The pivotal role of CTP synthetase in the metabolism of (deoxy)nucleosides in neuroblastoma
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Gemcitabine and cyclopentenyl cytosine: 
a promising combination for the treatment of neuroblastoma

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submitted for publication
Abstract

Neuroblastoma is the most common solid malignancy of childhood. Despite intensive chemotherapeutic regimens, the survival rate of children suffering from metastasized neuroblastoma remains very poor. Poor prognosis is often associated with amplification of the MYCN oncogene. In this paper, we demonstrate that 2',2'-difluorodeoxycytidine (Gemcitabine, dFdC) has potent anti-tumor activity against neuroblastoma in vitro. dFdC is a pro-drug that is activated by phosphorylation to its nucleotides, of which deoxycytidine kinase (dCK) catalyzes the first and rate-limiting step. dFdC was tested on a panel of human neuroblastoma cell lines consisting of MYCN-amplified and MYCN-single copy cell lines. In both types of cell lines, low ED$_{50}$ values (nM range) were observed. Despite the fact that the specific deoxycytidine kinase (dCK) activity was 60% higher in MYCN-amplified cell lines than in MYCN-single copy cell lines, this did not correlate with the ED$_{50}$ values observed. However, while treatment with dFdC induced cell death in MYCN-amplified cell lines, MYCN-single copy cell lines underwent neuronal differentiation. Shep21N cells underwent cell death after incubation with dFdC when MYCN was expressed, but no cytotoxicity was observed when MYCN was not expressed. This may be caused by the fact that the dCK activity was higher in MYCN expressing Shep21N cells than in Shep 21 cells that did not express MYCN. Pre-incubation with the CTP synthetase inhibitor cyclopentenyl cytosine (CPEC) significantly lowered the ED$_{50}$ values of 13 out of 15 cell lines. In SK-N-BE(2)c cells that had been incubated with 100 nM CPEC for 1-4 days, the anabolism of dFdC was increased to 6-44 times as compared to untreated control cells. This was paralleled by an significant increase in the expression of dCK-mRNA, dCK proteins and increase of dCK activity. We feel that the combination of dFdC and CPEC may hold promise for the treatment of high-risk neuroblastoma.

1. Introduction

Neuroblastoma is the most frequently occurring extra cranial solid malignancy of childhood and is responsible for 15% of all childhood cancer deaths [1]. The tumor arises most commonly from sympathetic precursor cells in the adrenal medulla, and to a lesser extend, from ganglion precursor cells in the spinal cord. The prognosis for patients suffering from metastasized neuroblastoma is very poor. The majority of these children die from disease progression despite intensive therapy. Therefore, new and effective strategies need to be developed and evaluated in an effort to increase the survival of patients suffering from high-risk neuroblastoma.

Poor prognosis is associated with amplification of the MYCN oncogene and is found in approximately 25% of primary, predominantly metastasized neuroblastomas[2]. Increased expression of MYCN increases the transcription of a large set of genes that function in
ribosome biogenesis and protein synthesis [3]. Moreover, increased expression of MYCN enhances the proliferation potential and tumorigenicity [4;5].

Gemcitabine (dFdC) is an analog of deoxycytidine and has proven anti-tumor activity in vivo against non-small-cell lung cancer [6] and pancreatic cancer [7;8]. dFdC is a pro-drug that has to be activated by phosphorylation to its nucleotide-diphosphate and nucleotide-triphosphate forms to be therapeutically effective. The first and rate-limiting enzyme in the activation of dFdC is deoxycytidine kinase (dCK) [9;10]. dFdC-monophosphate is subsequently phosphorylated to dFdC-diphosphate and dFdC-triphosphate by nucleoside monophosphate kinases and diphosphate kinases, respectively. dFdC interferes with nucleotide metabolism in a number of ways. dFdC-diphosphate inhibits ribonucleotide reductase (RR) [11], causing depletion of the deoxyribonucleotide pools, including dCTP. Since dCTP is a feedback inhibitor of dCK, the inhibition of RR by dFdC-diphosphate stimulates the activation of dFdC by dCK [11]. dFdC-triphosphate is an inhibitor of CTP synthetase [12], DNA synthesis, DNA repair and is incorporated into the DNA, causing DNA damage [13]. After one moiety is incorporated into DNA, one more nucleotide is added, after which chain elongation stops (masked DNA chain termination), rendering the dFdC moiety resistant to excision by DNA exonuclease activity [14].

