In Situ Cyclopentenyl Cytosine Infusion for the Treatment of Experimental Brain Tumors

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ABSTRACT
Cyclopentenylcytosine (CPEC; NSC 375575) is a pyrimidine nucleoside analogue that has potent antitumor effects when tested in vitro and also when tested in experimental tumors outside the central nervous system. CPEC exerts its antiproliferative effect through inhibition of CTP synthetase and consequent depletion of CTP and dCTP pools required for cell replication. Due to its poor penetration of the blood-brain barrier, CPEC has failed to demonstrate therapeutic efficacy in experimental brain tumors after systemic administration. We therefore examined the in vivo activation, distribution, and antitumor effect of CPEC after long-term regional infusion of the drug directly into experimental brain tumors in rats.

HPLC analysis of CPEC incubated with homogenized human brain and brain tumor tissue showed minimal degradation of the drug over 24 h. Analysis of rat cerebral 9L gliosarcoma infused with tritium-labeled CPEC demonstrated intratumoral accumulation of the active metabolite CPEC-triphosphate and concomitant depletion of CTP to a much greater extent in tumor tissue than in the adjacent brain. Tumor tissue UTP also decreased, but no significant effects on other ribonucleoside triphosphates were detected. Only trace amounts (<1%) of CPEC and its metabolites were detected. Only trace amounts (<1%) of CPEC and its metabolites reached peripheral sites, including the liver and kidneys, after intratumoral infusion. Rats treated with continuous intratumoral infusion of CPEC for 4 weeks using s.c. implanted osmotic pumps survived significantly longer than control rats receiving intratumoral saline or i.p. CPEC (P < 0.0001). Long-term intratumoral infusion of CPEC was not associated with any detectable toxicity.

Our results support the feasibility of using intratumoral administration of CPEC as a regional therapy for malignant brain tumors.

INTRODUCTION
CPEC2 is an analogue of the ribonucleoside cytidine and is activated through intracellular phosphorylation, predominantly to its active metabolite CPEC-TP (1, 2). CPEC-TP potently inhibits CTP synthetase in the de novo pyrimidine-biosynthetic pathway (2). Exposure of cells to CPEC results in depletion of CTP and dCTP pools and leads to inhibition of RNA and DNA synthesis, inducing a cytotoxic effect on tumor cells in vitro and in vivo (3, 4). The antitumor activity of CPEC has been demonstrated previously in a variety of human tumor xenografts (5), in cell lines from murine solid tumors (4), in leukemia and melanoma tumor implants (1), and in human glioblastoma cells in vitro (6). In studies of CPEC distribution after systemic administration in nonhuman primates, however, cerebrospinal fluid levels achieved were only 8% of plasma levels (7). Thus, because of poor i.e. penetration of the drug, brain tumors are unlikely to respond to systemically administered CPEC. Accordingly, we have chosen an alternative route of administration and evaluated the antitumor efficacy of CPEC by in situ delivery of the drug into experimental brain gliosarcomas as a potential new therapeutic approach for these highly invasive malignant tumors.

MATERIALS AND METHODS

Chemicals and Radiochemicals. CPEC (NSC 375575) was provided by Dr. Karl Flora, Developmental Therapeutics Program, National Cancer Institute. CPEC-MP was synthesized from CPEC and phosphorus oxychloride and purified and characterized as described previously (8). [5-3H]CPEC (specific radioactivity, 15 Ci/mmol) was prepared for the Developmental Therapeutics Program, National Cancer Institute, by Research Triangle Institute (Research Triangle Park, NC), and provided by Dr. R. Haugwitz, Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program. CPEC 5'-TP was synthesized in our laboratory.2 Chromatography reagents were all HPLC grade. HPLC mobile phases were filtered and degassed prior to use. Other chemicals and reagents used were of the highest quality obtainable.

