The cytostatic- and differentiation-inducing effects of cyclopentenyl cytosine on neuroblastoma cell lines

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Abstract

This paper describes the effects of cyclopentenyl cytosine (CPEC) on the proliferation and cell-cycle distribution of the SK-N-BE(2)c and SK-N-SH neuroblastoma cell lines, as well as their ability to recover from treatment with CPEC. The IC_{50} value of SK-N-BE(2)c for CPEC, determined after 48 hr was 80 nM. SK-N-BE(2)c cells showed a time- and concentration-dependent accumulation in the S-phase of the cell cycle after 2 and 3 days of incubation with 50–250 nM CPEC, followed by a G_0/G_1-phase arrest after 4 days. After incubation with 50 nM CPEC for 2 days, SK-N-BE(2)c cells fully recovered and resumed logarithmic proliferation. In contrast, a complete and persistent growth arrest occurred when SK-N-BE(2)c cells were incubated for 2 days with 100 or 250 nM CPEC. The IC_{50} value of SK-N-SH, determined after 48 hr, for CPEC was ≥ 1 μM. SK-N-SH cells incubated with 250 nM or 1 μM CPEC showed a time-dependent accumulation in the S-phase of the cell cycle, followed by an accumulation in the G_0/G_1-phase, which reached a maximum of 84.1% after 7 days of incubation with 1 μM CPEC. SK-N-SH cells did not resume proliferation after removal of the drug. In addition, CPEC strongly induced differentiation in SK-N-SH cells. After 48 hr incubation with 250 nM CPEC, 90% of the cell population was differentiated. Both neuronal type and Schwannian type cells were observed. We conclude that at very low concentrations, CPEC has profound cytostatic- and differentiation-inducing effects on the neuroblastoma cells studied. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Neuroblastoma; Cyclopentenyl cytosine; CTP synthetase; Differentiation; MYCN amplification; Chemotherapy

1. Introduction

Neuroblastoma is the most common extra cranial tumor of childhood. The tumors are derived from the neural crest and originate in the adrenal medulla and paraspinal sites where sympatic nervous system tissue is present. Neuroblastoma has a very diverse genetic makeup [1]. The most common genetic prognostic markers are amplification of *MYCN* and deletions in the distal arm of chromosome 1p. Both amplification of the *MYCN* oncogene and deletions of chromosome 1p are markers for a poor prognosis of the disease [2–4]. The clinical outcome of neuroblastoma is dependent on its stage. There are 5 distinct prognostic stadia known as stage 1 to 4 and 4S, in which S stands for special

[5]. This staging system is based on the degree of infiltration of the tumor into neighboring tissues. Unfortunately, the prognosis for stage 4 patients with metastatic disease is very poor. In order to increase the cure rate of neuroblastoma, effective novel chemotherapeutic therapies need to be developed.

A prominent characteristic of neuroblastoma cells, as in most tumor cells, is their excessive drive to proliferate, which requires vast amounts of nucleotides that are needed for energy, RNA and DNA synthesis. To sustain this high turnover of nucleotides, key enzymes of the purine and pyrimidine biosynthesis pathways are up-regulated.

CTP synthetase is the rate-limiting enzyme in the synthesis of cytidine nucleotides from both *de novo* and uridine salvage pathways. As CTP synthetase activity is up-regulated in malignant cells, the intracellular CTP concentrations are higher in malignant cells than in their non-transformed counterparts [6–8]. Decreasing the CTP pool by inhibition of CTP synthetase might therefore reduce the ability of neuroblastoma cells to proliferate. A drug that inhibits the synthesis of CTP is cyclopentenyl cytosine

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Abbreviations: CPEC, cyclopentenyl cytosine; and CPECTP, cyclopentenyl cytosine-5′-triphosphate.

(CPEC) a carbocyclic analog of cytidine. CPEC has been shown to have antitumor, as well as antiviral activity, both *in vitro* and *in vivo* models [9–12]. Recently, the cytostatic effect of CPEC in leukemic cells from pediatric patients suffering from acute non-lymphatic leukemia has been demonstrated [13].

