The cytotoxic drug cyclo-pentenyl cytosine: from manufacturing to anti-tumor activity and (cardio)toxicity
schimmel, K.J.M.
CHAPTER 5

LIMITED ANTITUMOR-EFFECT ASSOCIATED WITH TOXICITY OF THE EXPERIMENTAL CYTOTOXIC DRUG CYCLOPENTENYL CYTOSINE IN NOD/SCID MICE WITH ACUTE LYMPHOBLASTIC LEUKEMIA

Leukemia Research 2007; april 23, epub ahead of print

Kirsten Schimmel1, Bart Nijmeijer2, Marianke van Schie2, Frederik Falkenburg2, Henk-Jan Guchelaar2

1 Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, The Netherlands
2 Department of Hematology, Leiden University Medical Center, The Netherlands
ABSTRACT

The experimental cytotoxic drug cyclopentenyl cytosine (CPEC) is a non-competitive inhibitor of the enzyme cytidine triphosphate (CTP) synthethase. We evaluated the in vitro and in vivo antitumor activity of CPEC on human ALL cell lines. CPEC displayed anti-leukemic activity with IC50 (after 3 days of incubation) ranging from 6 to 15 nM. Subsequently the in vivo activity of CPEC against primary human ALL was evaluated in a xenogeneic model of human ALL using NOD/scid mice inoculated with primary human ALL cells. In the model, only a marginal anti-leukemic activity was observed at 1.5 mg/kg (5 days per week) and 5 mg/kg (2 days per week), however, this activity was associated with severe systemic toxicity. The observed toxicity was not specific for the NOD/scid model, as toxicity at comparable treatment intensity was also observed in Balb/c mice. In conclusion, although CPEC showed antitumor activity against human ALL cells in vitro, its activity in the in vivo human leukemia model was only marginal and accompanied by severe toxicity.

Keywords: cyclopentenyl cytosine, CPEC, NOD/scid, leukemia, ALL
Limited anti-tumor-effect associated with toxicity of the experimental cytotoxic drug cyclopentenyl cytosine in NOD/scid mice with acute lymphoblastic leukemia

INTRODUCTION

Acute lymphoblastic leukemia (ALL) generally has a poor prognosis in adults, with an overall leukemia-free survival of 30 to 40%. Current therapy strategies include chemotherapy and allogenic stem cell transplantation [1]. As prognosis decreases after the first relapse, agents improving first line therapy are still warranted.

In leukemic cells of adults with acute lymphoblastic leukemia (ALL), CTP (cytidine triphosphate) synthetase activity was found increased as compared to nonmalignant cells [2,3]. High enzyme activity has also been found in lymphoblasts of pediatric patients with ALL and cells of pediatric patients suffering from acute myeloid leukemia (AML) [4,5]. These findings suggest that CTP synthetase might be an attractive target enzyme for inhibition. Moreover, it has been shown that some malignant tissues synthesize CTP predominantly via the uridine pathway and thus via CTP synthetase [2].

The experimental cytotoxic drug cyclopentenyl cytosine (CPEC), is a non-competitive inhibitor of the enzyme CTP synthetase. CPEC is a pyrimidine analogue of cytidine in which the furan ring of the ribose sugar has been replaced by a carbocyclic cyclopentenyl moiety [6]. CPEC is activated by intracellular phosphorylation ultimately forming its 5’-triphosphate analogue (CPEC-TP) by subsequently uridine-cytidine kinase, nucleoside monophosphate kinase and nucleoside diphosphate kinase [7]. CTP-synthetase catalyzes the conversion of uridine triphosphate (UTP) into cytidine triphosphate (CTP), which is one of the only two cellular pathways for synthesizing CTP. CTP is a precursor for RNA, DNA and phospholipids.

