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ORIGINAL ARTICLE

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Cyclopentenyl cytosine-induced activation of deoxycytidine kinase increases gemcitabine anabolism and cytotoxicity in neuroblastoma

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Abstract The effect of the CTP synthetase inhibitor cyclopentenyl cytosine (CPEC) on the metabolism and cytotoxicity of 2′,2′-difluorodeoxycytidine (dFdC, gemcitabine) and the expression and activity of deoxycytidine kinase (dCK) was studied in human neuroblastoma cell lines. The cytotoxicity of dFdC and CPEC was studied in a panel of *MYCN*-amplified and *MYCN*-single-copy neuroblastoma cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-assays. dFdC-metabolism was studied in SK-N-BE(2)c cells using [^{3}H]-radiolabeled dFdC. dCK activity and expression were measured using enzyme assays, immunoblot and quantitative PCR, respectively. Both *MYCN*-amplified and *MYCN*-single-copy neuroblastoma cell lines were highly sensitive to dFdC, with concentration of the drug resulting in 50% effect when compared to untreated controls (ED_{50}) values in the nanomolar range after a 3-h exposure to dFdC. There was no correlation of the observed ED_{50} with the dCK activity. Treatment with dFdC induced cell death in *MYCN*-amplified cells whereas *MYCN*-single-copy-cell lines underwent neuronal differentiation. Pre-incubation with CPEC significantly increased dFdC-cytotoxicity from 1.3 to 5.3-fold in 13 out of 15 cell lines. [^{3}H]dFdC-anabolism increased 6–44 fold in SK-N-BE(2)c cells after incubation with CPEC and was paralleled by a significant increase in expression and activity of dCK. In conclusion, the combination of dFdC and CPEC is highly toxic to neuroblastoma in vitro.

Keywords Neuroblastoma · MYCN · Deoxycytidine kinase · Cyclopentenyl cytosine · Gemcitabine · CTP synthetase · Combination therapy

Abbreviations dFdC: Gemcitabine, 2′,2′-difluorodeoxycytidine · dFdCMP: 2′,2′-difluorodeoxycytidine-5′-monophosphate · dFdCDP: 2′,2′-difluorodeoxycytidine-5′-diphosphate · dFdCTP: 2′,2′-difluorodeoxycytidine-5′-triphosphate · CdA: 2-chloro-2′-deoxyadenosine · CdAMP: 2-chloro-2′-deoxyadenosine 5′-monophosphate · CI: Combination index · CPEC: Cyclopentenyl cytosine · CPECTP: Cyclopentenyl cytosine-5′-triphosphate · dCK: Deoxycytidine kinase · ED_{50}: Concentration of drug resulting in 50% effect when compared to untreated controls · MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide · Thd: Thymidine

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Introduction

Neuroblastoma is the most common extra cranial solid malignancy in children and is responsible for 15% of all childhood cancer deaths [13]. Despite extensive efforts to improve the treatment of patients suffering from neuroblastoma, the prognosis for most patients remains poor. Therefore, new and effective strategies need to be developed and evaluated.

Poor prognosis is associated with amplification of the *MYCN*-oncogene and is found in approximately 25% of primary, predominantly metastasized, neuroblastomas [5]. Increased expression of *MYCN* increases the transcription of multiple genes that function in ribosome

biogenesis and protein synthesis [4] and enhances proliferation and tumorigenicity [22, 24].

Gemcitabine (dFdC) is an analog of deoxycytidine and has proven anti-tumor activity in vivo against pancreatic cancer [21] and non-small-cell lung cancer [23]. 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) [17]. 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) [15] 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 phosphorylation of dFdC by dCK [15]. dFdC-triphosphate is an inhibitor of CTP synthetase [16], DNA synthesis, DNA repair and is incorporated into the DNA, causing DNA damage [26]. 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 [18].

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 [20, 31]. CPEC possesses anti-tumor activity against leukemia (in vitro and in vivo) [11, 20, 30] and in vitro against several solid tumors, including neuroblastoma [1, 28]. We have shown previously that CPEC causes a retardation in the S-phase of the cell cycle in neuroblastoma cells [1]. The combination of CPEC with an S-phase-active deoxycytidine analog, like dFdC, is an attractive strategy to explore. The interaction between CPEC- and dFdC-metabolism is depicted in Fig. 1. Thus far, 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 of dFdC and thereby an increase of cytotoxicity of dFdC is demonstrated.