Cyclopentenyl cytosine (CPEC) is an analog of cytidine, which in its triphosphate nucleotide form, is a potent inhibitor of CTP synthetase and causes depletion of both the cytidine ribonucleotide pools and the deoxycytidine nucleotide pools [15;15-17]. CPEC possesses anti-tumor activity against leukemia (in vitro and in vivo) [15;18;19] and in vitro against solid tumors such as colon carcinoma [20-22], glioblastoma [23] and neuroblastoma [24-26]. We have shown previously that CPEC causes a retardation in the S-phase of the cell cycle in neuroblastoma cells [25]. This makes the combination of CPEC with a deoxycytidine analog, which is toxic to cells in the S-phase of the cell cycle, e.g. dFdC, an attractive one to explore.

Hitherto, no reports have been published on the cytotoxic effects of dFdC on neuroblastoma. In the present paper, the cytotoxicity of dFdC towards a panel of neuroblastoma cell lines, consisting of MYCN-single copy and MYCN-amplified cell lines, is reported. Moreover, the modulating effect of CPEC on the anabolism dFdC and thereby an increase of cytotoxicity of dFdC is explored.

2. Materials and methods

2.1 Chemicals

CPEC (NSC 375575) was obtained from the Developmental Therapeutics Program National Cancer Institute (Bethesda, MD, USA). dFdC was obtained from Eli Lilly (Nieuwegein, The Netherlands). [3H]-2',2'-difluorodeoxycytidine 14 Ci/mmol), was purchased from Moravek Biochemicals (Brea, CA), [14C]Thymidine (2.04 GBq/mmol) was obtained from Amersham International (Buckinghamshire, UK). CdA and all nucleotide
standards and tetracycline were obtained from Sigma Chemicals (Zwijndrecht, The Netherlands). Dulbecco’s Modified Eagles Medium, Bovine Fetal Serum and Penicillin/Streptomycin/Fungizone-mix were obtained from BioWhittaker Europe (Verviers, Belgium). L-glutamine and gentamycin were obtained from Gibco BRL (Paisley, Scotland). All other chemicals were of analytical grade.

2.2 Cell culture

The SK-N-BE(2), SK-N-BE(2)c and SK-N-SH neuroblastoma cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). All the other neuroblastoma cell lines were a generous gift from Dr. R. Versteeg (Dept. Human Genetics, Academic Medical Center, Amsterdam, The Netherlands). The cells were routinely cultured in Dulbecco’s Modified Eagles Medium (DMEM), supplemented with 2 mM L-glutamine, 50 I.U./ml penicillin, 50 µg/ml streptomycin, 0.2 mg/ml gentamycin, 0.25 µg/ml fungizone and 10 % v/v bovine fetal serum at 37 °C in humidified (90 %) air with 5 % CO2. Shep2 and Shep21N cell lines were maintained in RPMI 1640 medium, under the same conditions as the cell lines cultured in DMEM, with the addition of 10 mM HEPES and 0.15 % (w/v) NaHCO3. Tetracycline was used at a concentration of 10 ng/ml to inhibit MYCN expression. The cells were maintained in 75 cm² loosely capped culture flasks (Co-star Corp, Cambridge, MA, USA) and maintained in logarithmic growth phase. Cell cultures were consistently free of mycoplasma (tested with Mycoplasma PCR ELISA, Boehringer Mannheim).

2.3 Extraction and analysis of radio-labeled nucleotides

Cells were seeded in 6-wells plates at a density of 0.5 ×10⁶ cells per well. The cells were pre-incubated with CPEC for 1-4 days, after which the medium containing CPEC was removed and replaced my medium containing 50 nM [³H]dFdC and 250 nM [¹⁴C]Thymidine. After 3 h, the cells were extracted and analyzed as described previously [27].

2.4 Chemo-sensitivity assay

Cells were plated in 24-wells plates at a density of 20-50 × 10³ cells per well, depending on the cell line used, in a total volume of 0.5 ml and the cells were allowed to adhere overnight. Subsequently, the medium was changed with normal medium or medium containing 100 nM CPEC. After 24 hrs, experiments were started by adding dFdC to a final concentration of 7.8 – 1000 nM. After an incubation period of 3 hr, the medium containing dFdC was removed and changed for normal culture medium. After 4 days the viability of the culture was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The MTT activity of the cultures at the start of the experiments was determined in order to be able to measure a decline in the number of cells (LD50 values). ED50 and LD50 values were determined from at least 4 experiments. The differences in ED50 values and LD50
values determined with and without pre-treatment with CPEC were analyzed using the Student's t-test for paired samples, using MS Excel computer program.

