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# Cyclopentenyl cytosine inhibits cytidine triphosphate synthetase in paediatric acute non-lymphocytic leukaemia: a promising target for chemotherapy

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**Abstract**

Cytidine triphosphate (CTP) synthetase is a key enzyme in the anabolic pathways of cytosine and uracil ribonucleotide metabolism. The enzyme catalyses the conversion of uridine triphosphate (UTP) into CTP, and has a high activity in various malignancies, which has led to the development of inhibitors of CTP synthetase for therapeutic purposes. We studied both CTP synthetase activity and ribonucleotide concentrations in leukaemic cells of 12 children suffering from acute non-lymphocytic leukaemia (ANLL), and performed incubation experiments with cyclopentenyl cytosine (CPEC), a nucleoside analogue that is capable of inhibiting CTP synthetase.The CTP synthetase activity in ANLL cells ($5.1 \pm 2.3$ nmol CTP/mg/h) was significantly higher compared with granulocytes of healthy controls ($0.6 \pm 0.4$ nmol CTP/mg/h, $P = 0.0002$), but was not different from the CTP synthetase activity in non-malignant CD34+ bone marrow cells ($5.6 \pm 2.4$ nmol CTP/mg/h). Major shifts were observed in the various ribonucleotide concentrations in ANLL cells compared with granulocytes: the absolute amount of ribonucleotides was increased with a substantial rise of the CTP (2.4 versus 0.4 pmol/μg protein, $P = 0.0007$) and UTP (8.7 versus 1.6 pmol/μg protein, $P = 0.0007$) concentrations in ANLL cells compared with granulocytes. Treatment of ANLL cells *in vitro* with CPEC induced a major depletion (77% with 2.5 μM of CPEC) in the concentration of CTP, whilst the concentrations of the other ribonucleotides remained unchanged. Therefore, the high activity of CTP synthetase in acute non-lymphocytic leukaemic cells can be inhibited by CPEC, which provides a key to a new approach for the treatment of ANLL. © 2000 Elsevier Science Ltd. All rights reserved.

*Keywords:* Acute non-lymphocytic leukaemia; Cytidine triphosphate synthetase; CTP; Cyclopentenyl cytosine; Ribonucleotides; UTP

## 1. Introduction

Cytidine triphosphate (CTP) synthetase (E.C. 6.4.3.2) is an important enzyme for the synthesis of cytosine nucleotides and is considered as the rate-limiting step for the biosynthesis of CTP in some malignant and non-malignant tissues [1,2]. CTP synthetase catalyses the conversion of uridine triphosphate (UTP) into CTP, which is one of only two mechanisms of synthesising CTP. The other mechanism is the cytidine salvage pathway catalysed by uridine/cytidine kinase and nucleoside mono- and diphosphate kinases (Fig. 1).

CTP synthetase activity has been analysed in various malignant and non-malignant tissues in humans and animals and a high activity has been found in rat hepatoma [1], human renal cell carcinoma [3], Hodgkin's disease and some types of non-Hodgkin's lymphomas (NHL) [4]. The *in vitro* enzyme activity proved to be increased in leukaemic cells of adults with acute lymphocytic leukaemia (ALL) [4], which has been confirmed by studying fluxes of ribonucleotides *in situ* in a MOLT-3 lymphoblastic cell line [5]. In MOLT-3, the preferential pathway for the synthesis of CTP proved to be via CTP synthetase, whereas proliferating T-lymphocytes (with

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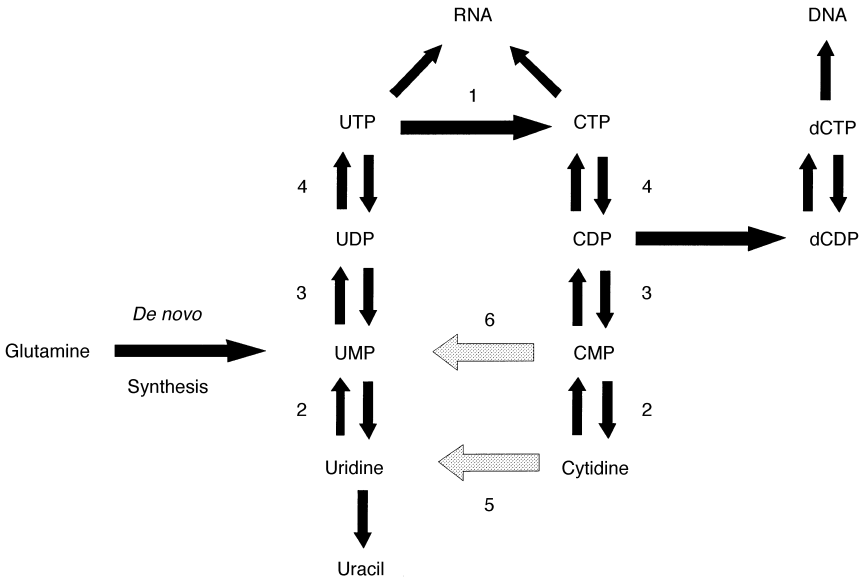