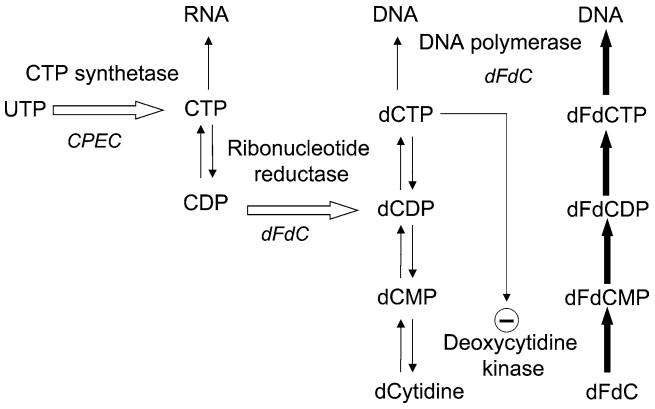

Fig. 1 The interaction between cyclopentenyl cytosine (CPEC) and dFdC metabolism. *Bold black arrows* indicate reactions that are stimulated by incubation with CPEC. *Large white arrows* indicate target enzymes of CPEC and dFdC, respectively, which are indicated with italics

Materials and methods

Chemicals

Cyclopentenyl cytosine (NSC 375575) was obtained from the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD, USA). dFdC was obtained from Eli Lilly (Nieuwegein, The Netherlands). [^{3}H]-2′,2′-difluorodeoxycytidine 14 Ci/mmol), was purchased from Moravek Biochemicals (Brea, CA), [^{14}C]Thymidine (Thd) (2.04 GBq/mmol) was obtained from Amersham International (Buckinghamshire, UK). 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 from BioWhittaker Europe (Verviers, Belgium). L-glutamine and gentamycin were obtained from Gibco BRL (Paisley, Scotland). All other chemicals were of analytical grade.

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 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% CO_2. Shep2 and Shep21N cell lines were maintained in RPMI 1640 medium with the same supplements plus 10 mM HEPES and 0.15% (w/v) $NaHCO_3$. Tetracycline was used at a concentration of 10 ng/ml to inhibit *MYCN* expression. It was checked in Shep2 cells that tetracycline did not influence sensitivity in CPEC or dFdC. Cell cultures were consistently free of mycoplasma (tested with Mycoplasma PCR ELISA, Boehringer Mannheim).

Extraction and analysis of radiolabeled nucleotides

Cells were seeded in six well plates at a density of 0.5 $\times 10^6$ cells per well and allowed to adhere overnight. The

cells were pre-incubated with CPEC for 1–4 days, after which the medium containing CPEC was removed and replaced by medium containing 50 nM [^{3}H]dFdC and 250 nM [^{14}C]Thd. Changing the medium removed any floating non-viable cells, leaving only adherent viable cells [1]. After 3 h of incubation, the cells were extracted and analyzed as described previously [2]. Briefly, after incubation with radiolabeled precursors, the cells were extracted with perchloric acid (0.4 M) on ice for 10 min and then centrifuged for 5 min at 4°C at maximum velocity in a tabletop centrifuge. The supernatant was neutralized using K_2CO_3 and was then centrifuged again. The resulting supernatant was used for the analysis of free nucleotides. The pellet obtained after the perchloric acid precipitation was taken up in a 0.2 M NaOH solution and precipitated again with perchloric acid (1.2 M). After centrifugation, the protein- and DNA-containing pellet was dissolved in NaOH and radioactivity was measured on a ß-counter.

Chemo-sensitivity assay

Cells were plated in 24-wells plates at a density of 20–50 $\times$ 10^3 cells per well and allowed to adhere overnight. Subsequently, the medium was exchanged with normal medium or medium containing 100 nM CPEC. After 24 h, experiments were started by adding dFdC to a final concentration of 7.8–1000 nM. After 3 h exposure, the medium was changed for normal medium. The viability of the culture was measured 4 days after exposure using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)-assays. Concentration of drug resulting in 50% effect when compared to untreated controls (ED_{50}) values were deduced from dose-effect curves. LD_{50} was defined as the dose causing a 50% decrease in MTT-activity after exposure to the cytotoxic compounds relative to the MTT-activity measured prior to the exposure to the cytotoxic compounds. ED_{50} and LD_{50} values were determined from at least four experiments. The statistical significance was determined using the Student's t-test for paired samples, and using the MS Excel computer program.