2.5 Differentiation

To assess the degree of differentiation induced by dFdC, cell cultures were examined by microscopy. Cells with neurites 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.

2.5 Fractional effect analysis

In order to determine whether the combination of CPEC and dFdC were synergistic, additive or antagonistic, the combination index (CI), as described by Chou and Talalay[28] was determined for each combination of CPEC and dFdC. The CI values were determined using the CalcuSyn computer program (Biosoft, Ferguson, MO). The qualitative interpretation was made according to Peters et al. [29].

2.6 dCK immuno-blot analysis

The anti-dCK antibody was a kind gift from Prof. dr. I. Talianidis (Institute of Molecular Biology and Biotechnology, Fo.R.T.H., Heraklion, Greece). Cell extracts (50 µg) were fractionated on a 15% (w/v) SDS-polyacrylamide gel and transferred to a nitrocellulose filter. Blocking of the membrane was performed for 16 h with TBS (25 mM Tris, 137 mM NaCl and 2.7 mM KCl, pH 7.4) containing 5% (w/v) nonfat dry milk. Subsequently, the membrane was incubated for 1 h with a 1:5000 dilution of rabbit anti-rat dCK monoclonal antibody in TBS, supplemented with 0.05% (v/v) Tween 20. The membranes were washed three times (5 min each) with TBS containing 0.05% (v/v) Tween 20 and incubated for 45 min with TBS containing 0.05% (v/v) Tween 20, 5% (w/v) nonfat dry milk and a 1:5000 dilution of a goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Dako, Copenhagen, Denmark). After rinsing the membrane three times (5 min each) with TBS containing 0.05% (v/v) Tween 20, detection of dCK was performed with NBT, nitroblue tetrazolium, and BCIP, 5-bromo-4-chloro-5-indolyl-phosphate (Biorad, Veenendaal, The Netherlands).

dCK mRNA expression

RNA was isolated from SK-N-BE(2)c cells by standard procedures using TRIZOL (Life Technologies, Breda, The Netherlands). Subsequently, cDNA was synthesized from 1µg RNA in 20 µl with oligo-dT primer using the First Strand cDNA synthesis Kit for RT-PCR, (Roche, Basel, Switzerland) according to the manufacturer's manual. dCK and glyceraldehyde dehydrogenase (GAPDH) cDNA were amplified with the Light Cycler-DNA Master SYBR Green kit (Roche, Basel, Switzerland) using 1 µl of the cDNA preparation, 5
mM MgCl₂ and 0.5 μM of each primer in the PCR reaction. The primers used to detect dCK (Genbank accession number XM 003471) were: forward: 5'-TGGATTAACCAGTCCAGACG-3', reverse: 5'-CAATGAGTGTAGCTCCACTG-3'. GAPDH (Genbank accession number: XM 006959) was detected using the following primers: forward: 5'-CAACGACCACTTTGTCAAGC-3', reverse: 5'-TGAGCACAGGGTACTTTATTG-3'. Amplification of cDNA was performed in a Light Cycler (Roche) for 35-40 cycles (dCK: 0" 95 °C, 0" 60 °C, 8" 72 °C; GAPDH: 0" 95 °C, 0" 60 °C, 12" 72 °C. The data were analyzed using the Light Cycler Data Analysis software (Roche) with the second derivative maximum module.