Fig. 1. A schematic overview of pyrimidine '*de novo*' synthesis and salvage pathways. Glutamine can be metabolised via several steps into uridine monophosphate (UMP), which in turn can be phosphorylated to uridine diphosphate (UDP) and uridine triphosphate (UTP). CTP synthetase (no. 1 in the figure) catalyses the conversion of UTP into CTP (a predominant pathway in various malignancies). The right trunk represents the cytidine salvage pathway, where cytidine is phosphorylated into CTP by three consecutive steps, catalysed by uridine/cytidine kinase (no. 2), nucleoside monophosphate kinase (no. 3) and nucleoside diphosphate kinase (no. 4). The conversion of cytidine into uridine (no. 5) is catalysed by cytidine deaminase, which has a lower activity in myeloid leukaemic cells compared with granulocytes. CMP deaminase (no. 6) has a variable activity in myeloid leukaemic cells.

an equal proliferation rate) mainly utilised the cytidine salvage pathway [5]. In addition, we have shown that a high CTP synthetase activity was also present in lymphoblasts of paediatric patients with ALL [6].

So far, in paediatric haematological malignancies of myeloid origin the CTP synthetase activity and pyrimidine ribonucleotide concentrations have not been investigated. However, the presence of a high CTP synthetase activity was suspected because of an increased concentration of CTP that has been observed in myeloblasts obtained from adults suffering from chronic myeloid leukaemia [7]. If the activity of CTP synthetase is increased in acute non-lymphocytic leukaemic (ANLL) cells, chemotherapy targeted against CTP synthetase might lead to more successful therapeutic regimens, as children suffering from ANLL have a moderate prognosis with the currently available combination therapy [8]. In this respect, it is noteworthy that cyclopentenyl cytosine (CPEC), which is an inhibitor of CTP synthetase, and which has been under clinical investigation [9], proved to have a growth-inhibiting effect on a HL-60 promyelocytic cell-line [10]. CPEC has not only a cytostatic effect *per se* [10–12] but might also enhance the cytotoxicity of cytarabine [13–15] which is one of the main drugs currently used for the treatment of ANLL.

Increased concentrations of ribonucleotides have been shown in a variety of malignancies [7,16–19], the most pronounced rise being found in the cytosine ribonucleotides. If this pattern also exists in leukaemic cells of patients suffering from ANLL, then inhibition of CTP synthetase will most probably lead to decreased cytosine ribonucleotide concentrations in the leukaemic cells with a consequent antiproliferative effect, comparable with the effect of CPEC on HL-60 cells [10].

For these reasons, we analysed the ribonucleotide concentrations and the CTP synthetase activity of bone marrow (BM) samples of paediatric patients suffering from ANLL at diagnosis, of non-malignant CD34+ BM cells and of neutrophilic granulocytes of healthy donors. *In vitro* treatment of the leukaemic cells with CPEC showed a major decrease in the concentrations of CTP in the leukaemic cells, reflecting the inhibition of CTP synthetase. Our study is the first to describe this CTP-depleting effect of CPEC on non-cultured human leukaemic cells.

## 2. Patients and methods

### 2.1. Patients

Samples of bone marrow or peripheral blood of paediatric patients suffering from ANLL were studied. They were classified according to the international FAB classification, based on morphology and immunophenotyping as assessed by flow cytometry. As control samples, two different cell populations were studied. One population consisted of non-malignant CD34+ BM cells, that represent control cells with a capacity to proliferate and at the same stage of differentiation as the ANLL cells. The CD34+ BM cells were obtained after

informed consent from healthy orthopaedic patients (ages 15, 17 and 33 years) or from paediatric patients (ages 2 and 3 years) with stage IV neuroblastoma, who had been treated with vincristine, teniposide, carboplatin and ifosfamide, after which an autologous bone marrow transplantation (ABMT) was intended, but who died before ABMT could be performed. The second control population consisted of granulocytes, that are non-proliferating myeloid cells, but that do need CTP for their RNA synthesis. The granulocytes were obtained from healthy adult volunteers chosen amongst the hospital personnel. For ethical reasons, these controls could not be age-matched, because 20 ml of blood was required for the isolation of the granulocytes, which might induce anaemia in small children.