CPEC is readily taken up by the cell and is rapidly metabolized to its triphosphate nucleotide form (CPECTP), which is a potent inhibitor of CTP synthetase [14,15]. CPEC itself is an alternative substrate of uridine/cytidine kinase, the first enzyme of the pyrimidine salvage pathway, and is a competitive inhibitor for uridine and cytidine phosphorylation [14,16].

The mechanism of action and the antitumor effect of CPEC has primarily been studied in myeloid cell line systems and leukemic cells obtained from patients [10,13,14, 17,18]. To date, only limited aspects of CPEC have been investigated in solid tumors [11,15,19] such as neuroblastoma [20,21]. CTP synthetase in particular might be an attractive target for chemotherapy in neuroblastoma, as the gene coding for CTP synthetase is located on the often-deleted part of chromosome 1 (1p34), hence causing loss of heterozygosity of this gene [22]. Furthermore, neuroblastoma cells can be induced to differentiate into more benign forms, both *in vitro* as *in vivo*, by various compounds such as retinoic acid. This characteristic also makes neuroblastoma an interesting object to study the effect of cytostatic drugs.

In this paper, we demonstrate that low concentrations of CPEC induce a long-lasting state of cytostasis and differentiation in human neuroblastoma cells.

2. Materials and methods

2.1. Chemicals

CPEC (NSC 375575) was obtained from the Developmental Therapeutics Program, National Cancer Institute. All nucleotide standards and propidiumiodide were obtained from Sigma Chemical Co. Spermine was obtained from Merck. Nonidet P40 was obtained from LKB-produkter AB. Annexin-V-Fluos, soybean trypsin inhibitor, and RNAse were obtained from Boehringer Mannheim. Dulbecco's modified Eagle's medium, bovine fetal serum, and penicillin/streptomycin/fungizone-mix were obtained from Bio-Whittaker Europe. L-glutamine and gentamycin were obtained from GIBCO BRL. Isoton II was obtained from Beckman Coulter. Triton X-100 and saponine were from BDH Laboratory Supplies. All other chemicals were of analytical grade.

2.2. Cell culture

SK-N-SH and SK-N-BE(2)c neuroblastoma cell lines were obtained from the American Type Culture Collection.

The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 2 mM L-glutamine, 50 IU/mL of penicillin, 50 μg/mL of streptomycin, 0.2 mg/mL of gentamycin, 0.25 μg/mL of fungizone, and 10% v/v bovine fetal serum at 37° in humidified (90%) air with 5% CO_2. The cells were maintained in 75-cm^2 loosely capped culture flasks (Co-star Corp.) and maintained in logarithmic growth phase. Cells were passaged and split in a 1:20 ratio once a week, the medium was changed after 3 or 4 days. SK-N-BE(2)c was used up to passage 45. SK-N-SH was used up to passage 65. Cell cultures were consistently free of mycoplasma (tested with Mycoplasma PCR ELISA, Boehringer Mannheim).

2.3. Proliferation assay

Cells were seeded into 6-well plates (Corning Co-star) at a density of 0.25–0.50 $\times$ 10^6 cells per well, and allowed to adhere overnight. The experiments were started by replacing the medium with medium containing CPEC. Adherent cells were harvested by trypsinization. Cell numbers were determined after solubilizing the cells in isoton II containing 2.7 $\times$ 10^{-7}% (v/v) Triton X-100 and 2.7 $\times$ 10^{-3}% (w/v) saponin. The nuclei were counted with a Coulter Counter Z 1000 with a 100-μM orifice (Coulter Electronics Ltd.). The viability of adherent cells was more than 99%, as determined by exclusion of Trypan blue.

