ± SEM) to 53 ± 9% (0.05 μM CPE-C) and 52 ± 13% of control (0.1 μM CPE-C), but was insufficient to rescue from 0.5 μM CPE-C (colony number 9% of control). Continuous exposure to CPE-C plus 10 μM Cyd increased the colony number to above 90% of control despite 0.05 and 0.1 μM CPE-C; with 0.5 μM CPE-C, 10 μM Cyd increased the colony number to 85 ± 3% of control.

The effect of Cyd on CPE-C-induced CTP depletion after a 24-hr exposure was then examined. Cyd at 1 μM increased CTP pools by 2.7-fold in the presence of 0.05 μM CPE-C, but did not replete CTP pools in the presence of 0.5 μM CPE-C (nmol/10^6 cells): 0.5 μM CPE-C alone, 0.66 ± 0.24 (mean ± SD, N = 3); CPE-C + 1 μM Cyd, 0.65 ± 0.25, mean ± range, N = 2). Coadministration of 10 μM Cyd with 0.5 μM CPE-C was associated with CTP repletion (1.95 ± 0.35 nmol/10^6 cells, mean ± range, N = 2).

Another potential factor in Cyd protection may be interference with CPE-C metabolism, either through competition for transport or phosphorylation. Following a 3-hr exposure to 0.5 μM CPE-C with 1 and 10 μM Cyd, CPE-CTP formation was decreased to 81 and 24% of CPE-C alone, respectively. Similarly, CPE-C RNA incorporation was reduced to 70 and 44% of CPE-C alone by 1 and 10 μM Cyd (Fig. 5). After a concurrent 24-hr exposure, CPE-CTP formation was unaffected by

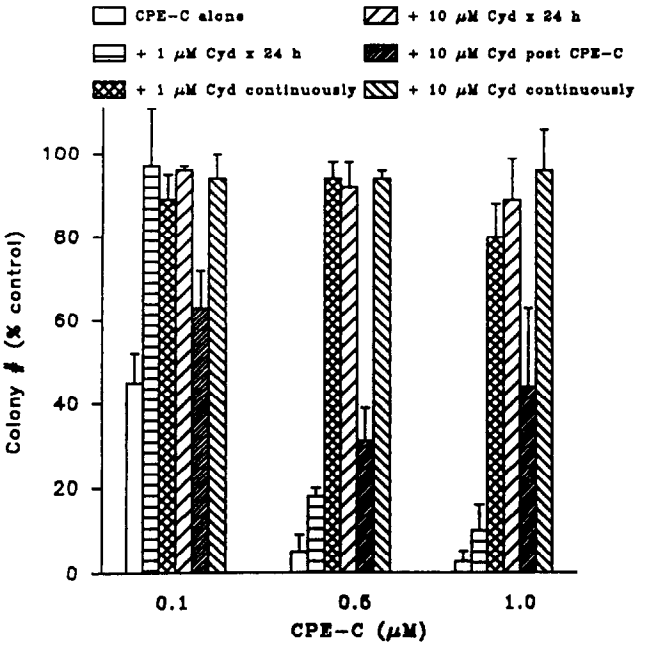

Fig. 6. Effect of Cyd on CPE-C lethality. Cloning studies were performed following a 24-hr exposure to either CPE-C alone or together with Cyd at the indicated concentrations. The cells were washed two times at hr 24, and then fresh medium was replaced containing either no drug or Cyd. Cyd was given either continuously, concurrently with CPE-C for only 24 hr, or following CPE-C removal. The data, expressed as percent control colony formation (mean ± SEM), are from three to four experiments done in duplicate. The control colony number was 140 ± 9.