### 2.2. Cell separation and purification

Leukaemic cells and granulocytes were isolated as previously described [6,20]. Patients' samples that initially contained less than 80% of blasts were further purified by magnetic cell sorting (MACS, Miltenyi, Germany) using anti-CD33 Microbeads [21,22] and washed with phosphate buffered saline (PBS) containing 5 mM glucose. The CD34+ BM samples of the orthopaedic patients were obtained by puncturing the posterior iliac crest until 50–75 ml of BM was harvested. The CD34+ cells were isolated by the same procedure as previously described for the leukaemic cells [6,20], with the final purification step being magnetic cell sorting using CD34 Multisort Microbeads [21]. The BM samples obtained from the 2 paediatric patients, were harvested by routine procedures [23], and the mononuclear fraction was isolated and cryopreserved as described by the group of Slaper-Cortenbach [23]. After thawing and washing these samples, the CD34+ cells were isolated by MACS procedures using CD34 Multisort Microbeads [21,22]. Cells were counted with a Coulter Counter ZF cell counter, in Isoton containing $3.3\times10^{-5}$% (v/v) Triton X-100 and $6.7\times10^{-3}$ (w/v) saponine. Vitality of the various cell types was assessed by the trypan blue exclusion test [24] and initial cell purity was assessed by microscopic examination of cytospin preparations stained with Jenner Giemsa and by flow cytometric analysis (FACScalibur, Becton Dickinson, USA). Cell samples for the determination of CTP synthetase activity ($5–10\times10^6$ cells) were briefly centrifuged (12 000 rpm), after which the supernatant was discarded and the cells frozen in liquid $N_2$ and stored in −80°C. Cell samples for the nucleotide extraction were kept on ice for 1 h after the isolation [25]. Ribonucleotides were extracted by adding 150 μl of ice-cold 0.4 N perchloric acid to a cell pellet (11 000×g, 4°C, 7 sec) of $5\times10^6$ cells. After 10 min at 0°C this suspension was centrifuged (11 000×*g*, 4°C, 5 min) after which the ribonucleotide containing supernatant was neutralised with 7.5 μl of 5.0 M $K_2CO_3$. These ribonucleotide samples were stored at −20°C. The protein content of the cell pellets after ribonucleotide extraction was measured according to Smith and colleagues [26], using human albumin as a standard.

### 2.3. CTP synthetase assay

Cells were solubilised by sonicating three times (10 sec, output 8.5 Watt, Vibracell, Sonics & Materials, Inc., USA) at a concentration of $40\times10^6$ cells/ml in a buffer containing 35 mM Tris-Mops (pH 7.9), 1 mM EGTA, 2.5 mM phenylmethylsulphonyl fluoride (PMSF) and 10 mM dithiothreitol (DTT). The samples were kept on ice, after which the homogenate was centrifuged and the CTP synthetase activity was measured in the supernatant by the method described by Van Kuilenburg and colleagues [27]. The nucleoside triphosphates were separated by anion-exchange high performance liquid chromatography (HPLC), on a Whatman Partisphere SAX 4.6×125 mm column (5 μm particles) and Whatman 10×2.5 mm SAX guard column, under isocratic elution conditions using a 0.594 M $NaH_2PO_4$ (pH 4.55) buffer at a flow rate of 1 ml/min [27]. The HPLC system consisted of a Gilson 231XL and 402 sampling device, a Perkin Elmer Binary LC 250 pump, a model 480 Waters Lambda-Max LC Spectrophotometer, a Nelson 900 series Interface and Nelson PC Integrator Software (version 5.1.5). The protein content of the cell homogenate was determined using the modified Lowry method [28] with human albumin as the standard.

### 2.4. Ribonucleotide analysis

The cell extracts were centrifuged for 5 min at 11 000*g*, 4°C, after which the supernatants were injected into the same HPLC system as described above. However, the elution conditions were different: after equilibrating the column with 100% buffer A (9 mM $NH_4H_2PO_4$, pH 3.50) for 10 min, the run was started with 100% buffer A for 2 min. A linear gradient was then started to reach a level of 80% of buffer B (325 mM $NH_4H_2PO_4$, 500 mM KCl, pH 4.70) after 25 min. This was followed by a change to 90% B to be reached in 10 min and then maintained for a further 10 min. The gradient was then changed back to 100% A over 1 min and this was kept for 10 min. Absorption was measured at a wavelength of 280 nm. Response factors were calculated using external standards.