2.4. Nucleotide extraction

Cells were seeded in 6-well plates at a density of 1–2 $\times$ 10^6 cells per well. After overnight adherence, the experiments were started by addition of CPEC to the culture medium. After the incubation, the cells were washed once with PBS. The adherent cells were extracted with 200 μL of ice-cold 0.4 M perchloric acid for 10 min on ice with intermittent scraping with a disposable cell scraper. The resulting suspension was centrifuged at 10,000 $\times$ g at 4° for 3 min. The supernatant was removed and neutralized with K_2CO_3 and used for HPLC analysis. The pellet containing total protein was dissolved in 500 μL of 0.2 M NaOH. Protein content was determined using bicinchonic acid solution containing 0.1% $CuSO_4$, as described previously [23].

2.5. HPLC analysis

Nucleotide profiles were determined by ion exchange HPLC, using a Whatman Partisphere SAX 4.6 $\times$ 125 mm column (5-μm particles) and a Whatman 10 $\times$ 2.5 mm AX guard column (Whatman Inc). The buffers used were: 9 mM $NH_4H_2PO_4$, pH 3.5 (buffer A) and 325 mM $NH_4H_2PO_4$, 500 mM KCl, pH 4.4 (buffer B). Nucleotides were eluted with a gradient from 100% buffer A to 90% buffer B in 60 min at a flow rate of 1 mL/min.

2.6. Cell cycle analysis

The following solutions were used in the cell cycle analysis protocol. Solution S: 0.1% (v/v) Nonidet P40, 1.5 mM spermine, 3.4 mM citrate, 0.5 mM Tris, pH 7.6. Solution A: 25 mg/mL of trypsin in solution S. Solution B: 0.5 mg/mL of soybean trypsin inhibitor and 0.1 unit RNAse in solution S. Solution C: 0.38 mg/mL of propidium iodide, 4.5 mM spermine, 3.4 mM citrate, 0.5 mM Tris, pH 7.6. Adherent cells were harvested by trypsinization and centrifuged for 7 min at $302 \times g$. Subsequently, the cells were re-suspended in 450 μL solution A ($\pm$ 1 $\times$ 10^6 cells/mL) and incubated for 10 min at room temperature. Subsequently, 375 μL of solution B was added and the sample was incubated for 10 min at room temperature. Then, 375 μL solution C was added and the sample was incubated in the dark for 30–60 min on ice. The nuclei were analyzed by using a FACScalibur flowcytometer (Becton Dickinson) and the data were fitted with the Modfit computer program.

2.7. The ability of neuroblastoma cells to recover from treatment with CPEC

Cells were incubated with CPEC (1, 2, or 3 days for SK-N-BE(2)c and 2, 4, or 7 days for SK-N-SH). Subsequently, the medium containing the drug was removed and the cells were allowed to recover in fresh medium (3 days for SK-N-BE(2)c and 7 days for SK-N-SH), while control cells received fresh medium containing CPEC. Proliferation and intracellular CTP and CPECTP levels were measured in adherent cells in separate experiments.

2.8. CTP synthetase assay

The CTP synthetase activities in crude homogenates were determined in a reaction mixture containing 1 mM UTP, 4 mM ATP, 1 mM GTP, 10 mM L-glutamine, 20 mM MgCl$_2$, 10 mM KCl, 15 mM phosphoenol pyruvate, 17 U/mL of pyruvate kinase, 1 mM [Bis-amino-ethyl]-glyco-ether-N,N,N',N'-tetra-acetic acid, 10 mM dithiothreitol, 35 mM Tris-Mops, pH 7.6. Separation of the nucleotides was performed isocratically (0.596 M NaH$_2$PO$_4$, pH 4.55) by ion-exchange HPLC on a Whatman Partisphere SAX 4.6 $\times$ 125 mm column (5 μm particles) and a Whatman 10 $\times$ 2.5 mm AX guard column (Whatman Inc), as described previously [24].