2.7 dCK activity assay

For determination of the *in vitro* dCK activity, cells were suspended in 50 mM Tris-HCl pH 7.4, 200 mM NaCl, 2.5 mM PMSF, and 10 mM DTT and disrupted by sonication. Subsequently, the lysates were centrifuged for 15' at 10,000g at 4 °C. The dCK activity was determined in the supernatant with CdA as the substrate. 25 μl supernatant (5-100 μg protein per assay) was added to 25 μl of the reaction mixture, containing (final concentrations): 500 μM CdA, 5 mM ATP, 5 mM MgCl₂, 200 mM NaCl, 10 mM NaF, and 6 mM DTT. After 60 minutes incubation at 37 °C, the reaction was terminated on ice by addition of an equal volume of ice-cold methanol. After centrifugation, CdA and CdAMP (the product) were separated by reversed-phase HPLC using a 250 × 4.6-mm Supelcosil LC-18-S column at a flow rate of 1 ml/min, using a gradient of 50 mM NH₄H₂PO₄ (buffer A) and 50% methanol in 50 mM NH₄H₂PO₄ (buffer B). After elution for 10 minutes with 90% buffer A, a linear gradient to 50% buffer A was applied in 10 minutes, and these conditions were maintained for 10 minutes. Detection of CdA was performed at a wavelength of 265 nm. CdA and CdAMP concentrations were calculated using pure CdA as a standard. The protein pellet obtained after the methanol precipitation was dissolved in 0.2 M NaOH and the protein content was determined as described before [30].

3. Results

3.1 Modulation of dFdC metabolism by CPEC

Pre-treatment of SK-N-BE(2)c cells with 100 nM CPEC for 1-4 days, followed by a 3-hr incubation with 50 nM [³H]dFdC, dramatically increased the anabolism of dFdC as compared to cells treated with dFdC only. The increase in the amount of [³H]dFdC metabolized was dependent on the length of pre-incubation with CPEC. Pre-incubation of SK-N-BE(2)c cells with 100 nM CPEC for 1-3 days increased the anabolism of dFdC 17 to 40-fold (table 1) as compared to cells that had not been pre-incubated with CPEC. After 4 days of pre-incubation with 100 nM CPEC, however, the increase in [³H]dFdC-anabolism was 7-fold as compared to cells that had not been pre-incubated with CPEC.
The \(^{[3]}\text{H}\)dFdCMP pool increased up to 54-fold after 3 days of pre-incubation with 100 nM CPEC. The predominant \(^{[3]}\text{H}\)dFdC metabolite, however, was \(^{[3]}\text{H}\)dFdCTP, with or without pre-incubation with CPEC. A maximum of 39-fold increase in \(^{[3]}\text{H}\)dFdCTP accumulation was observed after 3 days of pre-incubation with 100 nM CPEC. \(^{[3]}\text{H}\)dFdCDP was the smallest pool of the \(^{[3]}\text{H}\)dFdC metabolites, regardless of the presence or absence of CPEC. CPEC, however, did not alter the relative distribution of \(^{[3]}\text{H}\)dFdC over the mono- (5-9%), di- (2-4%), tri-phosphates (58-63%) and DNA-incorporated \(^{[3]}\text{H}\)dFdCTP (25-33%). The increase in incorporation of \(^{[3]}\text{H}\)dFdCTP into DNA (6-44 fold), after pre-incubation with CPEC, paralleled the increase observed for the free \(^{[3]}\text{H}\)dFdC metabolites. After a 4-day pre-incubation with 100 nM CPEC, the phosphorylation of \(^{[3]}\text{H}\)dFdC decreased when compared to 1-3 days of pre-incubation. The observed decrease in phosphorylation of \(^{[3]}\text{H}\)dFdC, coincided with an approximately 50% decrease of the intracellular pools of UTP, ATP and GTP (data not shown).

No deaminated metabolites of dFdC were observed in extracts of non-CPEC and CPEC-treated SK-N-BE(2)c cells. Hence, dCMP deaminase activity was not considered to be significant in this cell line.

The incorporation of \(^{[14]}\text{C}\)TTP into DNA, was inhibited by 50-77% by 100 nM CPEC in SK-N-BE(2)c cells, depending on the length of the incubation and by 70% by 50 nM dFdC after 3 hr of incubation (table 1). DNA synthesis was profoundly inhibited (83-95%) by the combination of 100 nM CPEC and 50 nM dFdC.

