Modulation of the Cytotoxic Effect of Cyclopentenylcytosine by Its Primary Metabolite, Cyclopentenyluridine

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Abstract

Cyclopentenylcytosine (CPE-C), a synthetic cytidine analogue with significant preclinical antitumor activity against both solid tumor xenografts and 1-β-d-arabinofuranosylcytosine resistant murine leukemia cell lines, will soon enter phase I clinical trials. Unlike 1-β-d-arabinofuranosylcytosine which is activated by deoxycytidine kinase, the enzyme responsible for the phosphorylation of CPE-C is uridine/cytidine kinase. Preclinical pharmacokinetic studies of CPE-C in nonhuman primates revealed that the primary route of elimination in this species was deamination to cyclopentenyluridine (CPE-U), an inhibitor of uridine/cytidine kinase. Since CPE-C is likely to be deaminated in humans, we investigated the modulating effect of CPE-U on the in vitro cytotoxicity of CPE-C in Molt-4 lymphoblasts. Concurrent exposure of cells to cytotoxic concentrations of CPE-C and 50 μM CPE-U resulted in the rescue of 50% of cells and exposure to CPE-U concentrations in excess of 100 μM resulted in the rescue of greater than 90% of cells. Progressive attenuation of the rescue effect was observed with delayed administration of CPE-U and no cells were rescued when addition of CPE-U was delayed for more than 2 h. At the intracellular level it was observed that the formation of the cytotoxic metabolite, cyclopentenylcytosine triphosphate, was blocked by increasing concentrations of CPE-U presumably secondary to inhibition of uridine/ cytidine kinase by CPE-U. Although CPE-U can modulate the cytotoxic effects of CPE-C in vitro, the minimum CPE-U levels that are required for modulation coupled with the available preclinical pharmacokinetic data from nonhuman primates suggests that this modulation is not likely to impact on the antitumor effects of CPE-C in humans.

Introduction

CPE-C2, a synthetic cytidine analogue in which a cyclopentenyl moiety replaces the furan ring of the ribose sugar (1), has undergone extensive preclinical testing and will soon enter phase I clinical trials. This compound is active in vivo against human lung cancer, melanoma, and breast cancer xenografts grown in athymic mice and against the P388 and L1210 murine leukemias (2, 3). Collateral sensitivity to CPE-C has also been demonstrated in ara-C resistant murine leukemia cell lines (3, 4). Like ara-C, CPE-C must be intracellularly phosphorylated to a nucleotide (CPE-CTP) before expressing cytotoxicity. However, unlike ara-C which is activated by deoxycytidine kinase, the enzyme responsible for the phosphorylation of CPE-C is uridine/ cytidine kinase.

Extensive preclinical pharmacokinetic studies of CPE-C in rodents, dogs, and nonhuman primates have revealed significant interspecies differences in drug catabolism. Rodents and dogs eliminate CPE-C primarily by renal excretion (5), but the primary route of elimination in monkeys is by deamination of CPE-C to the noncytotoxic uridine metabolite, CPE-U (6). However, CPE-U is known to inhibit uridine/cytidine kinase (1), the enzyme required for the formation of CPE-CTP. In the present study the modulating effect of CPE-U on CPE-C cytotoxicity in vitro was investigated since CPE-C is likely to be deaminated in humans. The implications of these findings for the planned clinical trials with this agent in humans are discussed.

Materials and Methods

Drugs. CPE-C and CPE-U were obtained from the Pharmaceutical Resources Branch, National Cancer Institute, Bethesda, MD. [3H]CPE-C (specific activity, 15.0 Ci/mmol), labeled at position 5 of cytosine, was provided by the Division of Cancer Treatment, National Cancer Institute.

Cell Culture. Molt-4 leukemia cells were propagated continuously in a drug-free suspension culture in RPMI 1640 (Mediatech, Washington, DC) supplemented with 10% heat inactivated fetal bovine serum (Bio-Fluids, Inc., Rockville, MD) at 37 °C in a humidified atmosphere of 5% CO2 in air. Cells were passaged every 3–4 days and maintained in logarithmic growth phase.