3. Results

3.1. Effect of CPEC on proliferation

The effect of CPEC on the proliferation of SK-N-BE(2)c and SK-N-SH cells is shown in Fig. 1. The doubling time of untreated SK-N-BE(2)c cells was $\pm$ 24 hr. Incubation with 20 and 35 nM CPEC slightly inhibited the proliferation of
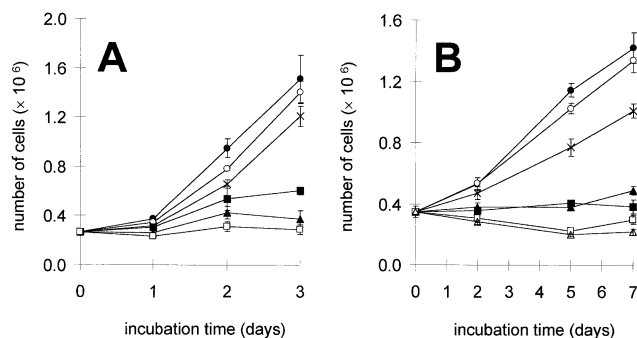

Fig. 1. The effect of CPEC on the proliferation of neuroblastoma cells. Panel A: SK-N-BE(2)c; ● 0 nM; ○ 20 nM; × 35 nM; ■ 50 nM; ▲ 100 nM; ❑ 250 nM CPEC. Panel B: SK-N-SH; ● 0 nM; ○ 35 nM; × 50 nM; ■ 150 nM, ▲ 250 nM, ❑ 500 nM, △ 1000 nM CPEC. The values shown are the mean of 3 experiments $\pm$ SD.

SK-N-BE(2)c cells, while the effect of 50 nM CPEC was more pronounced. Proliferation was completely inhibited at CPEC concentrations of 100 and 250 nM. No other effects than cytostasis were observed up to 5 μM CPEC (data not shown). The doubling time of untreated SK-N-SH cells was 30–36 hr. In SK-N-SH cells, growth was completely arrested with drug concentrations between 150 and 1000 nM. The IC$_{50}$ values, deduced from the growth curves, at the 48-hr time point were 80 nM and $\geq$1 μM for SK-N-BE(2)c and SK-N-SH, respectively.

3.2. Effect of CPEC on intracellular CTP levels

In both cell lines, the depletion of CTP and the accumulation of CPECTP were concentration- and time-dependent. Fig. 2A shows the effect of various CPEC concentrations on intracellular CTP levels. The concentration-dependence was assessed by incubating neuroblastoma cells for 6 hr with CPEC concentrations between 50 nM and 5 μM. A CTP depletion of 50% (ED$_{50}$) was observed at 110 and 170 nM CPEC for, respectively, SK-N-BE(2)c and SK-N-SH cells. After a 6-hr incubation with 1 μM CPEC, the remaining amount of CTP was 7% in SK-N-BE(2)c and 15% in SK-
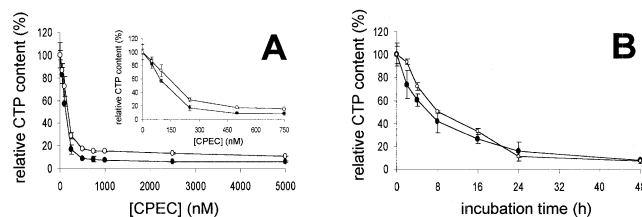

Fig. 2. The effect of CPEC on the intracellular CTP concentration. Panel A: concentration-dependence. Cells were incubated for 6 hr with various concentrations of CPEC. Subsequently, the cells were extracted and the intracellular CTP content was determined. The insert shows the concentration dependence between 0 and 750 nM CPEC. Panel B: time-dependence. Cells were incubated with 100 nM CPEC and the cells were extracted at various time points. ● SK-N-BE(2)c; ○ SK-N-SH. The values shown are the means of 3 experiments $\pm$ SD.

N-SH. CPEC was readily phosphorylated to its triphosphate form, which at 5 μM reached 99.7% and 86.0% of the normal CTP pool size in SK-N-BE(2)c and SK-N-SH cells, respectively (data not shown). When cells were incubated with 100 nM CPEC, the intracellular CTP pool was depleted by 50% after 6 hr in SK-N-BE(2)c and 8 hr in SK-N-SH cells (Fig. 2B). The rate of CTP depletion during the first 4 hr was 9.9% per hour in SK-N-BE(2)c cells and 6.9% per hour in SK-N-SH cells. Although after a 48-hr incubation with 100 nM CPEC, the final degree of CTP depletion was the same in both cell lines, and the initial rate of CTP depletion was 1.4 times higher in SK-N-BE(2)c than in SK-N-SH. After 48 hr, CTP levels were depleted to 8% of the initial concentration in SK-N-BE(2)c and to 7% in SK-N-SH cells. Under these conditions, the CPECTP concentration did not accumulate to more than 7% of the normal CTP concentration (data not shown) in either cell line indicating that low concentrations of CPECTP fully inhibited the *in situ* CTP synthetase activity.