CPEC has shown to have activity in human mammary, colon carcinoma and melanoma xenograft models [6,8]. In humans, CPEC has been studied in a single phase I clinical trial in adults with a variety of solid tumors. Twenty six patients suffering from predominantly colorectal cancer were treated every 3 weeks with increasing doses of CPEC. Treatment was associated with dose-dependent and dose-limiting decreases in granulocyte and platelet counts. Non dose-limiting nausea, vomiting, mucositis and diarrhea were also reported. The most severe toxicity observed was cardiovascular in the form of hypotension. From the 26 patients treated with CPEC, 5 patients experienced a hypotensive episode (dose range: 3-4.7 mg/m²/h for 24 h) which was in two cases fatal. Toxicity seemed to be dose-related as no hypotension was seen in patients receiving doses equal or below 2.5 mg/m²/h [9]. However, these cardiotoxic effects could not be reproduced in animal experiments and it remains unclear whether or not the observed hypotension was a true cardiotoxic side effect [10].

In vitro experiments on leukemic cell lines showed reduction of RNA- and DNA-synthesis and growth inhibiting effects of CPEC [7,11,12,13]. Moreover, CPEC also seemed to increase the phosphorylation of the cytostatic drug arabinofuranosyl cytosine (AraC) in a T-lymphoblastic
(MOLT-3) and a myeloid (HL-60) leukemic cell line, enhancing the cytotoxicity of AraC [13]. In vivo anti-leukemic activity of CPEC was demonstrated in mice inoculated with murine leukemias by Moyer et al [12].

Considering the results of the various in vitro and in vivo experiments with lymphocytic and myeloid leukemia, and the hematotoxic side effects in humans with solid tumors, CPEC might have potential in the treatment of ALL. However, the in vivo experiments providing this evidence were carried out in mice inoculated with murine leukemic cells from leukemia-derived cell lines [12] whereas no data are available with human leukemic cells.

We therefore studied the antitumor effect of CPEC on human acute lymphoblastic leukemia cell lines in vitro, as well as on corresponding human primary acute lymphoblastic leukemia cells in a xenogeneic in vivo model. Although CPEC exerted activity on human leukemic cell lines, CPEC displayed no detectable activity at tolerated (i.e. non-lethal) doses, suggesting a minimal therapeutic window in ALL.

**METHODS**

**Leukemic cells**

We established a number of continuously proliferating ALL cell lines. These cell lines were generated from primary cells by culturing these cells in a serum-free medium that was previously developed [14]. The emerging cell lines displayed similar phenotype as compared to the primary cells. Karyotype analysis revealed similarity with primary cells although additional aberrations were observed in some, but not all, cell lines. The leukemic cells were obtained by leukapheresis from 5 patients with ALL after informed consent. Patient and cell line characteristics are presented in Table 1.

The serum-free medium consisted of IMDM (BioWhittaker, Verviers, Belgium), supplemented with 3mM L-glutamine (BioWhittaker), antibiotics, 0.4% human serum albumin (HSA) (wt/vol) (Sanquin, Amsterdam, The Netherlands), 20 μg/ml cholesterol (Sigma-Aldrich, Zwijndrecht, The Netherlands), 2 μg/ml transferrin (Serva, Heidelberg, Germany), 5x10^{-6} M β-mercaptopethanol (Sigma-Aldrich), and 10 μg/ml insulin (Sigma-Aldrich).
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Table 1 Patient and cell line characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Karyotype of primary cells</th>
<th>Cell line</th>
<th>Karyotype of cell line**</th>
</tr>
</thead>
<tbody>
<tr>
<td>VBK</td>
<td>pro-B ALL</td>
<td>46.XY,t(9;22)(q34;q11),der17t (1;17)(q21;p11.2)</td>
<td>LeidenALL- BV</td>
<td>46.XY(t(9;22)(q34;q11),der17t(1;17)(q21;p11.2)</td>
</tr>
<tr>
<td>COA</td>
<td>common- ALL</td>
<td>46,XY,del(7)(q22),dup(8)(q24q21),del(20)(q11),der(22)t(9;22)(q34; q11)</td>
<td>LeidenALL- CM*</td>
<td>47,XY,del(7)(q22),dup(8)(q24q21),del(20)(q11),der(22)t(9;22)(q34; q11),+der(22)t(9;22)(q34; q11)</td>
</tr>
<tr>
<td>MHX</td>
<td>common- ALL</td>
<td>47,XX,+i(21)(q10)</td>
<td>LeidenALL- HP</td>
<td>45,XX, 47,XY, -13,add(16)(q2?4),i(21)(q10)</td>
</tr>
<tr>
<td>WKD</td>
<td>common- ALL</td>
<td>48,XY,+5,+8,t(9;22)(q34;q11)</td>
<td>LeidenALL- KW</td>
<td>48,XY,+5,+8,t(9;22)(q34;q11)</td>
</tr>
<tr>
<td>MRJ</td>
<td>pre-B ALL</td>
<td>46,XX,i(7)(q10),der(19)t(1;19)(q23;p13)</td>
<td>LeidenALL- RL</td>
<td>46,XX,i(7)(q10),t(9;10)(q10;q10),der(19)t(1;19)(q23;p13)</td>
</tr>
</tbody>
</table>