<table>
<thead>
<tr>
<th>pre-incubation time (days)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{[3]}\text{H})dFdCMP (fmol/μg protein)</td>
<td>0.025 ± 0.002</td>
<td>0.361 ± 0.002</td>
<td>1.040 ± 0.025</td>
<td>1.320 ± 0.151</td>
<td>0.273 ± 0.034</td>
</tr>
<tr>
<td>(^{[3]}\text{H})dFdCTP (fmol/μg protein)</td>
<td>0.018 ± 0.006</td>
<td>0.167 ± 0.002</td>
<td>0.222 ± 0.060</td>
<td>0.380 ± 0.058</td>
<td>0.067 ± 0.005</td>
</tr>
<tr>
<td>(^{[3]}\text{H})dFdCDP (fmol/μg protein)</td>
<td>0.248 ± 0.031</td>
<td>4.180 ± 0.129</td>
<td>7.180 ± 2.100</td>
<td>9.660 ± 0.857</td>
<td>1.870 ± 0.082</td>
</tr>
<tr>
<td>(^{[3]}\text{H})dFdC in DNA (fmol/μg protein)</td>
<td>0.120 ± 0.006</td>
<td>2.330 ± 0.660</td>
<td>3.280 ± 0.260</td>
<td>5.260 ± 0.490</td>
<td>0.735 ± 0.013</td>
</tr>
<tr>
<td>(^{[14]}\text{C})TTP in DNA (pmol/μg protein)</td>
<td>0.911 ± 0.021</td>
<td>0.454 ± 0.012</td>
<td>0.243 ± 0.016</td>
<td>0.276 ± 0.015</td>
<td>0.211 ± 0.010</td>
</tr>
<tr>
<td>CPEC only</td>
<td>0.276 ± 0.009</td>
<td>0.070 ± 0.004</td>
<td>0.051 ± 0.012</td>
<td>0.155 ± 0.009</td>
<td>0.049 ± 0.003</td>
</tr>
</tbody>
</table>

Table 1: Modulation of dFdC metabolism in SK-N-BE(2)c cells by CPEC. SK-N-BE(2)c cells were incubated with 50 nM \(^{[3]}\text{H}\)dFdC with or without prior exposure to 100 nM CPEC for 1-4 days. \(^{[3]}\text{H}\)dFdC metabolites and DNA synthesis were measured as described in materials and methods. The results shown are the mean of three experiments ± SD.
3.2 dFdC cytotoxicity and its modulation by CPEC

ED$_{50}$ values for dFdC were determined in a panel of neuroblastoma cell lines, consisting of MYCN-single copy and MYCN-amplified neuroblastoma cell lines. A total of 15 cell lines were tested and in 14 cell lines an ED$_{50}$ value for dFdC could be determined (table 2). ED$_{50}$ values ranged from 12 nM to 175 nM, with no apparent difference between MYCN-single copy and MYCN-amplified cell lines. Only in the LAN6 cell line, which is a MYCN-single copy cell line, no ED$_{50}$ could be determined. When the neuroblastoma cell lines were pre-incubated with 100 nM CPEC for 24 hr, followed by a 3-hr incubation with dFdC and subsequent washout of the drugs, the ED$_{50}$ for dFdC was significantly lowered in 13 cell lines. The LAN6 cell line remained resistant to dFdC, while the SJNB8 cell line became less sensitive towards dFdC. In the remaining cell lines, ED$_{50}$ values for dFdC were lowered by 21-80%, again with no apparent difference between MYCN-single copy and MYCN-amplified cell lines.

LD$_{500}$ values were determined in the same panel of neuroblastoma cell lines (table 2). A striking difference became clear between MYCN-single copy and MYCN-amplified cell lines. LD$_{50}$ values could not be established in all MYCN-single copy cell lines, with or without pre-incubation with 100 nM CPEC. In 6 out of 9 MYCN-amplified cell lines, LD$_{50}$ values could be determined without pre-incubation with 100 nM CPEC. LD$_{50}$ values varied between 16 and 202 nM dFdC. After pre-incubation for 24 hr with 100 nM CPEC, the LD$_{50}$ values were lowered by 8-84%. The SK-N-BE(2) cell line, in which no LD$_{50}$ value could be determined without pre-incubation with 100 nM CPEC, was sensitized towards dFdC by CPEC. No LD$_{50}$ values could be established in the SJNB6 and SJNB8 cell lines. Further examination of the cell lines revealed that all MYCN-single copy cell lines differentiated after treatment with dFdC. Of the MYCN-amplified cell lines differentiation was observed in SK-N-BE(2) cells, which were sensitized by CPEC, the dFdC-sensitive cell line SJNB10 and the dFdC-resistant cell line SJNB8.