3.3. CTP synthetase activity

We determined the *in vitro* CTP synthetase activity in crude homogenates of both cell lines. The CTP synthetase activity of SK-N-BE(2)c was 16.6 ± 2.1 nmol CTP/mg protein/hour ($N = 6$) and of SK-N-SH it was 11.4 ± 1.1 nmol/mg protein/hour ($N = 3$). Expressed per number of cells, the CTP synthetase activity of SK-N-BE(2)c was 4.5 ± 0.7 nmol CTP/10^6 cells/hour ($N = 3$) and of SK-N-SH it was 0.7 ± 0.1 nmol CTP/10^6 cells/hour ($N = 3$). Thus, the CTP synthetase activity, expressed as nmol CTP/mg protein/hour, was 1.5 times higher in SK-N-BE(2)c than in SK-N-SH cells. When expressed in nmol CTP/10^6 cells/hour, the CTP synthetase activity of SK-N-BE(2)c cells was 6.4 times higher than that of SK-N-SH cells.

3.4. Effect of CPEC on the cell cycle distribution

Upon incubation with CPEC we observed a time- and concentration-dependent accumulation in the S-phase of the cell cycle of SK-N-BE(2)c cells, which after three days of incubation reached a maximum of 52% with 50 nM CPEC and 75–77% with 100 and 250 nM CPEC. Surprisingly, after incubation for 4 or 5 days with CPEC, 61–75% of the cells finally arrested in the G_0/G_1 phase. When CPEC was removed after 1 or 2 days of incubation, the cells still continued to accumulate in the S-phase, followed by a G_0/G_1 phase accumulation (data not shown). To rule out the possibility that the cells accumulated in the G_0/G_1-phase because of the salvage of cytidine from fresh medium, we tested if cytidine from the medium (600 nM endogenous concentration) influenced the effect of CPEC on the cell cycle distribution. After three days of incubation with 250 nM CPEC, the medium was changed for normal medium, cytidine-depleted medium (0 nM cytidine, obtained by a 24-hr incubation of medium with normal cells) or cytidine-

depleted medium to which 600 nM cytidine was added (fresh CPEC was also added). In all three cases, the cells accumulated in the G_0/G_1-phase, indicating that cytidine from the medium did not influence the shift in cell cycle distribution.

The effect of CPEC on the cell cycle distribution of SK-N-SH cells was less pronounced. CPEC moderately increased the number of cells in the S-phase from $38 \pm 1.2\%$ to $51 \pm 0.8\%$ after 2 days incubation with 150 nM CPEC. This was followed by a slight increase in the number of cells in the G_0/G_1-phase ($68 \pm 0.9\%$) after 5 days of incubation with 150 nM CPEC. When SK-N-SH cells were incubated with 1 μM CPEC, the cells were retained longer in the S-phase. After 5 days of incubation, $50 \pm 1.3\%$ of the cells were in the S-phase. After 7 days of incubation with 1 μM CPEC, $84 \pm 1.7\%$ of the cells were arrested in the G_0/G_1-phase.

3.5. The ability of neuroblastoma cells to recover from treatment with CPEC

The ability of neuroblastoma cells to recover from treatment with CPEC was dependent on both concentration and duration of incubation with CPEC. SK-N-BE(2)c cells were incubated for 48 hr with various concentrations of CPEC, after which the drug was removed and the cells were allowed to recover in normal medium. After 48 hr of incubation with 50 nM CPEC, SK-N-BE(2)c cells resumed logarithmic proliferation after removal of CPEC. However, after 48 hr of incubation with 100 or 250 nM CPEC, proliferation was still arrested three days after removal of the drug, and remained arrested at least 7 days (data not shown).