*CML in lymphoid blast crisis. **Additional aberrations as compared to the primary cells are underlined.

In vitro proliferation assay

The effect of CPEC (NCI, Maryland, USA) and cytarabine (AraC) (Pfizer, Capelle a/d IJssel, The Netherlands) on proliferation of leukemic cell lines was tested by [3H]thymidine incorporation assay. Leukemic cell lines were cultured in the absence or presence of the agents in 96-well tissue culture plates (Corning Costar, Schiphol-Rijk, The Netherlands) at 4 x 10^4 cells/well in serum-free medium. After 72 hours of incubation, 1 μCi [3H]thymidine (Amersham, Roosendaal, The Netherlands) was added to each well. After 18 h of further culture [3H]thymidine incorporation was measured as described earlier [15]. By using the isobologram method [16] we analyzed whether the combination of CPEC and AraC was synergistic, additive or antagonistic. We determined the concentration combinations that caused 50% survival for the different cell lines. For each pair of drug concentrations producing 50% survival (IC50), the combination index (CI, based on Loewe additivity [16, 17] was calculated as follows: CI = (CPEC concentration/IC50 CPEC) + (AraC concentration/IC50 AraC). IC50 CPEC and AraC in this formula represent the concentrations of the individual drugs that would result in 50% survival.
In vivo NOD/scid mouse leukemia model

Female NOD/scid mice, aged 5-6 weeks (Charles River, les Oncins, France) were housed in sterile cages supplied with sterile filtered air and were supplied with sterile food and sterile water containing ciprofloxacin. Mice were engrafted with primary human leukemic cells by injecting $10^6$ leukemic cells intravenously in a lateral tail vein. Engraftment and progression of leukemia was monitored twice a week by flowcytometric analysis of peripheral blood samples of individual animals as described before [18]. Briefly, blood samples were taken from a lateral tail vein by using capillary blood collection tubes (Sarstedt, Nümbrecht, Germany). Total nucleated cell counts (NCC) were determined on a Sysmex F 820 automated cell counter (Sysmex Corporation, Kobe, Japan). After lysis of red blood cells, murine leukocytes and human ALL cells were stained using PE-conjugated anti-murine CD45 (Ly5; Pharmingen, San Diego, CA) and FITC-conjugated anti-human CD45 (Becton Dickinson, San Jose, CA) respectively, allowing determination of the percentage of murine and human cells (%Hu). Samples were analyzed on a Becton Dickinson FACScan flow cytometer. Leukemic cell counts (LCC, $10^6$/ml) were calculated as LCC = NCC ($10^6$/ml) x %Hu.