Determination of Drug Sensitivity. A modified MTT assay (7, 8) was used to determine the sensitivity of the Molt-4 lymphoblasts to CPE-C and CPE-U. One hundred thirty-five μl of 1 × 105 cells/ml were plated into 96-well microtiter plates and incubated at 37 °C. Twenty-four h later, drug, at specified concentrations, was added to each well, in replicates of six. Cells were exposed to drug for 24 h, at which time the number of surviving cells was quantitated with MTT. 15 μl of MTT (5 mg/ml) was added to each well and the plates were protected from light and agitated for 10 min. Following incubation at 37 °C for 4 h, the plates were centrifuged at 400 × g for 10 min, the medium was aspirated to waste, and 150 μl of dimethyl sulfoxide were added to each well to solubilize the formazan. The plates were then shaken for 10 min, and the absorbance was measured at 540 and 690 nm using a microplate spectrophotometer (Bio-tek EL 312, Bio-tek Instruments, Winooski, VT). Cell survival was calculated by subtracting the background absorbance of media alone and then dividing the absorbance of test wells by the absorbance of the control (untreated) wells. The concentration producing 90% inhibition of growth was determined by regression analysis using points on the steep portion of the dose-response curve.

CPE-U Effects on CPE-C Cytotoxicity. The modified MTT assay was also used to determine the effect of CPE-U on the cytotoxicity of CPE-C against Molt-4 lymphoblasts in vitro. After incubation of 135 μl of 1 × 105 cells/ml in 96-well microtiter plates, 0.5 μM CPE-C was added to all cell wells excluding the control. Specified concentrations of CPE-U (range, 10-10–10-6 M) were then added in replicates of six at 0, 1, 2 and 4 h after the addition of CPE-C. Cells were exposed to drug for 24 h, at which time the number of surviving cells was quantitated with MTT as described above.

Effect of CPE-U on the Cellular Pharmacology of CPE-C. 5 ml of 1
× 10⁵ cells/ml of Molt-4 lymphoblasts were incubated for 1 h with 1.0 μM [³H]CPE-C and either 5, 50, or 500 μM CPE-U. The intracellular metabolism following exposure of cells to 1.0 μM CPE-C for 1, 2, and 4 h in the absence of CPE-U was also determined. Cells were washed once with 5 ml iced phosphate buffered saline and the cell suspension was centrifuged for 10 min at 800 rpm. Two ml of 60% iced methanol were then added to the washed pellet. The methanol precipitable fraction was washed three times with 1 ml iced methanol. An aliquot of the extracted sample was placed in a scintillation vial and the sample was counted in a Packard 2500 TR liquid scintillation counter (Packard Instrument Company, Meriden, CT) following the addition of scintillation fluid. Another aliquot of the sample (200–600 μl) was analyzed for CPE-C nucleotide content according to the previously published anion exchange high performance liquid chromatography method of Yee et al. (9). A Waters 600E multisolvent delivery system (Waters Chromatography, Division of Millipore Corporation, Milford, MA) was used with an online Flo-One beta radioactive flow detector (Radiomatic Instruments, Tampa, FL). A SAX Radial-Pak column (Waters) was used with two buffers as the mobile phase: buffer A was 0.007 M ammonium phosphate, pH 4.5; buffer B was 0.75 M ammonium phosphate, pH 4.5. The flow rate was 2.4 ml/min using the following gradient: 100% buffer A 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 for 15 min between separations. Four tritium labeled peaks were identified, the first (2.3 min) coeluted with cold CPE-C, and the next three peaks which were presumed to be the mono-, di-, and triphosphate metabolites of CPE-C coeluted with CMP (7.6 min), CDP (20.3 min), and CTP (39.7 min), respectively.

Results

The cell survival curves for Molt-4 cells exposed to varying concentrations of either CPE-C or CPE-U for 24 h are shown in Fig. 1. The concentration of CPE-C that inhibited cell growth by 90% was 0.5 μM, whereas CPE-U failed to produce cytotoxicity at concentrations as high as 1 mM. When varying concentrations of CPE-U were coincubated with 0.5 μM CPE-C for 24 h, Molt-4 cells were rescued from the cytotoxic effects of CPE-C. Fifty % of the cells were rescued at a CPE-U concentration of 50 μM and 90% of the cells were rescued at CPE-U concentrations of 100 μM when CPE-C and CPE-U were added simultaneously. Delayed administration of CPE-U for 1, 2, and 4 h following CPE-C exposure resulted in progressive attenuation of rescue from CPE-C cytotoxicity (Fig. 2).