The intracellular CTP concentration of SK-N-BE(2)c cells, incubated for 48 hr with 50 nM CPEC, was $40.7 \pm 0.6\%$ ($N = 3$) of untreated control cells and restored to $70.4 \pm 1.1\%$ ($N = 3$) three days after removal of CPEC. Because these cells had reached confluence, the CTP content was compared to that of untreated confluent cells (2.03 ± 0.17; $N = 3$). A very small quantity of CPECTP (0.03 ± 0.01 pmol/μg protein, $N = 3$) was still present, which was apparently not enough to completely inhibit CTP synthetase. After 48-hr incubation with 100 or 250 nM CPEC, the intracellular CTP concentrations of SK-N-BE(2)c cells were $10.4 \pm 2.2\%$ ($N = 3$) and $1.1 \pm 0.3\%$ ($N = 3$), respectively, and three days after removal of CPEC these were $34.3 \pm 3.0\%$ ($N = 3$) and $1.6 \pm 0.6\%$ ($N = 3$), respectively. The amount of CPECTP that remained 3 days after removal of CPEC was 0.09 ± 0.02 pmol/μg protein ($N = 3$) for 100 nM CPEC and 0.30 ± 0.07 pmol/μg protein ($N = 3$) for 250 nM CPEC respectively. The ability of SK-N-SH cells to recover from treatment with CPEC was dependent on the concentration of CPEC in a similar fashion; however, we did not observe logarithmic proliferation after removal of CPEC.

We also determined the effect of incubation time on the ability of neuroblastoma cells to recover from treatment
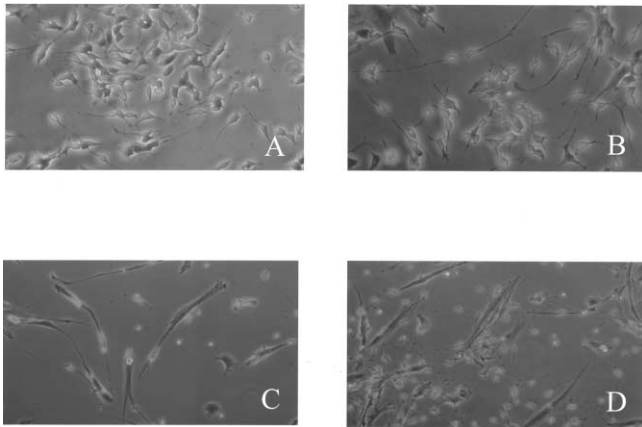
Fig. 3. The effect of CPEC on the morphology of SK-N-SH cells. Untreated cells (panel A), cells treated for 24 hr with 500 nM CPEC (panel B), cells treated for 8 days with 500 nM CPEC (panel C), and cells treated with 500 nM CPEC for 2 days, followed by 6 days of recovery in normal culture medium.
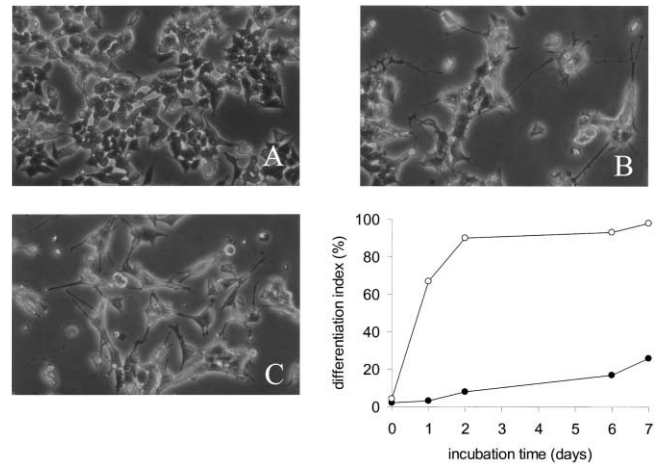