Treatment was performed by administrating CPEC (in 200 μl normal saline) or normal saline (control group) intravenously as a bolus injection in the tail vein. Animals were further monitored for response or tumor progression. Toxicity was evaluated by determining weight loss, hemaglobin (Hb) and visual aspects (breathing, activity, shaking). At the end of the experiment, or after observing severe toxicity (>20% weight loss), animals were euthanized by CO$_2$ inhalation. Blood, bone marrow and spleen were tested for leukemic cells. Heart, spleen, liver and kidney were further processed for histological evaluation. For this purpose paraplast embedded organs were mounted on slides and stained with haematoxylin-azophloxin.
RESULTS

In vitro experiments

To evaluate the activity of CPEC on human leukemic cells, we incubated five different cell lines with CPEC (1-1000 nM) for 72 hours. The mean IC50 of CPEC was 12 nM (range 6-15 nM). Total cell death was observed at 50 nM and higher. By comparison, the IC50 of AraC was 2.4 nM. (Fig. 1).

As earlier studies reported a possible synergistic effect of Ara-C and CPEC, we also studied anti-leukemic activity of combinations of CPEC and AraC. In figure 2 the isobologram of the co-incubation of CPEC and AraC (concentration CPEC 0-63 nM, AraC 0-32 nM) is presented. Most of our data are slightly above the diagonal that defines Loewes additivity. We also calculated the average CI from all the concentration combinations giving 50% survival. The average CI was 1.09 (SD 0.13) and as defined by Chou, CI values between 0.90 and 1.10 may be considered as nearly additive [19].

Figure 1 Activity of CPEC on proliferation of ALL cells

To study the in vitro activity of CPEC and AraC, five different leukemic cell lines (derived from primary leukemic cells from 5 patients with ALL) were exposed to serial dilutions of CPEC and AraC (1-1000 nM). A thymidine incorporation assay was performed after 3 days of incubation. The results shown in the figure are representative of three separate experiments. The IC50 of AraC was observed at 0.002-0.004 μM, IC50 of CPEC at 0.006-0.015 μM. Dotted lines (...) represent the AraC curves, CPEC is represented by the solid lines (-).
To study a possible synergistic effect of CPEC and AraC, 4 leukemic cell lines (derived from primary leukemic cells from patients with ALL) were exposed to serial dilutions of CPEC and AraC. The thymidine incorporation assay was performed after 3 days of coincubation. Data points represent the drug combinations leading to 50% survival. The diagonal represents Loewe additivity. On the axis percentages of the IC50 concentrations of the drugs are plotted. The isobologram indicates a nearly additive and no synergistic effect.

In vivo experiments

The five ALL cell lines displayed similar sensitivity to CPEC in vitro. As the in vitro experiments did not show a superior effect of CPEC and AraC co-incubation over CPEC alone, we chose to study CPEC mono-therapy in vivo. Primary leukemic cells from patients MHX and COA, from which the HP and CM cell lines were previously generated, were selected for evaluation in the animal model. These patients were selected in order to include both a Philadelphia (Ph) positive (COA) as well as a Ph negative sample (MHX) in the in vivo studies. Mice inoculated with cells from patient MHX first showed quantifiable leukemic cell counts (LCC) 84 days after inoculation. Mice inoculated with cells from patient COA first showed quantifiable LCC 17 days after inoculation.

In the phase I study CPEC was tested in a dose range from 0.7 – 4 mg/kg/day as a 24 h infusion every three weeks [9]. As in vivo studies on murine leukemia demonstrated activity of CPEC in a more frequent dose schedule [12], we started with administration of CPEC on 2 days/week (2 consecutive days followed by 5 days of rest). Three dosages were tested: 0, 0.5 and 5 mg/kg (3 mice per group). Only the highest dose was associated with marginal activity, however, this was accompanied by severe toxicity. We therefore tested whether a more intense schedule with a lower maximum dose would result in more effect with less toxicity. We administrated respectively 0, 0.5 and 1.5 mg/kg for 5 times a week (4 mice per group). Still neither effect nor toxicity was seen in the lower dose ranges. A marginal effect was associated with 1.5 mg/kg, however, again severe toxicity, expressed by more than 20% decrease in weight or Hb, was observed. The logLCC counts for the two dose schedules tested are represented in figure 3.
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Figure 3a logLCC counts for MHX ALL cells in NOD/scid mice

In figure 3a data from mice inoculated with MHX primary leukemic cells are shown. CPEC administration was started in this group 104 days after inoculation. Mice received CPEC on two consecutive days with a 5-day interval for a maximum of four weeks (time points of the first and last injection are indicated by the arrow). Each curve represents the mean logLCC counts for each group during the study.