Differentiation

Differentiation induced by dFdC and/or CPEC was assessed microscopically. Cells with neurites with a length of approximately twice the diameter of the cell body as well as cells that were connected to one another by means of neurites were scored as being differentiated.

Fractional effect analysis

The mode of interaction (synergy, antagonism, and additivity) was determined by the combination index (CI), as described by Chou and Talalay [8] and was calculated for each combination of CPEC and dFdC using the Calcusyn computer program (Biosoft, Ferguson, MO). The qualitative interpretation was made according to Peters et al. [25].

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) non-fat 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) non-fat 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 1 µg 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 glyceraldehydedehydrogenase (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 $MgCl_2$ 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, in which 0″ means that upon reaching the programmed temperature, the apparatus was programmed to proceed immediately to the next programmed temperature). The data were

analyzed using the Light Cycler Data Analysis software (Roche) with the second derivative maximum module.

dCK activity assay

The dCK activity of the neuroblastoma cell lines used was determined in an reaction mixture containing 500 μM 2-chloro-2′-deoxyadenosine (CdA), 5 mM ATP, 5 mM $MgCl_2$, 200 mM NaCl, 10 mM NaF, and 6 mM DTT . Separation of CdA and 2-chloro-2′-deoxyadenosine 5′-monophosphate (CdAMP) was performed using reversed-phase HPLC [3].

Results

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 [^{3}H]dFdC, strongly increased the anabolism of dFdC in a time-dependent manner. Pre-incubation with 100 nM CPEC for 1–3 days increased dFdC-anabolism 17 to 40-fold, while 4 days of pre-incubation with CPEC increased dFdC metabolism seven fold (Fig. 2).

The 2′,2′-difluorodeoxycytidine-5′-monophosphate ([^{3}H]dFdCMP) pool increased 54-fold after 3 days of pre-incubation with 100 nM CPEC. However, the predominant [^{3}H]dFdC metabolite was 2′, 2′-difluorodeoxycytidine-5′-triphosphate ([^{3}H]dFdCTP). A 39-fold increase in [^{3}H]dFdCTP accumulation was observed after 3 days of pre-incubation with 100 nM CPEC. 2′, 2′-difluorodeoxycytidine-5′-diphosphate ([^{3}H]dFdCDP) was the smallest pool of the [^{3}H]dFdC metabolites, regardless of the presence or absence of CPEC. CPEC did not alter the relative distribution of [^{3}H]dFdC over the mono- (5–9%), di- (2–4%), tri-phosphates (58–63%) and DNA-incorporated [^{3}H]dFdCTP (25–33%). The increased incorporation of [^{3}H]dFdCTP into DNA (6–44 fold), after pre-incubation with CPEC, paralleled the increase in [^{3}H]dFdC nucleotides. After 4 days pre-incubation with 100 nM CPEC, phosphorylation of [^{3}H]dFdC decreased when compared to 1–3 days of pre-incubation. The observed decrease in phosphorylation of [^{3}H]dFdC, coincided with a decrease of approximately 50% of the intracellular UTP-, ATP- and GTP-pools (data not shown).