### 2.5. Incubation experiments

The leukaemic cells were incubated in DMEM-Nut F12 culture medium containing 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin,

Table 1
Patients' characteristics and cytidine triphosphate (CTP) synthetase activity of the leukaemic cells at time of diagnosis

| Patients | Age (months) | Gender | FAB-type | WBC ($\times 10^9$/l) | Blast % (peripheral ) | CTP synthetase activity (nmol CTP/mg protein/h) |
|---|---|---|---|---|---|---|
| 1 | 26 | F | M5 | 54.7 | 57 | 2.3[a] |
| 2 | 124 | F | M5 | 35.4 | 85 | 9.2[a] |
| 3 | 182 | M | M4 | 52.7 | 85 | 4.3[a] |
| 4 | 29 | F | M4 | 126.0 | 71 | 9.1[a] |
| 5 | 16 | M | M4 | 48.7 | 61 | 3.1[a] |
| 6 | 28 | M | M5 | 23.8 | 23 | 5.2 |
| 7 | 15 | M | M6 | 40.9 | 29 | 4.1[a] |
| 8 | 28 | F | M7 | 13.2 | 10 | 4.1 |
| 9 | 27 | F | M7 | 48.7 | 69 | 6.2 |
| 10 | 16 | F | M7 | 12.4 | 3 | 3.3[a] |
| 11 | 62 | F | M4 | 50.0 | 86 | 6.0[a] |
| 12 | 65 | M | M5 | 39.0 | 85 | 6.7[a] |

[a] Ribonucleotide concentrations were determined. WBC, white blood cells.

200 μg/ml gentamycin, 0.125 μg/ml amphotericin B, 2 mM L-glutamine, 5 μg/ml transferrin and 5 ng/ml sodium selenite. Cells were incubated in T25 culture bottles at a density of $2\times10^6$ cells/ml in humidified air containing 5% $CO_2$ at 37°C for 18 h with concentrations of CPEC, ranging between 0.63 and 5 μM, using a 750 μM stock solution of CPEC in 0.9% NaCl. CPEC was kindly provided by the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, USA. The samples were harvested, centrifugated ($200\times g$, 7 min, 20°C), washed with PBS containing 5 mM glucose and kept on ice for 1 h after the isolation [20].

### 2.6. Statistics

The differences between ANLL cells and granulocytes of the mean activities of CTP synthetase and the ribonucleotide concentrations were analysed by the non-parametric Mann–Whitney test for two independent samples, using the Statistical Package for the Social Sciences (SPSS 6.0.1 software version), as were the differences in CTP synthetase activity between the ANLL cells and the non-malignant BM cells. The correlation between the CTP synthetase activity and the CTP concentration or UTP/CTP ratio was studied by determination of Pearson's correlation coefficients. $P$ values of $<0.05$ were considered as statistically significant.

## 3. Results

### 3.1. Patients' characteristics

Material from 7 girls and 5 boys was obtained, their age ranging from 15 months to 15 years and 2 months (mean: 5 years and 2 months). Using the FAB classification there were 4 cases of M4, 4 cases of M5, 1 case of M6 and 3 cases of M7 ANLL. White blood cell counts varied from 12.4 to $126\times10^9$/ml (mean = 31.8). The patients' characteristics are shown in Table 1.

### 3.2. CTP synthetase activity

The patients' leukaemic cells and the non-malignant CD34+ BM fractions ($n=5$) showed a cell purity of at least 80%. The granulocytes of healthy volunteers ($n=9$) were at least 95% pure. The viability of all cells was higher than 85%.

The CTP synthetase activity is expressed as the specific enzyme activity, i.e. the amount of CTP that is produced per hour and per mg protein. The CTP synthetase activity in the cells of each patient is shown in Table 1 and ranged from 2.3 to 9.2 nmol CTP/mg/h. As shown in Fig. 2, the mean specific activity of CTP

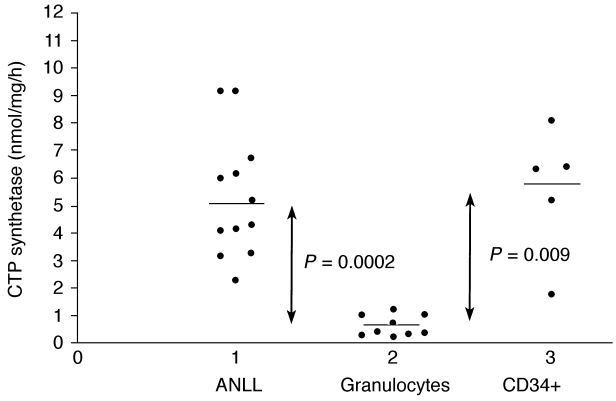


Fig. 2. The cytidine triphosphate (CTP) synthetase activity of the patients' leukaemic cells (left), granulocytes of healthy volunteers (middle) and the non-malignant CD34+ bone marrow (BM) cells (right).

synthetase in the leukaemic cells proved to be 5.1 ± 2.3 nmol CTP/mg/h, which was significantly higher ($P = 0.0002$) than the mean enzyme activity detected in granulocytes of healthy volunteers (0.6 ± 0.4 nmol CTP/mg/h). Although the series is not sufficiently large to be conclusive, the enzyme activity did not seem to be influenced by age, gender or leukaemic subtype. In addition, there was no correlation between the enzyme activity and the white blood cell count (Pearson's correlation coefficient ($r = 0.444$, $P = 0.231$), nor with the blast percentage of the peripheral blood mononuclear cells ($r = 0.425$ , $P = 0.254$).