Figure 3b logLCC counts for COA ALL cells in NOD/scid mice

In figure 3b data from mice inoculated with COA primary leukemic cells. CPEC administration was started 46 days after inoculation. Mice received CPEC for five days per week for a maximum of four weeks.

In both figures the highest dose group demonstrate a possible treatment effect of CPEC. However, these were the only groups with unintentional death during treatment (indicated by †).
Toxicity

In animals treated with more than 1.5 mg/kg CPEC/day, analysis of blood samples revealed induction of pancytopenia and anemia. After 1.5 mg/kg CPEC for 5 days/week hemoglobin levels decreased to 5.7 mmol/l (SD 0.74) as compared to 8.6 mmol/l (SD 0.49) in control treated animals within 14 days after treatment start. Diarrhoea and significant weight loss (>25%) were observed. Hematological toxicity of CPEC was confirmed microscopically. The bone marrow compartment of treated animals was void of hematopoietic cells (Fig. 4). In the kidneys tubular necrosis, and signs of proteinuria and bleeding were observed (Fig.5). Gut toxicity of CPEC presented as atrophy of ilear epithelium. No signs of cardiotoxicity were found.

No toxicity was observed in the lower dosage regimens (equal or below 1.0 mg/kg CPEC, 5 days/week). To investigate whether susceptibility to CPEC-mediated toxicity was a strain-specific trait of NOD/scid mice, we administrated CPEC to non-leukemic Balb-c mice. Although 1.5 mg/kg (5 days/week) was well tolerated, daily administration of 5 mg/kg CPEC resulted in severe toxicity and death.

Figure 4 Severe aplasy in the bone marrow compartment after CPEC treatment
Details of the femur of a CPEC treated animal (revealing severe aplasy in the bone marrow) (4a), whereas no abnormalities were observed in the femur of an untreated animal (figure 4b).

Figure 5 Kidney toxicity after CPEC treatment
Details of the kidney showing tubular necrosis, signs of proteinuria and bleeding after treatment with CPEC (figure 5a). In figure 5b details of the kidney of an untreated animal are shown.
DISCUSSION

With this study we demonstrated that CPEC has *in vitro* activity on human leukemic cells. However, in our NOD/scid model CPEC showed no detectable activity at tolerated doses whereas at higher doses limited activity was associated with severe toxicity. Balb/c mice were also susceptible to CPEC toxicity, suggesting a minimal therapeutic window in ALL.

CPEC has been evaluated clinically in the phase I study by Politi *et al* in patients with solid tumors [9]. In this study hematological toxicity was dose-limiting and the most severe side effect was cardiotoxic. The cardiotoxicity only occurred in the higher dose levels and could not be reproduced in animal experiments [10]. The hematological toxicity however, suggested a potential antitumor effect in leukemia. Various *in vitro* and *in vivo* experiments with CPEC on lymphoid and myeloid leukemia seemed to confirm this hypothesis [7,11,12,13]. These *in vivo* experiments however, were murine tumor-based models. Therefore, the activity of CPEC against primary human leukemia cells in vivo remained unclear.

*In vitro*, on previously established LeidenALL acute lymphoblastic leukemia cell lines, CPEC exerted antileukemic activity in the nanomolar range, comparable to other reports. Previously, Verschuur *et al* reported a synergistic effect between AraC and CPEC because co-incubation with AraC and CPEC increased cell death in a T-lymphoblastic cell line (Molt-3) as compared to either agent alone[20]. However, in our B-lineage ALL cell lines, we detected a nearly additive and no synergistic relationship between the activity of CPEC and cytarabine. Therefore, we investigated monotherapy with CPEC in the *in vivo* model for primary human ALL.