Stability of CPEC in Saline and Brain Tissues. Since for optimal distribution locally administered drugs need to be stable within the target tissue, we first evaluated the levels of CPEC at body temperature after coincubation for an extended period with normal saline, human brain, and tumor tissues. Solutions of CPEC in 0.9% sodium chloride in concentrations ranging from 1–100 µM were incubated at 37°C in sealed Falcon tubes for 14 days. Aliquots were removed for analysis before incubation and after 2 weeks at 37°C. CPEC was quantified by narrow-bore HPLC using a LKB 2150 pump/LKB 2152 controller and a Gilson 231 auto sampler with a mobile phase of 0.1 M ammonium formate (pH 5) and a flow rate of 0.15 ml/min. A Gilson 116 UV detector at 278 nm was used to determine CPEC concentrations based on an individual peak area as compared to an authentic CPEC standard at 2.5 µM. Frozen brain tumor tissue which had been removed at surgery, together with normal tissue from adjacent areas, was homogenized and incubated at 37°C with solutions of CPEC in 0.9% NaCl at 10, 100, and 500 µM. CPEC quantitation after 5 and 24 h was performed as described above.

Inoculation of 9L Brain Tumor and Infusion of CPEC. In vivo studies were approved by the NIH Animal Care and Use Committee and performed in accordance with the NIH guidelines for the care and use of laboratory animals. A syngeneic Fischer rat 9L gliosarcoma tumor was used (9). Implantation i.e. of this highly malignant tumor results in the death of all animals within 3–4 weeks. Fischer 344 rats (n = 39) weighing 230–350 g were anesthetized i.p. with ketamine (90 mg/kg; Fort Dodge Laboratories, Inc., Fort Dodge, IA) and xylazine (10 mg/kg; Mobay Corporation, Shawnee, KS) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Syngeneic 9L gliosarcoma cells (4 × 10^6 cells in 5 µl HBSS) were injected into the deep white matter of the right cerebral hemisphere. Seven days later the rats were reanesthetized and an Alzet brain infusion cannula (Alza Corporation, Palo Alto, CA) was stereotaxically placed at the site of tumor inoculation and fixed to the skull using methy1 methacrylate (Kerr Division of Sybron Corporation, Romulus, MI). The brain infusion cannula was then connected s.c. to an Alzet 2ML4 osmotic pump (Alza Corporation) which delivers 2.5 µl/h for 4 weeks. The osmotic pumps were filled with normal saline (0.9% NaCl solution; n = 13), 50 µM CPEC in normal saline (n = 13), or 200 µM CPEC in normal saline (n = 13) and then implanted s.c. in the flank. Rats received an i.m. injection of 30,000 units of penicillin G (Roering Division of Pfizer Corporation, New York, NY) and were returned to the animal care facilities for observation. The infusion pumps remained in place for 4 weeks. Death was
recorded as the end point. The brains from all animals were inspected for the presence of tumor and then processed for histological evaluation.

To evaluate the antitumor effect of systemically administered CPEC, ten additional Fischer 344 rats weighing 230–250 g were inoculated with 9L gliosarcoma as described previously. Seven days later, the rats were reanesthetized, and Alzet 2ML4 osmotic pumps were filled with 2 ml of either 0.9% NaCl solution (n = 5), or 42 nmol CPEC in 0.9% NaCl solution (a concentration calculated to give a dose of 2 mg/kg/day for a 300-g animal; n = 5) were placed s.c. and connected to a catheter that was implanted into the peritoneal cavity. Survival of these rats was then recorded.

**Determination of Distribution and in Vivo Activation of CPEC and of Tissue Content of CTP.** Three Fischer 344 rats weighing 230–250 g were reanesthetized i.p. with ketamine/xylazine and underwent stereotaxic high-dose inoculation of 9L gliosarcoma cells (8 × 10^4 cells in 5 µl HBSS) into the deep white matter of the right cerebral hemisphere. Fourteen days later the rats were reanesthetized, a brain infusion cannula was placed at the inoculation site, and inoculation of 9L gliosarcoma cells (8 X 10^4 cells in 5 µl HBSS) into the deep s.c. and connected to a catheter that was implanted into the peritoneal cavity. Survival of these rats was then recorded.