The activity of CTP synthetase in non-malignant CD34+ bone marrow cells proved to be 5.6 ± 2.4 nmol CTP/mg/h (Fig. 2), which was significantly higher ($P = 0.009$) than the enzyme activity of granulocytes of healthy persons, but was not statistically different from the activity of CTP synthetase encountered in the ANLL cells ($P = 0.84$).

### 3.3. Ribonucleotide concentrations

From only 9 patients (Table 1) could we obtain a sufficient number of cells to analyse the ribonucleotide concentrations. These ribonucleotide concentrations were also determined in granulocytes, but not in the CD34+ bone marrow fractions, due to a shortage of cells. The total amount of ribonucleotides expressed per μg protein is significantly higher in leukaemic cells compared with granulocytes (78 ± 10 pmol/μg protein versus 41 ± 8 pmol/μg protein, $P = 0.0008$). A significant increase of both purine and pyrimidine ribonucleotides was observed in comparison with granulocytes. Since the relative increase in pyrimidine ribonucleotide concentrations is more pronounced than in the purine ribonucleotides, a significantly ($P = 0.0008$) decreased purine/pyrimidine ratio of 4.6 was found in ANLL cells compared with 16.7 in granulocytes. The cytosine ribonucleotide concentrations were 6-fold higher in ANLL cells (2.4 ± 1.3 pmol/μg protein) compared with granulocytes (0.4 ± 0.1 pmol/μg protein, $P = 0.0007$), which was paralleled by a more than 5-fold increase of uracil ribonucleotides in ANLL cells (8.7 ± 2.8 pmol/μg protein) compared with granulocytes (1.6 ± 0.4 pmol/μg protein, $P = 0.0007$), leading to a comparable uracil/cytosine ribonucleotide ratio in leukaemic cells compared with granulocytes (3.6 versus 4.0). Considering the purine ribonucleotide concentrations, an increase in both ATP (43 ± 10 versus 27 ± 7 pmol/μg protein, $P = 0.0009$) and GTP (7.8 ± 2.1 versus 6.4 ± 1.3 pmol/μg protein, $P = 0.08$) is observed in leukaemic cells compared with granulocytes, but the increase in ATP is slightly higher. Thus, an increase in adenine/guanine ribonucleotide ratio is observed in leukaemic cells (5.5), compared with granulocytes (4.2), which was significant ($P = 0.002$).

In leukaemic cells, no significant correlation could be observed between the CTP synthetase activity and the CTP concentration (Pearson's coefficient $r = 0.47$, $P = 0.20$) (Fig. 3a), although a trend seemed to exist. Nevertheless, the CTP synthetase activity and the UTP/CTP ratio are inversely correlated ($r = -0.70$, $P = 0.035$) (Fig. 3b).

### 3.4. CPEC incubation experiments

Cell samples of 9 other patients (Table 2) were used for incubation experiments with various concentrations of CPEC (0.63–5 μM). Of these patients, 6 suffered from ANLL, 2 had a relapse of ALL with a biphenotypic immunological aspect and 1 was a patient with Down's syndrome suffering from a leukaemoid reaction with a myeloid phenotype (CD13+/CD33+/CD34+). CPEC induced a depletion of CTP in all of the leukaemic samples (Fig. 4 and Table 2). After incubating the cells with 2.5 μM of CPEC for 18 h ($n = 6$), we observed a mean CTP depletion of 77 ± 13%, leaving a concentration of CTP of 23% compared with the samples that were not treated with CPEC. There were no significant differences between the effect of 0.63, 1.25, 2.5 or 5 μM of CPEC, inducing a CTP depletion of 47 ± 42, 59 ± 32,