1 μM Cyd (112 ± 10% of CPE-C alone), and was inhibited by 30% with 10 μM Cyd (70 ± 23% of CPE-C alone). Cyd at 1 and 10 μM did not diminish CPE-C RNA incorporation significantly after a 24-hr exposure (Table 4). Similarly, following a 24-hr exposure to 1 μM Cyd and 0.05 μM CPE-C, no interference with CPE-CTP formation or RNA incorporation was seen. With brief incubations, therefore, it appears that Cyd at a ratio of 20 to 1 can interfere with CPE-C metabolism and RNA incorporation. With longer incubations, however, CPE-C metabolism is minimally affected, presumably because the cells have reached a steady-state equilibrium.

Of note, 10 μM Cyd did not appear to alter the distribution of [^{3}H]CPE-C into various RNA species (Table 4), suggesting that CTP depletion *per se* did not account for [^{3}H]CPE-C accumulation in low molecular weight RNA species. Finally, 10 μM Cyd restored total [^{3}H]Ado incorporation into RNA to control values (Table 5) (CPE-C alone vs CPE-C + 10 μM Cyd, $P_2 = 0.03$). As observed with the [^{3}H]CPE-C distribution experiments, 10 μM Cyd did not normalize the distribution of Ado incorporation into RNA species: a disproportionate increase in Ado-labeled low molecular weight RNA species was still seen, confirming that the accumulation of low molecular weight RNA species is not dependent on CTP depletion.

DISCUSSION

The major objectives of this study were to determine the cytotoxicity of CPE-C in human colorectal carcinoma cell lines, to characterize its intracellular metabolism and RNA incorporation, and to correlate the biochemical effects with the formation of active metabolites. CPE-C appears to be highly potent against all three cell lines tested in both growth inhibition and clonogenic assays. This finding is noteworthy in view of resistance of NCI-H630 cells to several other antimetabolites. For example, the sensitivity to 5-fluorouracil (IC_{50} 2.9 to 49 μM) and trimetrexate (IC_{50} 7 to 93 μM) in clonogenic assays varies by 17- and 14-fold, respectively, among the three cell lines following a 24-hr drug exposure.

We also wished to determine if the cytotoxicity of CPE-C was dependent on duration of exposure, and whether lethality occurred at pharmacologically relevant concentrations. A comparison of the IC_{50} of CPE-C expressed as μM or AUC for various durations of exposure suggests that the total drug exposure is more important than peak drug concentration. These findings are consistent with a cell cycle-phase specific inhibitor. Following administration of maximally tolerated doses of CPE-C every 3 hr for four doses to mice, the AUC was 80 μM·hr [23]. Preclinical pharmacokinetic studies performed in rhesus monkeys using a single intravenous bolus injection of a non-toxic dose of CPE-C revealed that the AUC was 11 μm·hr [18]. The AUCs at the IC_{50} for a 24-hr exposure in our tissue culture model ranged from 7–18 μM·hr, achievable levels *in vivo*. While Glazer and coworkers reported no lethality with a 2-hr exposure to 10 μM CPE-C in HT29 cells [1], we found that exposure to 5–10 μM CPE-C for a slightly longer period, 3 hr, reduced viability of each of the three colon cancer cell lines by at least 50%.

The depletion of CTP pools observed in the three cell lines correlates with inhibition of RNA and DNA synthesis. We found that CPE-C markedly decreased dCTP pools in HCT 116 cells; thus, inhibition of DNA synthesis is presumably a consequence of dCTP depletion. Similar effects on dCTP pools have been reported with other antimetabolites such as *N*-(phosphonacetyl)-L-aspartate that inhibit *de novo* pyrimidine synthesis [24]. Pronounced S phase accumulation was evident at 48 hr following both continuous exposure and with exposure to CPE-C for only the initial 24 hr. In clonogenic studies, concurrent exposure to 10 μM Cyd, capable of repleting CTP pools, protected cells from concentrations up to 1.0 μM CPE-C. In contrast, with 1 μM Cyd, rescue was observed at the higher CPE-C concentrations only if it were administered both during and after CPE-C exposure. These observations support inhibition of CTP synthetase as an important mechanism of cytotoxicity.