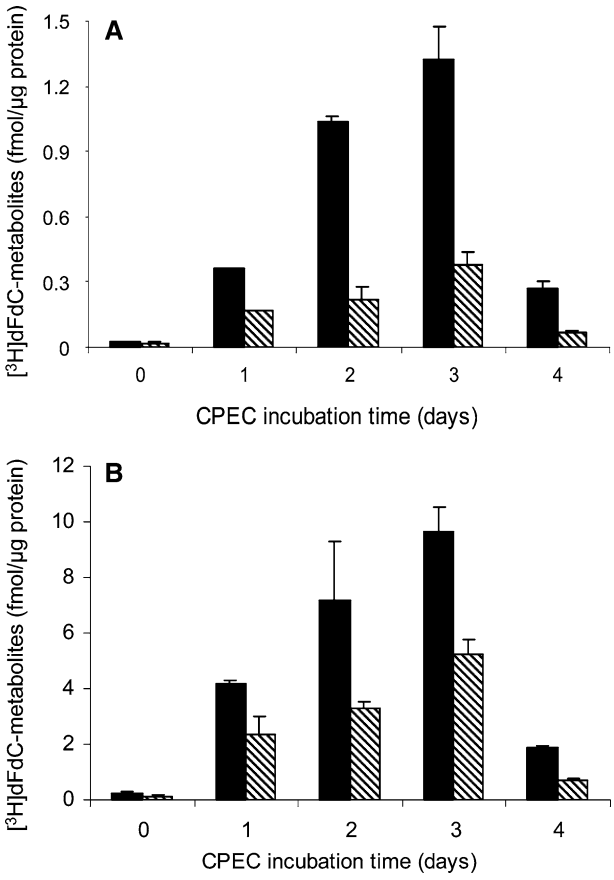

Fig. 2 The effect of pre-incubation with CPEC on the metabolism of dFdC. SK-N-BE(2)c cells were incubated with 100 nM CPEC and, subsequently at the indicated time-points incubated with [^{3}H]dFdC as described in materials and methods. *panel A*: intracellular 2′,2′-difluorodeoxycytidine-5′-monophosphate ([^{3}H]dFdCMP) (*black bars*) and 2′,2′-difluorodeoxycytidine-5′-diphosphate ([^{3}H]dFdCDP) pools (*hatched bars*). *panel B*: intracellular 2′,2′-difluorodeoxycytidine-5′-triphosphate ([^{3}H]dFdCTP) pools (*black bars*) and [^{3}H]dFdC incorporated in DNA (*hatched bars*). The results show the mean of three experiments ± SD

No deaminated metabolites of dFdC were observed in the 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}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 h of incubation (Table 1).

Table 1 The effect of 100 nM cyclopentenyl cytosine (CPEC) and 50 nM [^{3}H]dFdC on DNA synthesis as measured by [^{14}C]TTP incorporation. The values shown are the mean of three experiments ± SD

Pre-incubation time with 100 nM CPEC (days)	No pre-incubation	1	2	3	4
[^{14}C]TTP in DNA (pmol/μg protein) CPEC only	0.911 ± 0.021 100%	0.454 ± 0.012 50%	0.243 ± 0.016 27%	0.276 ± 0.015 30%	0.211 ± 0.010 23%
[^{14}C]TTP in DNA (pmol/μg protein) CPEC + dFdC	0.276 ± 0.009 30%	0.070 ± 0.004 8%	0.051 ± 0.012 6%	0.155 ± 0.009 17%	0.049 ± 0.003 5%

DNA synthesis was profoundly inhibited (83–95%) by the combination of 100 nM CPEC and 50 nM dFdC.

dFdC cytotoxicity and its modulation by CPEC

Concentration of drug resulting in 50% effect when compared to untreated controls values for dFdC were determined in a panel of neuroblastoma cell lines, consisting of *MYCN*-single copy and *MYCN*-amplified neuroblastoma cell lines. In 14 out of 15 cell lines ED_{50} values could be determined (Table 2). ED_{50} values ranged from 12 to 175 nM, with no apparent difference between *MYCN*-single copy and *MYCN*-amplified cell lines. After a 24-h pre-incubation with 100 nM CPEC, the ED_{50} values were significantly lowered by 21–80% in 13 cell lines with no apparent difference between *MYCN*-single copy and *MYCN*-amplified cell lines.

LD_{50} values were determined in the same panel of neuroblastoma cell lines (Table 2). The LD_{50} values of *MYCN*-amplified cell lines varied between 16 and 202 nM dFdC. After pre-incubation for 24 h 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. In none of the *MYCN*-single-copy cell lines LD_{50} values could be established, with or without pre-incubation with CPEC. All *MYCN*-single copy cell lines differentiated after treatment with dFdC. Differentiation was also observed in the *MYCN*-amplified cell lines SK-N-BE(2), SJNB10 and SJNB8.

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 100 nM CPEC for 1–3 days and are shown in Table 3. Fractional effect analysis of these data revealed a synergistic interaction between dFdC and CPEC after 1 day of pre-incubation with CPEC (Fig. 3). 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.