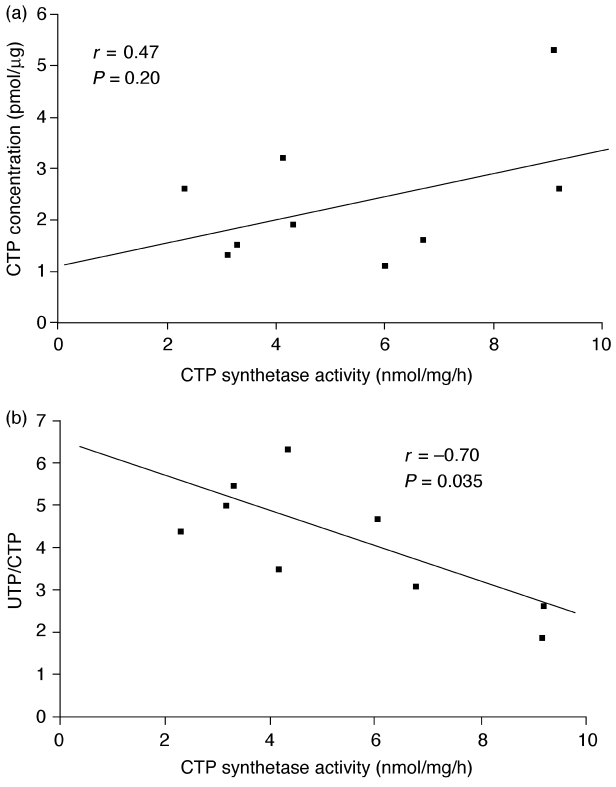


Fig. 3. (a) Correlation (non-significant) between the cytidine triphosphate (CTP) synthetase activity and CTP concentration of the patients' leukaemic cells. (b) Inverse correlation between the CTP synthetase activity and the uridine triphosphate (UTP)/CTP ratio, that was observed in the leukaemic cells. Each square represents the data of 1 patient.

Table 2
Patients' samples that were incubated with various concentrations of CPEC

| Patients | Diagnosis | CTP-concentration after treatment with CPEC of: | | | |
|---|---|---|---|---|---|
| | | 0.63 μM | 1.25 μM | 2.5 μM | 5 μM |
| 5 | M4 | | | | 17% |
| 11 | M4 | 50% | 39% | 32% | 32% |
| 12 | M5 | | 26% | 31% | 28% |
| 13 | M1 | | | 41% | |
| 14 | M3 | | | 9% | |
| 15 | M5 | | | 13% | |
| 16 | Rec. ALL | | | | 52% |
| 17 | Rec. ALL | 97% | 86% | | |
| 18 | Leukaemoid reaction | 13% | 14% | 13% | |
| Mean ± S.D. | | 53 ± 42% | 41 ± 32% | 23 ± 13% | 31 ± 13% |

Cytidine triphosphate (CTP) concentrations are percentages of CTP in untreated samples (see Fig. 4). Patients 16 and 17 had recurrent acute lymphocytic leukaemia (ALL) with myeloid features. CPEC, cyclopentenyl cytosine.

77 ± 13 and 69% ± 13 respectively (Fig. 5a), but the numbers of treated samples were too low to provide sufficient statistical power. We could unfortunately not use every concentration of CPEC for each sample due to a shortage of cells. At a concentration of 2.5 μM of CPEC, there were practically no changes in the concentrations of UTP (89%), ATP (93%) and GTP (98%) (Fig. 5b). The cells of the patient suffering from a leukaemoid reaction proved to be very sensitive to CPEC, showing a depletion of CTP (87%) after 0.63 μM of CPEC.

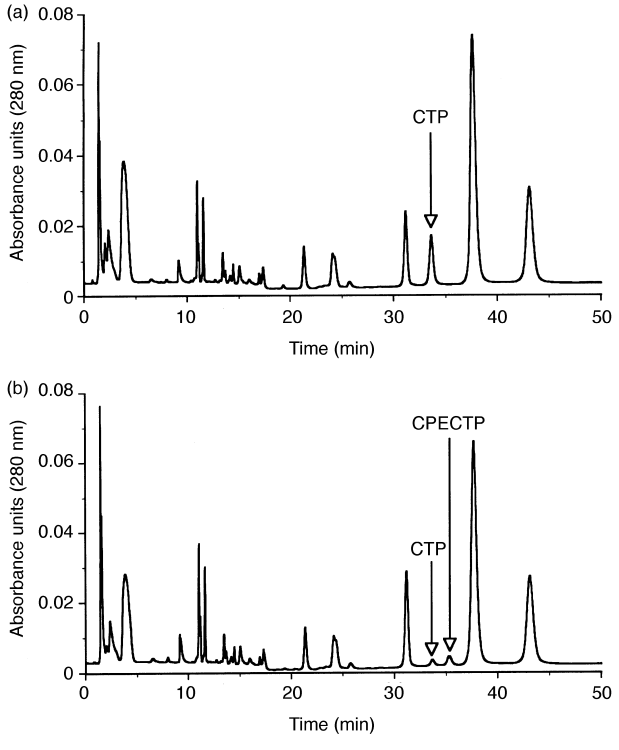


Fig. 4. A chromatogram (a) showing a ribonucleotide profile of an acute non-lymphocytic leukaemia (ANLL) sample at diagnosis. (b) Ribonucleotide profile of the same ANLL sample after incubation with 0.63 μM of cyclopentenyl cytosine (CPEC) for 18 h, showing the CPEC-triphosphate (CPECTP) peak after 35.3 min. The CTP peak (33.6 min) was reduced by 87% compared with the untreated sample (a).