Formation of CPE-CTP, the major anabolite, was relatively linear over a 3-hr exposure with increasing extracellular concentrations of CPE-C up to 5 μM. However, no further accumulation of CPE-CTP occurred at concentrations above 5 μM. The percentage of CPE-CTP for the total metabolite pool decreased from over 80% to less than 30% at 100 μM CPE-C, while the percentage of parent drug increased proportionally, suggesting that CPE-C anabolism to the monophosphate form becomes rate-limiting. Because CTP is a feedback inhibitor of Urd/Cyd kinase, one might expect that CPE-C-dependent depletion of CTP would lessen the degree of feedback inhibition, thus resulting in increased activation of CPE-C. We have reported previously that CPE-C-mediated depletion of CTP and consequently dCTP pools was associated with enhanced formation of cytosine arabinoside and arabinosyl-5-azacytosine nucleotides, presumably as a result of decreased feedback inhibition of dCyd kinase [6]. In contrast, our present results suggest that CPE-C-mediated CTP depletion is not accompanied by enhanced accumulation of CPE-C nucleotides. CPE-C is a competitive inhibitor of Urd/Cyd kinase [4]; it is possible that CPE-CTP at sufficient intracellular concentrations may act as a feedback inhibitor of Urd/Cyd kinase, thus self-limiting its further accumulation. CPE-CTP was relatively stable once formed, and therefore does not appear to be readily susceptible to enzymatic degradation by phosphatases or nucleotidases in the intracellular environment. Despite the presence of Cyd deaminase and dCMP deaminase in these colon cancer cell lines, CPE-U nucleotide formation was negligible compared to CPE-CTP levels. Presumably, a cell-free assay system may overestimate the activity of the deaminases in intact cells in which activating enzymes may compete more efficiently for the substrate. In addition, CPE-C may be a relatively poor substrate for the deaminases compared with the physiological substrates used to assess deaminating activity in these assays. Alternatively, CPE-CTP may directly or indirectly (through depletion of dCTP) affect the activity of dCMP deaminase.

Comparison of the metabolism of [^{3}H]CPE-C in human colon cancer and Molt-4 leukemia cells reveals several interesting differences. Ford *et al.* [5] reported that following a 2-hr exposure to 0.2 μM CPE-C, the CPE-CTP pool size was approximately 25 pmol/10^6 cells. In the HCT 116 cells, in contrast, the CPE-CTP pool size was one order of magnitude higher, 321 pmol/10^6 cells, after a 3-hr exposure to 0.2 μM CPE-C. In addition, the reported intracellular half-life of CPE-CTP following drug removal was shorter, 9.3 hr in Molt 4 cells, compared to 24 hr in the HCT 116 cell lines [5]. Ford and coworkers [5] did not find saturation of CPE-CTP formation with CPE-C concentrations $\leq 1\,\mu$M, but higher concentrations were not tested.