3.3 The time-dependence of modulation of dFdC cytotoxicity by CPEC

The modulating effect of CPEC on the efficacy of dFdC proved to be time-dependent. LD$_{50}$ and ED$_{50}$ values for dFdC were determined in SK-N-BE(2)c cells that had been pre-incubated with 100nM CPEC for 1 to 3 days. The values obtained after 1, 2 and 3 days of pre-incubation were: 1 day pre-incubation: ED$_{50}$ = 34 ± 4 nM (p<0.001), LD$_{50}$ = 725 ± 185 nM (p<0.001); 2 days pre-incubation: ED$_{50}$ = 20 ± 4 nM (p<0.001), LD$_{50}$ = 51 ± 24 nM (p<0.001); 3 days pre-incubation: ED$_{50}$ = 84 ± 9 nM (p<0.001), LD$_{50}$ > 500 nM.

Fractional effect analysis of these data revealed a synergistic interaction between dFdC and CPEC after 1 day of pre-incubation with CPEC (figure 1). After pre-incubation with 100 nM CPEC for 2 or 3 days, however, the CI versus dFdC concentration plots indicates synergistic interaction between CPEC and dFdC for low concentrations of dFdC, but for higher concentrations of dFdC the calculated CI indicates antagonism.
Table 2: Anti-tumor effects of dFdC towards a panel of neuroblastoma cell lines and modulation by CPEC.

ED_{50} and LD_{50} values for dFdC were determined four days after 3 hr of exposure to dFdC, with or without prior exposure to 100 nM CPEC for 24 hr. ED_{50} values determined after exposure to CPEC are corrected for CPEC toxicity. Differentiation was appreciated by microscopic examination of dFdC-only treated cells at the end of the incubation period. The values shown are the mean of 4-6 experiments ± SD. * p < 0.05, ** p < 0.01. - No differentiated cells observed, + 20-40 % differentiation, ++ 40-60% differentiation, +++ 75-100 % differentiation. n.a. not applicable.

3.4 The effect of MYCN expression of dFdC sensitivity

ED_{50} and LD_{50} values for dFdC were determined in the Shep21N cell line, in which MYCN expression is regulated via a tetracycline sensitive promoter. As a control, the mock-transfected parent cell line Shep2 was used. No ED_{50} and LD_{50} values could be established in Shep21N cells with and without expression of MYCN, after a 3 hr incubation with dFdC. After 24 hr pre-incubation with CPEC, the ED_{50} value for dFdC in MYCN expressing Shep21N cells was 44 ± 11 nM. When MYCN was not expressed, CPEC did not sensitize Shep21N cells for dFdC. Shep2 cells had ED_{50} values of 202 ± 49 nM and 184 ± 68 nM dFdC, with and without pre-incubation with CPEC, respectively. No LD_{50} values could be determined in Shep2 cells. The presence of tetracycline did not affect dFdC toxicity in Shep2 cells. Both Shep21N and Shep2 cells readily differentiated to a neuronal phenotype upon incubation with dFdC, CPEC, or the combination thereof.

Because 4 days after a 3 hr exposure to dFdC no ED_{50} and LD_{50} values were established in Shep21N cells, Shep21N cells were incubated for 3 days with dFdC concentrations between 2 and 250 nM. The ED_{50} values were 25 ± 1 nM without tetracycline (MycN on) and 14 ± 0 nM
with tetracycline (Myc off). In the case that MycN was expressed, the LD$_{50}$ value was $63 \pm 6$ nM dFdC, while without MycN expression the ED$_{50}$ value was greater than 250 nM dFdC.

![Graph showing combination index for dFdC and CPEC](image)

**Figure 1:** Combination index for 7.8-500 nM dFdC and 100 nM CPEC after 1-3 days pre-treatment with CPEC. Solid diamonds: 1 day pre-treatment with CPEC, solid squares: 2 days pre-treatment with CPEC, solid triangles: 3 days pre-treatment with CPEC.

### 3.5 Regulation of dCK expression and activity by CPEC

When SK-N-BE(2)c cells were incubated with 100 nM CPEC for 1-4 days, the amount of dCK protein increased in a time-dependent fashion (figure 2A). This increase in dCK protein was paralleled by a 1.8- to 2.5-fold increase in dCK-mRNA expression (figure 2B). Furthermore, the dCK activity increased 2 to 4-fold after incubation with 100 nM CPEC, when compared to non-CPEC treated cells (figure 2C).