## 4. Discussion

An increased CTP synthetase activity has been observed in adults [4] and children [6] suffering from ALL, but so far the activity of CTP synthetase has never been studied in paediatric patients with ANLL. The present study provides firm evidence of a high CTP synthetase activity in blasts of children suffering from ANLL, which is substantially higher compared with that observed in neutrophilic granulocytes. The latter are non-dividing cells but possess protein-synthesising capacities, requiring ribonucleotides such as CTP for RNA synthesis. A high CTP synthetase activity in ANLL is in line with the increased activities that have been found in various malignancies, e.g hepatoma [1],

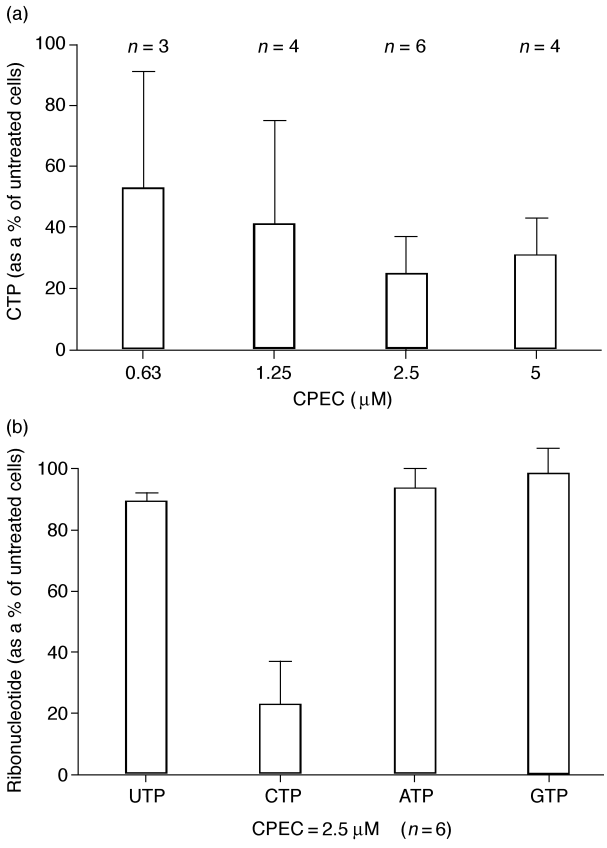


Fig. 5. (a) Mean cytidine triphosphate (CTP) concentration in the leukaemic cells as a function of the concentration of cyclopentenyl cytosine (CPEC) that was used. (b) Effect of 2.5 μM of CPEC on the concentrations of uridine triphosphate (UTP), CTP, ATP and GTP in the leukaemic cells of 6 patients.

renal cell carcinoma [3], Hodgkin's disease and NHL [4], ALL [4,6] and colon carcinoma [29]. A high CTP synthetase activity might be due to an increased anabolic state of the tumour cell, for increased activities of pyrimidine *de novo* and salvage enzymes have been observed, reflecting an increased synthesis of nucleic acid precursors [29]. However, the high activity of CTP synthetase that we found in the leukaemic cells of myeloid origin is not higher compared with the activity encountered in non-malignant CD34+ BM cells, that represent the most undifferentiated BM fraction and the best obtainable non-malignant counterpart of leukaemic cells. Therefore, the high activity of CTP synthetase in ANLL cells could also be due to the proliferative and/or immature state of the leukaemic cells and may not be related to the process of malignant transformation.

CTP synthetase inhibition might be obtained by experimental drugs such as 3-deazauridine and CPEC. Inhibition of CTP synthetase decreases the conversion of UTP into CTP, leading to a CTP depletion. When a promyelocytic HL-60 cell line was incubated with 3-deazauridine, an impressive differentiation occurred, which was preceded by a CTP depletion and a decrease in c-Myc mRNA expression [30]. A similar effect was observed when HL-60 cells were incubated with CPEC, which induced growth inhibition and induction of differentiation [10]. CPEC also proved to have an anti-leukaemic effect on cell lines of human and murine lymphocytic leukaemia [11,12], increasing the lifespan of mice with ALL [11]. Mice inoculated with tumour cells derived from a colon carcinoma cell line and that were subsequently treated with CPEC showed a reduction in the size of their tumours [31]. Our study provides the first evidence that non-cultured human leukaemic cells are capable of metabolising CPEC to its active triphosphate form, which induces a depletion of CTP in the blasts. We were not yet able to demonstrate a dose–response effect, but this is probably due to the small number of samples, for a trend seemed visible.