Previous experiments in mice bearing murine lymphoid leukemia reported an antitumor effect of CPEC when administrating 1 mg/kg CPEC for a maximum of 9 days [12]. In these experiments CPEC activity was evaluated by measuring the increase in life span, giving no indication on the actual activity of CPEC during treatment. Moreover, since both treated and untreated mice died within 20 days after inoculation, no discrimination between death due to toxicity of CPEC or leukemic progression was made. In our tumor model, leukemic progression is continuously monitored, allowing the identification of the actual response during treatment [17]. Because of the application of this technique, leukemic progression can be excluded as a possible cause of death. This allows precise discrimination between activity and toxicity of therapeutic interventions. Moreover, the model employs a curative setting (i.e. treatment is started after confirmation of leukemic engraftment ), whereas in the earlier experiments treatment is applied simultaneously with, or very shortly after tumor inoculation.

In the present studies, CPEC at highest doses, appeared to induce a small decrease in leukemic progression. However, this activity was accompanied by severe anemia. There were no indications of preferential toxicity of leukemic cells. Histological findings confirmed hematological toxicity as
severe aplasy was observed in the bone marrow. No cardiotoxicity was detected upon histological examination. Besides hematological toxicity, renal toxicity was apparent. It remains unclear however, whether renal toxicity was primary and caused anemia through hematuria, or whether renal necrosis was secondary and caused by anemia and the resulting hypoxia.

Non-hematological toxicity consisted of weight loss and diarrhea. Upon histological examination the ileum showed abnormalities that might be related to the diarrhea. Moreover, in the phase I trial patients experienced (non dose-limiting) grade 2 diarrhea, occurring more frequently in the higher dosage levels. It is conceivable that a possible hematological effect in humans will be associated with diarrhea as well.

To exclude the possibility that the small therapeutic window that was observed in our in vivo studies was a specific artifact of the NOD/scid mice model, we also administered CPEC to non-leukemic Balb/c mice. Although Balb/c mice tolerated CPEC at a dose of 1.5 mg/kg/day, administration of 5 mg/kg/day induced similar and severe toxicity (both hematological and non-hematological) as was observed in NOD/scid mice. Although these observations suggest that Balb-c mice may be slightly less sensitive to CPEC-mediated toxicity, it has to be noted that the coping ability of the hematopoietic system of leukemic NOD/scid mice may be impaired due to leukemic engraftment. Moreover, the highest dose tolerated by Balb-c mice (1.5 mg/kg 5 days/week) was not associated with a clear anti-leukemic activity in the NOD/scid mice.

Our in vitro results with CPEC are in line with previous studies and although in vitro less active that AraC, CPEC showed promising activity. The fact that we were not able to demonstrate this activity in vivo, might have been partly caused by CPEC induced toxicity which may have overwhelmed anti-leukemic activity. However, other in vivo models with solid and lymphoid tumors showed activity of CPEC before severe toxicity occurred [12]. It is possible that cytidine is involved in the low activity of CPEC in mice. In vitro results in leukemic and colorectal cells have shown an increase in survival after administration of cytidine to CPEC treated cells. The mechanism of action is supposed to involve competition for transmembrane transport and phosphorylation [7]. It is conceivable that high endogenous cytidine levels could protect tumor cells against CPEC. However, administration of cytidine has also been shown to protect against toxicity, without changes in activity [11]. The results of our study are not suggestive for an important role of cytidine, as we studied the effects of CPEC on a systemic single cell leukemic tumor and we experienced systemic toxicity. If high endogenous cytidine levels were responsible for the lack of activity in our model, this would most likely also have resulted in lower toxicity, which was not the case.

We conclude that, although CPEC shows antitumor activity against human ALL cells in vitro, its antitumor activity in the human in vivo leukemia model as a single agent is only marginal and is accompanied by severe toxicity.
REFERENCES


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