**Measurement by HPLC of CPEC Metabolites and Ribonucleotide Pools in Brain Tissue.** Frozen brain tissues (tumor and ipsilateral and contralateral hemispheres) were homogenized in 4 volumes of 10% trichloroacetic acid and centrifuged, and the resulting clear supernatant was neutralized with 1 volume of trioctylamine:trifluorotrichloroethane (1:3). Aliquots of 250 µl (equivalent to 50 µg of tissue) were chromatographed by anion exchange HPLC (8, 10). Fractions were collected at 1-min intervals for 45 min, and the eluted radioactivity was determined by scintillation counting of each individual fraction. The column was calibrated with reference standards of nucleotide triphosphates. The retention times were: UTP, 26.9 min; CTP, 28.3 min; ATP, 29.7 min; GTP, 32.8 min; and CPEC-TP, 29.0 min. Ribonucleotide pools were determined by the HPLC method described previously (8).

**Statistical Analysis.** The Mantel-Haenszel test (11) was used to compare survival between CPEC-treated and normal saline-treated rats in the survival experiment.

**RESULTS**

**i.c. Administration of CPEC.** Survival was significantly extended when 200 µM CPEC was administered by continuous i.c. infusion for 4 weeks compared to normal saline-treated and 50 µM CPEC-treated rats (32.2 days versus 24.7 and 23.9 days, respectively; P < 0.0001; Ref. 11) (Fig. 1). All rats died from tumor progression. No adverse effects of CPEC were detected. Histological evaluation of nontumoral brain, including the brain contiguous to the tumor, showed no abnormalities.

**i.p. Administration of CPEC.** In rats with intracerebral 9L tumors, continuous i.p. administration of CPEC at 2 mg/kg/day [twice the daily dose per body weight that had been shown previously to extend survival in human tumor xenografts in mice after i.p. administration (5)] resulted in no significant difference in survival compared to saline controls (29 ± 0 days versus 26.8 ± 3.3 days). All deaths were due to tumor progression and no toxicity was observed in any of the treated rats.

**Stability of CPEC in Saline, Brain, and Tumor Tissue.** CPEC solutions in concentrations ranging from 1 to 100 µM were chemically stable in saline for 2 weeks, with less than 8% degradation (data not shown). CPEC was stable when mixed and incubated with normal human brain tissue or tumor homogenates for 5 and 24 h. Less than 8% degradation was observed at the examined concentrations (10–500 µM) at both time points.

**In Vivo Activation of CPEC in Tumor and Adjacent Brain.** i.t. infusion of CPEC for 6 days at a concentration of 200 µM resulted in significant accumulation of CPEC-MP, CPEC-DP, and CPEC-TP in the tumor, with a cumulative total of phosphorylated metabolites of 11 nmol/g (~11 µM) (Fig. 2). Significant concentrations of CPEC and its metabolites were found in both cerebral hemispheres, although ^3H]CPEC levels in brain adjacent to the tumor were <27% of the levels in the tumor (Table 1). In contrast to the results seen with human glioblastoma cells in tissue culture (6), the active metabolite, CPEC-TP, was not the predominant metabolite in this analysis, presumably due to its rapid degradation to CPEC-MP and CPEC-DP during the extraction and weighing of the rat brains prior to freezing of the tissue. The radiolabeled-CPEC phosphates coeluted with the authentic, nonlabeled standards of these metabolites indicating that all radioactivity represented the infused drug and its phosphate metabolites. No cyclopentenyluracil or cyclopentenyluracil metabolites were detectable (7).