The effect of *MYCN* expression on dFdC sensitivity

The effect of *MYCN* on the cytotoxicity of dFdC was investigated in the Shep 21N cell line, in which the expression of *MYCN* is regulated by a tetracycline dependent promotor. Shep21N cells were incubated

Table 2 Anti-tumor effects of dFdC towards a panel of neuroblastoma cell lines and modulation by a 24-hr pre-incubation with 100 nM CPEC

	Cell line	Concentration of drug resulting in 50% effect when compared to untreated controls (ED_{50}) (dFdC) (nM)	ED_{50}(CPEC + dFdC) (nM)	ED_{50}(dFdC)/ ED_{50}(CPEC + dFdC)	LD_{50} (dFdC)(nM)	LD_{50} (CPEC + dFdC)(nM)	LD_{50} (dFdC)/ LD_{50} (CPEC + dFdC)	Differentiation
MYCN-single copy	SK-N-FI	174 ± 55	111 ± 37*	1.56	> 1,000	> 500	not applicable	75–100%
	SK-N-SH	148 ± 37	72 ± 13**	2.04	> 1,000	> 500	not applicable	75–100%
	SK-N-AS	52 ± 7	39 ± 3**	1.33	> 1,000	> 500	not applicable	40–60%
	GI-M-EN	51 ± 15	28 ± 5**	1.82	> 1,000	> 500	not applicable	75–100%
	LAN6	> 1,000	> 500	not applicable	> 1,000	> 500	not applicable	20–40%
	SJNB12	105 ± 23	38 ± 1**	2.78	> 1,000	> 500	not applicable	40–60%
MYCN-amplified	KCNR	58 ± 12	31 ± 10**	1.89	123 ± 28	77 ± 50	1.59	not observed
	NMB	12 ± 1	8.5 ± 0.5**	1.41	26 ± 3	16 ± 2**	1.61	not observed
	SK-N-BE(2)	120 ± 19	38 ± 5**	3.13	> 1,000	192 ± 30	sensitization	40–60%
	SK-N-BE(2)c	175 ± 20	34 ± 4**	5.26	725 ± 185	160 ± 21**	4.55	not observed
	SJNB6	136 ± 24	106 ± 24**	1.28	> 1,000	> 500	not applicable	not observed
	AMC106	170 ± 42	90 ± 12**	1.89	248 ± 58	201 ± 51	1.23	not observed
	SJNB8	38 ± 4	59 ± 16**	0.65	> 1,000	> 500	not applicable	20–40%
	N206	24 ± 2	12 ± 3**	2.00	53 ± 7	8 ± 2**	6.67	not observed
	SJNB10	57 ± 14	17 ± 8**	3.33	253 ± 65	197 ± 19	1.09	40–60%

ED_{50} and LD_{50} were determined 4 days after a 3 hr exposure to dFdC, with or without prior exposure to CPEC. ED_{50} values for dFdC obtained after exposure to CPEC are corrected for CPEC-toxicity. Differentiation was estimated by microscopic examination. The values shown are the mean of 4–6 experiments (* $p < 0.05$, ** $p < 0.01$).

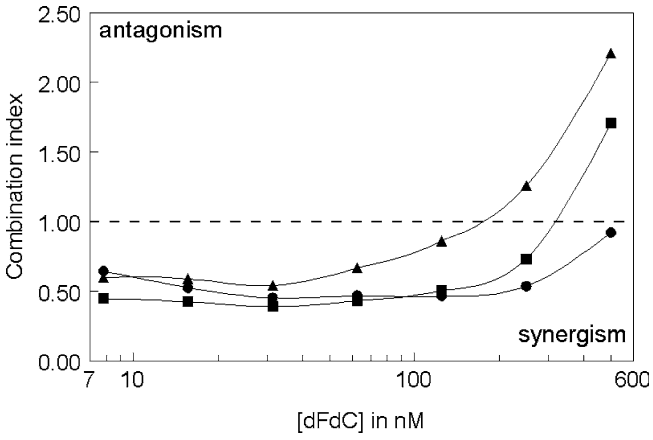

Fig. 3 Combination index for 7.8–500 nM dFdC and 100 nM CPEC after 1–3 days pre-treatment of SK-N-BE(2)c cells with CPEC. *Solid circles*: 1 day pre-treatment with CPEC, *solid squares*: 2 days pre-treatment with CPEC, *solid triangles*: 3 days pre-treatment with CPEC

continuously for 3 days with dFdC concentrations between 2 and 250 nM. The ED_{50} values were 25 ± 1 nM without tetracycline (*MYCN* is expressed) and 14 ± 0 nM with tetracycline (*MYCN* is not expressed). In the case that *MYCN* was expressed, the LD_{50} value was 63 ± 6 nM dFdC, while without *MYCN* expression no LD_{50} value could be established (> 250 nM dFdC). With or without the expression of *MYCN*, Shep 21N cells underwent neuronal differentiation upon exposure to dFdC (data not shown).