Fig. 4. The effect of CPEC on the morphology of SK-N-BE(2)c cells. Untreated cells (panel A), cells treated for 8 days with 500 nM CPEC (panel B), and cells treated with 500 nM CPEC for 2 days, followed by 6 days recovery in normal culture medium (panel C). Panel D: Induction of differentiation of neuroblastoma cells by CPEC. Neuroblastoma cells were incubated with 250 nM CPEC. To assess the degree of differentiation in the cell cultures, 400 cells were examined at the indicated time points. The relative number of differentiated cells, the differentiation index, is plotted against the incubation time. ● SK-N-BE(2)c; ○ SK-N-SH.

with CPEC. SK-N-SH cells were incubated for 2, 4, or 7 days with 500 nM CPEC after which they were allowed to recover for 7 days. After 2 days of incubation with 500 nM CPEC, we observed minimal proliferation 7 days after removal of CPEC. A persisting growth arrest ($\geq$7 days) was observed when cells were treated with 500 nM CPEC for 4 or 7 days. At all CPEC concentrations tested, CTP pools did not restore to 100% of the value of untreated log-phase cells. Intracellular CTP concentrations 7 days after removal of CPEC were 73 $\pm$ 11%, 45 $\pm$ 4%, and 59 $\pm$ 4%, for cells incubated with 500 nM CPEC for, respectively 2, 4, and 7 days. In SK-N-SH cells, CPECTP was not detectable 7 days after removal of CPEC.

3.6. Differentiation

Upon incubation with CPEC, we found a profound induction of differentiation of SK-N-SH cells. Neuronal type cells (N-type) as well as cells with the fibroblast-like morphology of the Schwannian type or substrate adherent type (S-type) were observed.

Untreated SK-N-SH cells are relatively small cells with small vestiges of neurites (Fig. 3A). After a 24-hr incubation with 500 nM CPEC, the cell bodies flattened and the neurites became elongated and thicker. Cellular contact through neurites also became more extensive (Fig. 3B). After 8 days of continuous incubation with 500 nM CPEC, large fibroblast-like cells were present which were connected to one another by means of long neurites. In addition, small cells bearing long neurites were also present (Fig. 3C). When the medium containing CPEC was removed after 2 days of incubation with 500 nM CPEC, cells with an identical morphology were observed (Fig. 3D).

When compared with SK-N-SH, induction of differentiation of SK-N-BE(2)c cells was remarkably less pronounced (Fig. 4). SK-N-BE(2)c cells were rather roughly

shaped, somewhat triangular cells (Fig. 4A) that flattened and became rounder and larger after 8 days of continuous incubation with 500 nM CPEC. Some neurites were also observed (Fig. 4B). After removal of the CPEC-containing medium, the cells remained flattened and enlarged (Fig. 4C). Only after 21 days of continuous culturing in the presence of 100 or 250 nM CPEC was a mature neuronal network formed by the remaining SK-N-BE(2)c cells (data not shown).

The extent of differentiation of cultures during incubation with 250 nM CPEC was assessed in both cultures of SK-N-BE(2)c and SK-N-SH cells, by scoring the morphology of 400 cells per culture per day (Fig. 4D). Cells bearing neurites (N-type) with a length of approximately twice the diameter of the cell body were scored as being differentiated, as well as cells that were connected to one another by means of neurites. Fibroblast-like cells were considered as being differentiated to S-type cells.

4. Discussion

The main objective of this study was to gain more insight into the effects of CPEC on neuroblastoma cell lines. The effects of CPEC on both proliferation and CTP levels were stronger in SK-N-BE(2)c than in SK-N-SH, which might reflect the differences in growth rate. SK-N-BE(2)c has 150 copies of MYCN whereas SK-N-SH has only two [25]. As *MYCN* amplification is accommodated by stimulation of proliferation because of enhancement of transcription of genes that favor proliferation, including key enzymes of the

purine and pyrimidine metabolism, SK-N-BE(2)c probably proliferates faster than SK-N-SH [26–28]. It is thus conceivable that SK-N-BE(2)c relies even more on the *de novo* synthesis of CTP than SK-N-SH. This would be in line with the fact that a higher CTP synthetase activity was present in SK-N-BE(2)c compared to SK-N-SH, despite loss of heterozygosity of the gene coding for CTP synthetase in SK-N-BE(2)c.