We documented incorporation of CPE-C into RNA with several methods. When CPE-C-RNA incorporation was compared to that seen after exposure to a toxic concentration of FUra, the levels were less than 2% of that attained with FUra. The effect of CPE-CTP versus FUTP on RNA function, however, may be quantitatively and/or qualitatively different. Under control conditions, distribution of [^{3}H]Cyd into 28 S and 18 S rRNA was 10-fold greater than incorporation into low molecular weight RNA species; at roughly equitoxic concentrations, the ratio of 28 S + 18 S to low molecular weight species for [^{3}H]FUra was 2, and the ratio was less than 1 for [^{3}H]CPE-C. CPE-C localized predominantly in low molecular weight RNA even in the presence of replete CTP pools (achieved with exogenous Cyd). This observation suggests that substrate depletion, *per se*, does not account for accumulation of low molecular weight RNA, although CPE-C exposure itself may affect the synthesis or turnover of low molecular weight RNA species. The possibility of increased degradation of CPE-C-RNA or chain termination as an explanation of low molecular weight RNA accumulation seems less likely given the distribution of tritium after gel electrophoresis: an actual peak was evident, rather than a smear of [^{3}H]RNA of various sizes; in the [^{3}H]Ado distribution experiments, background counts were not higher in the gels using RNA purified from CPE-C-treated cells compared to control. It is of interest that other investigators have reported a disproportionate accumulation of FUra in low molecular weight RNA species compared to 28 S and 18 S rRNA [25–28]. For example, Herrick and Kufe [26] found (in Friend erythroleukemia cells) that following a 3-hr exposure to 10 μM FUra, the synthesis of 28 S and 18 S rRNA was inhibited by over 80%, but [^{32}P] incorporation into 4 S RNA was inhibited by only 25%. Further, following a 3-hr exposure to 100 μM [^{3}H]FUra, incorporation into 4 S RNA was 2.4-fold greater than incorporation into 28 S plus 18 S rRNA [26]. Glazer and Lloyd found that the synthesis of low molecular weight nuclear RNA was not affected by toxic exposures to FUra or FUrd in HT29 colon cancer cells, and that 5-fold more [^{3}H]FUrd incorporated into 4 S RNA than into 28 S plus 18 S rRNA [25]. Armstrong *et al.* [28] found a large dose- and time-dependent accumulation of [^{3}H]Cyd-labeled U1 small nuclear RNA following FUrd exposure.

Cyd at concentrations capable of protecting cells

from CPE-C lethality inhibited CPE-CTP formation and RNA incorporation during the initial 3 hr; after 24 hr, however, the absolute levels of CPE-CTP and CPE-C-RNA were similar. Armstrong compared (in murine S-180 cells) the cytotoxicity and RNA incorporation of four different fluoropyrimidines given at equal concentrations; the author concluded that the rate of incorporation into RNA appeared to be more important than the total amount incorporated, and that the species into which FUra incorporated was also important [29]. It is possible, that the rate of CPE-C incorporation into RNA might also be important. The functional consequences of CPE-C incorporation into RNA and the mechanism of RNA synthetic inhibition will be examined in future experiments. Incorporation into methanol-precipitable materal over a broad range of CPE-C concentrations paralleled the CPE-CTP levels. Saturation of RNA incorporation at higher drug concentrations may be explained, in part, by inhibition of RNA synthesis due to further depletion of CTP pools and a plateau in the formation of CPE-CTP. While CPE-C incorporation into RNA may not be the critical cytotoxic event in the HCT 116 cell line following a 24-hr exposure to 0.5 μM CPE-C, the observation that protection in cells continuously exposed to CPE-C was incomplete despite the presence of sufficient exogenous Cyd to replete CTP pools raises the possibility that it may contribute to cytotoxicity with more prolonged exposures.

We did not detect deoxynucleotide metabolites of CPE-C, and no incorporation of [^{3}H]CPE-C into DNA was observed at concentrations of 0.05 and 0.5 μM for 24 hr. It is possible that incorporation below the limits of sensitivity of our assay might occur. Glazer *et al.* [1] reported that a short exposure to a non-lethal concentration of CPE-C does not alter the size of DNA by alkaline agarose electrophoresis. Further investigation will be needed to determine whether longer exposures to CPE-C at cytotoxic concentrations are associated with DNA incorporation or damage.

In summary, CPE-C is a highly potent compound against human colon cancer cells. Inhibition of CTP synthesis with consequent depletion of CTP and dCTP pools appears to be of primary importance. In our cell lines, an extracellular concentration of 0.5 μM produced near maximal CTP depletion, and saturation of anabolism occurred above 5 μM. CPE-CTP remains detectable for up to 96 hr following a 24-hr exposure to a toxic concentration, which appears to account for prolonged depletion of CTP pools. RNA incorporation occurs at a low level, but its contribution to cytotoxicity is unclear. CPE-C merits further evaluation as a therapeutic agent for colon cancer.

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