Our results showed high CTP and UTP concentrations in blasts of paediatric patients suffering from ANLL, concurrent with a decreased purine/pyrimidine ratio. Alterations in ribonucleotide pools have been observed in various malignancies [16–19], including lymphoblasts of paediatric patients with ALL [7]. One of our most striking observations is the proportionally similar increase of the CTP and UTP concentrations in leukaemic cells, giving rise to a comparable mean UTP/CTP ratio in leukaemic cells and granulocytes. The increased activity of CTP synthetase in ANLL cells is, therefore, linked to an efficient salvage of uridine. The observation that inhibition of CTP synthetase in HL-60 cells does lead to CTP depletion and an increased UTP/CTP ratio [10,30] proves that CTP synthetase is essential for the synthesis of CTP in malignant myeloid cells. Whether the pathway via CTP synthetase or the salvage pathway of cytidine is the most essential pathway for the synthesis of CTP in non-malignant CD34+ BM cells could not be determined, because of the difficulties in isolating a sufficient number of CD34+ BM cells to perform multiple experiments (75–125 ml of BM was already required to analyse the activity of CTP synthetase in non-malignant CD34+ cells). Our results suggest that CTP synthetase plays a major role in the synthesis of CTP in these non-malignant BM cells, and that CPEC might be toxic to CD34+ BM cells. However, the low to moderate haematological toxicity that was observed in the phase I trial of CPEC [9] may indicate that these non-malignant BM progenitor cells are capable of using an alternative pathway, that is the cytidine salvage pathway, for synthesising CTP. The *in vitro* myelotoxicity of CPEC has been studied by Volpe and colleagues [32], by analysing the colony-forming capacity of human colony forming unit granule macrophage (CFU-GM). They observed a 70% inhibition of colony formation at an area-under-the-curve (AUC) of 40.8 μMh [32]. In our experiments, we achieved a comparable AUC of 45 μMh, when incubating the leukaemic cells with 2.5 μM. However, our results indicate that with lower concentrations of CPEC (e.g. 0.63 μM leading to an AUC of 11.2 μMh), that would have a low haematological and cardiovascular toxicity according to the phase I trial with CPEC [9], a comparable CTP depletion of 47% was observed. In myeloid (HL-60) and T-lymphoblastic (Molt-3) leukaemic cell-lines, a CTP depletion of 54 and 36%, respectively was sufficient to induce growth inhibition (data not shown).

Several biochemical consequences might ensue from an increased CTP synthetase activity. Although we were not as yet able to demonstrate a direct correlation between the enzyme activity and the CTP concentration in each patient, an inverse relationship existed between the CTP synthetase activity and the UTP/CTP ratio. Furthermore, an overall increased CTP concentration was demonstrated in our patient population. An increased CTP concentration influences the intracellular CDP pools, which, in turn can be converted to deoxycytidine diphosphate (dCDP) by the enzyme ribonucleotide reductase. dCDP is subsequently converted into deoxycytidine triphosphate (dCTP). These conversions all represent steady-state equilibriums and, therefore, an increased CTP concentration will lead to a higher dCTP concentration [33]. Alterations in dCTP pools were found to be related to increased mutation rates *in vitro* by means of disturbing the normal deoxyribonucleotide equilibrium [33]. Furthermore, the dCTP concentration influences the sensitivity of tumour cells to some cytotoxic drugs that are frequently used in the treatment of ANLL. For example, an increased dCTP concentration proved to be correlated with cytosine arabinoside (cytarabine, AraC) resistance in a T-lymphocytic cell

line [34] by feedback inhibition of the enzyme deoxycytidine kinase. This enzyme is not only responsible for the phosphorylation of deoxycytidine but also for the first phosphorylating step of AraC (that needs three phosphorylating steps to become AraCTP, the active AraC metabolite). Furthermore, competition between AraCTP and dCTP arises in matters of affinity for DNA polymerase [34]. Therefore, by decreasing the intracellular dCTP pools in the blasts, the sensitivity of the leukaemic cells to AraC might be increased by either of two ways, and this was shown to occur in murine lymphocytic leukaemia and a human colon tumour cell line [13–15]. AraC is still one of the key drugs for the treatment of paediatric ANLL, and *in vitro* resistance to AraC is related to a poorer prognosis [35].

In conclusion, this study demonstrates a high CTP synthetase activity and high pyrimidine ribonucleotide concentrations in leukaemic cells in paediatric ANLL. We showed that the inhibition of CTP synthetase by CPEC is feasible in ANLL cells, and we provide the first evidence that CPEC has a CTP-depleting effect in non-cultured human leukaemic cells. CTP synthetase inhibition might form an approach in the future combination regimens for ANLL, as it could bear a direct cytotoxic effect or modulate the cytotoxicity of AraC.

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