### 3.6 dCK activity in neuroblastoma cell lines

The dCK activity was measured in the panel of cell lines (table 3). There appeared to be no correlation between dCK activity and the ED$_{50}$ values for dFdC. In fact, the LAN6 cell line, which is resistant to dFdC, had the highest dCK activity. However, when the dCK activities of the dFdC-sensitive cell lines were compared, a marked difference between MYCN-single copy and MYCN-amplified cell lines became clear. While the mean dCK activity of dFdC-sensitive MYCN-single copy cell lines was $19.0 \pm 3.76$ pmol/μg protein/hr, the mean dCK activity of MYCN-amplified cell lines was $29.8 \pm 5.4$ pmol/μg protein/hr ($p < 0.01$). The dCK activity in MYCN expressing Shep 21N cells was slightly higher (12.5 %) than in Shep 21N cells that did not express MYCN.
Figure 2: The effect of CPEC on dCK expression and activity. SK-N-BE(2)c cells were incubated with 100 nM CPEC for 0-4 days. At the indicated time-points the dCK protein content of the cell cultures was estimated by Western blot analysis (panel A). The expression of dCK-mRNA is shown in panel B. The results shown are the mean of 4 experiments ± SD. White bars: uncorrected relative dCK-mRNA expression, black bars: relative dCK-mRNA expression corrected for GAPDH expression. ** p < 0.01. In panel C, the relative dCK activity of SK-N-BE(2)c cells incubated for 0-4 days with 100 nM CPEC is shown. 100 % dCK activity = 41.9 ± 1.5 nmol CdAMP/µg protein/ hr. The results shown are the mean of three determinations ± SD.
### Table 3: dCK activity of neuroblastoma cell lines. The results shown are the mean of three determinations ± SD.

<table>
<thead>
<tr>
<th>MYCN-single copy</th>
<th>dCK activity (pmol/μg protein/hr)</th>
<th>MYCN-amplified</th>
<th>dCK activity (pmol/μg protein/hr)</th>
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<tr>
<td>cell line</td>
<td></td>
<td>cell line</td>
<td></td>
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<tr>
<td>SK-N-FI</td>
<td>18.1 ± 0.4</td>
<td>KCNR</td>
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<tr>
<td>SK-N-SH</td>
<td>16.3 ± 0.4</td>
<td>NMB</td>
<td>27.7 ± 1.4</td>
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<tr>
<td>SK-N-AS</td>
<td>23.1 ± 1.3</td>
<td>SK-N-BE(2)</td>
<td>23.8 ± 0.8</td>
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<tr>
<td>GI-MEN</td>
<td>14.8 ± 4.3</td>
<td>SK-N-BE(2)c</td>
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<tr>
<td>LAN6</td>
<td>42.0 ± 1.3</td>
<td>SJNB6</td>
<td>32.6 ± 1.1</td>
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<td>SJNB12</td>
<td>24.1 ± 1.5</td>
<td>AMC106</td>
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<td>SJNB8</td>
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<td></td>
<td></td>
<td>SJNB10</td>
<td>34.8 ± 3.6</td>
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<td>Shep21N (NMYC off)</td>
<td>16.7 ± 1.6</td>
<td>Shep21N (NMYC on)</td>
<td>18.8 ± 0.9</td>
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</table>