**In Vivo Depletion of CTP Pools.** HPLC analysis of the tumor and adjacent brain after i.t. infusion of 200 µM CPEC for 6 days showed complete depletion of CTP in the treated tumor when compared to the untreated tumor (13.5 nmol CTP/g tissue) (Table 2). In normal adjacent (ipsilateral) brain, CTP decreased significantly (75%) but was still measurable, while the CTP level remained at 42% of control in the contralateral hemisphere. Analysis of the effect of CPEC on other ribonucleoside triphosphate pools in rat brain tumor showed UTP depletion to 30% of control and a lesser (25%) decrease in tumor ATP. No effect on GTP was seen. The effects on UTP and ATP contrasted with an increase in these nucleotides seen in human glioblastoma cells in culture (data not shown) and were, perhaps, secondary to destruction of tumor cells by CPEC, because no significant effects on UTP, ATP, and GTP levels were detected in the adjacent ipsilateral or in the contralateral hemisphere (Table 2).

**Systemic Distribution of CPEC after i.t. Infusion.** The amount of CPEC that crosses the blood-brain barrier after i.t. infusion was evaluated by determining the radioactive CPEC content in the liver and kidneys of treated rats. The extracted radioactive CPEC phosphates coeluted in the same fractions as the standards of these metabolites. Only trace amounts (<0.2%) of the total administered dose...
Fig. 2. Average (n = 2) concentration of CPEC metabolites in tumor and brain after continuous i.t. infusion of 200 μM [3H]-CPEC (2 μCi/ml) for 6 days at 5 μl/h.

Table 1  Cumulative tissue levels of CPEC and CPEC metabolites in rats after i.t. CPEC infusion

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Tissue radioactivity (dpm/mg)</th>
<th>Fraction of total administered [3H]-CPEC (%)</th>
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<tbody>
<tr>
<td>Tumor</td>
<td>589</td>
<td>15.2</td>
</tr>
<tr>
<td>Ipsilateral hemisphere</td>
<td>162</td>
<td>4.2</td>
</tr>
<tr>
<td>Contralateral hemisphere</td>
<td>133</td>
<td>3.4</td>
</tr>
<tr>
<td>Liver</td>
<td>&lt;10</td>
<td>&lt;0.2</td>
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<tr>
<td>Kidneys</td>
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reached these organs, while 15% of the total radioactive CPEC was recovered from the tumor tissue (Table 1).

DISCUSSION

Glioblastomas are locally invasive and infiltrative malignant neoplasms but are not typically metastatic and thus are candidates for selective regional therapy. Since the majority of cells in the central nervous system are not actively dividing, selective targeting of tumor cells can be achieved by an agent that interferes with DNA replication. Approaches for selective local therapy using this principle include regional delivery of targeted immunotoxins (12) or delivery of a gene conferring drug sensitivity by a retroviral vector (9). CPEC selectively affects replicating cells by interfering with RNA and DNA synthesis. A Phase I study of CPEC for the treatment of systemic solid tumors has recently been completed (13). However, severe hypotension of unknown etiology occurred in three patients after systemic administration of CPEC. The hypotensive episodes observed in cancer patients after i.v. administration of CPEC are of major concern. However, our studies of systemic distribution of radiolabeled CPEC after regional i.t. administration detected only trace amounts of the drug in peripheral organs. The low dose administered into the tumor along with the poor "reverse" penetration of the drug across the blood-brain barrier may thus circumvent this adverse effect of CPEC.

In small experimental tumors, such as those used in the 9L rat model of brain tumors, simple diffusion from the infusion source during slow microinfusion can efficiently distribute the drug into the tumor, as well as into adjacent and remote brain regions. For treatment of large malignant brain tumors in humans, diffusion would not provide adequate distribution for meaningful antitumor activity. However, a novel technique capable of distributing molecules within brain and tumor tissue has recently been described (14, 15). This convective-mediated perfusion uses slow, continuous infusion into the interstitial space of the brain to enhance the distribution of large and small molecules via bulk fluid flow. Although more extensive in vitro
experience with freshly obtained human glioblastoma cells and other human brain neoplasms will be necessary, the small size and stability of CPEC would appear to merit its consideration as an agent for i.t. distribution using this technique.

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REFERENCES


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