Following exposure of cells to 1 μM [³H]CPE-C, CPE-CTP levels in Molt-4 cells were 4.5 pmol/10⁶ cells at 1 h and increased to 63 pmol/10⁶ cells by 2 h. After 4 h of exposure, CPE-CTP levels had essentially reached a plateau (73 pmol/10⁶ cells). The effect of CPE-U on the intracellular phosphorylation of CPE-C in Molt-4 lymphoblasts was then investigated following simultaneous exposure of cells to 1.0 μM [³H]CPE-C and either 5, 50, or 500 μM CPE-U for 1 h. As shown in Table 1, concurrent exposure of cells to CPE-C with a 50- and 500-fold excess of CPE-U resulted in a 26 and 31% decrease in parent drug accumulation, respectively. This is presumably a result of direct competition for uptake by the facilitated nucleoside transport inhibitor. The formation of CPE-CMP, however, was inhibited by 80% or more and the formation of CPE-CTP by 56 and 83% with concurrent exposure to 50 and 500 μM CPE-U, respectively. These observations suggest that the primary mechanism for decreased CPE-CTP formation is interference by CPE-U with the initial phosphorylation of CPE-C to CPE-CMP by uridine/cytidine kinase.

Discussion

In this study we demonstrated that CPE-U, the deamination product of CPE-C, could protect cells from the cytotoxic effects of CPE-C in a time and concentration dependent fashion (Fig. 2). Following concurrent exposure of cells to a 100-fold excess of CPE-U, CPE-C lethality was decreased from 100% to less than 10%. The primary mechanism of this rescue appears to be CPE-U inhibition of the initial phosphorylation of CPE-C by uridine/cytidine kinase. Preclinical pharmacokinetic studies in nonhuman primates demonstrated rapid deamination of CPE-C to CPE-U (6), analogous to the metabolism of ara-C to ara-U. Following a bolus dose of CPE-C to nonhuman primates, the half-life of CPE-U (270 min) was considerably longer than that of CPE-C (36 min) such that by 2 h after the dose, the concentration of CPE-U (11.7 μM) was more than 40-fold higher than that of CPE-C (27 μM) (6). In contrast, when CPE-C was administered by continuous intravenous infusion to nonhuman primates there was only a 4-fold difference in the
MODULATION OF CPE-C BY CPE-U

CPE-U (7.2 µM) to CPE-C (1.9 µM) steady state concentration (6). Based on the in vitro data presented here (Fig. 2) these differentials in CPE-U to CPE-C concentrations are unlikely to result in significant modulation of the cytotoxic effects of CPE-C, particularly after administration by continuous infusion. An important factor in the potential modulation of CPE-C cytotoxicity by CPE-U in humans will be the extent of deamination that the parent compound undergoes. Since humans reportedly have lower levels of cytidine deaminase than nonhuman primates (10), the likelihood that CPE-U will modify the cytotoxicity of CPE-C is reduced since less CPE-U will be formed. More definitive information regarding the relative rate of deamination of CPE-C in humans will be obtained by measurement of steady state CPE-C and CPE-U concentrations in planned phase I clinical trials.

The observation that neoplastic cells could be rescued from the cytotoxic effects of CPE-C by CPE-U might suggest that this protective effect could potentially be utilized to protect host cells following CPE-C administration, analogous to leucovorin rescue after methotrexate. As shown in Fig. 2 the maximum reversal of in vitro cytotoxicity of CPE-C by CPE-U occurs when the agents are added to cells simultaneously. When the addition of CPE-U is delayed by 1 h, however, 10-fold higher concentrations of CPE-U are required to rescue cells, and the rescue effect is essentially eliminated when CPE-U addition is delayed for more than 2 h. This time dependent rescue effect presumably results from the ability of the cells to synthesize sufficient levels of CPE-CTP to produce the cytotoxic effect during the delay. Therefore, administering CPE-U as a rescue agent after a prolonged CPE-C infusion would probably not protect host cells from the toxic effects of CPE-C. In contrast, delayed administration of cytidine has been shown to rescue cells in vitro from the cytotoxic effects of CPE-C (11).

In summary, CPE-U (an in vivo noncytotoxic metabolite of CPE-C) can modulate the cytotoxic effects of CPE-C. However, the minimum CPE-U levels that are required for in vitro modulation coupled with the available preclinical pharmacokinetic data suggest that this modulation is not likely to have an impact on the antitumor effects of this drug, particularly if CPE-C is administered by continuous infusion.

Table 1 Effect of CPE-U on the biochemical pharmacology of CPE-C in Molt-4 cells

<table>
<thead>
<tr>
<th>CPE-C (µM)</th>
<th>CPE-U (nM)</th>
<th>pmol/10^6 cells (% CPE-C alone)</th>
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<td></td>
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<tr>
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<tr>
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<td>12.0 (69)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.
* ND, not detected.

References

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