4. Discussion

Gemcitabine has been shown to be a potent agent for the treatment of various solid tumors such as non-small cell lung cancer and pancreatic cancer. In this paper, we demonstrate that dFdC also has profound cytotoxic and differentiation inducing effects in neuroblastoma in vitro. Both MYCN-amplified and MYCN-single copy cell lines were highly sensitive to dFdC. However, the effect of dFdC was significantly different between the two types of cell lines. While, after incubation with dFdC, irreversible differentiation was induced in 6 out of 6 MYCN-single copy cell lines, cell death was induced in 6 out of 9 MYCN-amplified cell lines. In MYCN expressing Shep21N cells, incubation with dFdC was lethal, while no cytotoxicity was observed in Shep21N that did not express MYCN, indicating that dFdC induces cell death in neuroblastoma cells that express MYCN. This may be explained by the fact that MYCN sensitizes neuroblastoma cells for drug induced apoptosis[31]. The induction of differentiation in MYCN-single copy cell lines cannot be explained by absence of MYCN alone, as dFdC also induced differentiation in some MYCN-amplified cell lines and in MYCN-expressing Shep21N cells. Shep21N was created from a MYCN-single copy cell line[5] and, therefore, behaved both as a MYCN-single copy cell line in the sense that differentiation was induced by dFdC, as well as a MYCN-amplified cell line in the sense that cell death was induced by dFdC. In general, MYCN-single copy neuroblastomas are relatively more differentiated than MYCN-amplified neuroblastomas. It may be that interference in the nucleotide metabolism and DNA synthesis triggers the progression of differentiation in these relatively differentiated neuroblastoma cells. The fact that the dCK activity in MYCN-amplified neuroblastoma cell lines was 60% higher than in MYCN-single copy cell lines may also, in part, explain the observed cytotoxicity. An increased activity is associated with an
increased phosphorylation, and thus increased cytotoxicity of dFdC. Hence, dFdC is more toxic to MYCN-amplified neuroblastoma cell lines than to MYCN-single copy cell lines. Also in MYCN expressing Shep21N cells, the dCK activity was higher than in Shep21N cells that did not express MYCN. Expression of MYCN is thought to increase the expression of genes involved in protein synthesis [3]. Thus the increased expression of dCK in MYCN-amplified neuroblastoma cells is in line with the increased protein synthesis.

The ED₅₀ value for dFdC was significantly decreased in 13 out of 15 cell lines, after pre-incubation with CPEC. This increased sensitivity was caused by an increase in the anabolism of dFdC and concomitant incorporation into the DNA, as was demonstrated in SK-N-BE(2)c cells. A dramatic increase in the uptake and phosphorylation of dFdC was observed in SK-N-BE(2)c cells after pre-incubation with 100 nM CPEC for 1-3 days. However, after 4 days, this effect declined, and was paralleled by depletion of the UTP and ATP pools. Because UTP and ATP are the phosphate donors utilized by dCK[32], a decrease in the UTP and ATP pools may cause a decrease of the in situ activity of dCK. This observation is supported by fractional effect analysis, which indicated a tendency towards antagonism for prolonged exposure to CPEC or dFdC concentrations greater than 200 nM. Apparently, high concentrations of dFdC or prolonged exposure to CPEC result in a maximum effect that can be only marginally increased. The effects of low concentrations of dFdC or short incubations with CPEC, however, are rather small and can strongly be increased, hence the observed synergism under these conditions.

During incubation with CPEC, the mRNA expression of dCK, dCK protein level and the specific dCK activity increased in SK-N-BE(2)c cells. dCK activity is regulated via feedback inhibition by dCTP [33], while expression of dCK is increased in response to inhibition of DNA synthesis and DNA damage [34-36]. By depleting the CTP pool, CPEC also depleted the dCTP pool and, hence, inhibited DNA synthesis. These combined effects may very well have caused the observed increase in dCK mRNA and protein expression and activity.

With or without prior exposure to CPEC, dFdCTP and dFdC incorporated into DNA were the major metabolites of dFdC, with an approximate ratio of 2:1. This is in accordance with the results of Heinemann and colleagues obtained in Chinese hamster ovary cells [10]. The fact that no relative accumulation of dFdCMP was observed indicated that, when the feedback inhibition of dCK is relieved, the rate-limiting step in the anabolism of dFdC was its incorporation into DNA. In this respect, dFdC differs from AraC, of which nucleoside monophosphate kinase activity is the rate-limiting enzyme in its anabolism, once the feedback inhibition of dCK is relieved [37]. This is in accordance with the observation of van Rompay and colleagues who showed that dFdCMP is a better substrate for human UMP/CMP kinase than AraCMP [38].

Despite the fact that the LAN6 cell line had a very high dCK activity, it was resistant to dFdC. The mechanism of resistance of LAN6 cells to dFdC remains to be elucidated. Possible causes could be high (d)Cyd deaminase activity or deletion of nucleoside transporters for which dFdC is a substrate.
In conclusion, dFdC is a highly potent drug against both MYCN-amplified and MYCN-single-copy neuroblastoma cells. Both the cell-death and differentiation inducing properties of dFdC are of great interest in the treatment of neuroblastoma. We feel that dFdC, deserves further development towards clinical application in the treatment of patients suffering from neuroblastoma.

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