After a 48-hr incubation with CPEC (100 or 250 nM), SK-N-BE(2)c showed a pronounced accumulation of the cells in the S-phase of the cell cycle. A decreased synthesis of cytosine ribonucleotides, due to inhibition of CTP synthetase, hampers the synthesis of dCTP via ribonucleotide reductase as well [29]. Thus, a depletion of the endogenous CTP pool is accompanied by a depletion of the dCTP pool [19,30,31]. Previous studies only reported cell cycle distributions after maximally 48 hr of incubation [11,19]. In our experiments, we observed that, after prolonged incubation with CPEC, cells finally arrested in the G_0/G_1-phase of the cell cycle. Depletion of CTP or the other ribonucleotides, in the absence of DNA damage, can cause a reversible G_0/G_1 arrest through the p53 pathway [32]. CPEC inhibits DNA synthesis without causing major DNA damage, as it is not incorporated into DNA [19]. Hence the p53 pathway, which is functional in most neuroblastomas, allows an arrest in the G_0/G_1-phase of the cell cycle [33–35]. In SK-N-SH cells, the G_0/G_1-phase arrest coincided with a strong induction of differentiation. Within 24 hr of CPEC incubation, a substantial part of the cell population was morphologically differentiated to either N-type cells with long neurites or to the fibroblast-like Schwannian type. When SK-N-SH cells were incubated with 1 μM CPEC, the percentage of differentiated cells increased rapidly and reached almost 100% in 2 days. The morphological signs of differentiation preceded accumulation in the G_0/G_1-phase of the cell cycle. It is possible that the decreased endogenous CTP pool, caused by CPEC, mimics the situation in differentiated cells, as pyrimidine metabolism is down-regulated in differentiated cells [36,37]. Until now, the differentiation-inducing properties of CPEC have only been reported for HL-60 myeloid cells [38]. Here, we demonstrate that CPEC is a powerful inductor of differentiation in neuroblastoma. Neuroblastoma cells can be induced to differentiate *in vitro* by natural compounds such as *all-trans* retinoic acid and other physiological compounds. As a result of physiologically induced differentiation, cells usually lose their ability to proliferate. In our experiments, however, the loss of the ability to proliferate by depletion of cytidine nucleotides may be the cause rather than the consequence of differentiation in SK-N-SH. The S-phase is a critical phase of the cell cycle in which the cell is highly susceptible to induction of differentiation by agents, such as CPEC, that disturb the fine balance that regulates synthesis and utilization DNA precursors [39]. In most cases, SK-N-SH did not resume logarithmic proliferation after removal of CPEC. It appears that CPEC is able to reprogram SK-N-SH cells by irreversibly

inducing differentiation and thereby reducing its malignant characteristics. This would correspond with our finding that after removal of CPEC, CTP levels were partially restored while proliferation remained arrested. The induction of differentiation by CPEC might be a useful aspect in a possible future clinical setting.

The ability of SK-N-BE(2)c cells to recover from treatment with CPEC depended on the concentration used and the duration of incubation. At a low concentration of CPEC (50 nM), SK-N-BE(2)c cells resumed logarithmic proliferation, probably due to the small amount of CPECTP which is accumulated under these circumstances. After incubation with 100 or 250 nM CPEC, the high concentration of CPECTP, combined with its high retention, likely caused a continuous inhibition of CTP synthetase. As a result, SK-N-BE(2)c cells remained quiescent in the G_0/G_1-phase of the cell cycle, even after removal of CPEC.

We conclude that at very low concentrations, CPEC had different, but profound and long-lasting effects on the SK-N-BE(2)c and SK-N-SH neuroblastoma cell lines. The concentrations that induced these effects were considerably lower than the plasma concentrations that caused toxicity in a phase I clinical trial with patients suffering from solid tumors [40].

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