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 (Fig. 4A). This increase in dCK protein was paralleled by a 1.8 to 2.5-fold increase in dCK-mRNA expression (Fig. 4B). Furthermore, the dCK activity increased two to four fold after incubation with 100 nM CPEC, when compared to non-CPEC treated cells (Fig. 4C).

dCK activity in neuroblastoma cell lines

The dCK activity was measured in the panel of cell lines (Table 4). There appeared to be no correlation between dCK activity in the untreated cells and the ED_{50} values for dFdC. In fact, the LAN6 cell line, which is resistant to dFdC, had the highest dCK activity. However, the dCK activities of the dFdC-sensitive cell lines differed markedly between *MYCN*-single copy and *MYCN*-amplified cell lines. While the mean dCK activity of dFdC-sensitive *MYCN*-single copy cell lines was 19.0 ± 3.76 pmol/μg protein/h, the mean dCK activity of

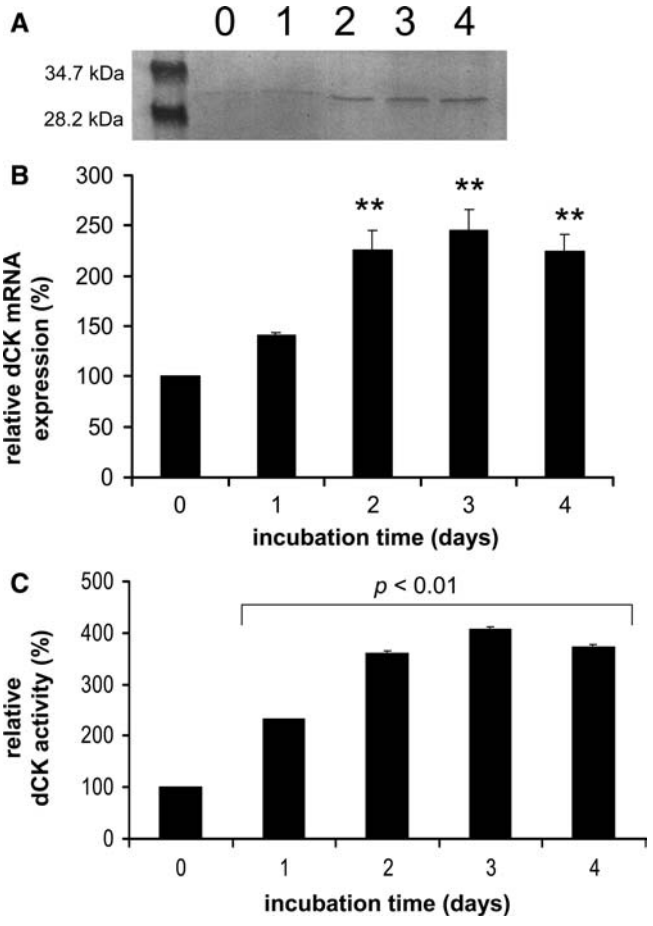

Fig. 4 The effect of CPEC on Deoxycytidine kinase (dCK) expression and activity. SK-N-BE(2)c cells were incubated with 100 nM CPEC for 0–4 days. At the indicated time-points the dCK protein content of the cell cultures was estimated by Western blot analysis (panel A). The expression of dCK-mRNA was corrected for GAPDH expression and is shown in panel B. The results shown are the mean of four experiments ± SD. ** $p < 0.01$. 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 pmol 2-chloro-2′-deoxyadenosine 5′-monophosphate (CdAMP)/μg protein/h. The results shown are the mean of three determinations ± SD

MYCN-amplified cell lines was 29.8 ± 5.4 pmol/μg protein/h ($p < 0.01$).

Discussion

dFdC is an effective 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 has profound cytotoxic and differentiation inducing effects in both *MYCN*-amplified and *MYCN*-single-copy neuroblastoma cell lines *in vitro*. These effects were observed after very short exposures (3 h) to dFdC and at very low concentrations (nanomolar range).

Table 3 Time-dependence of modulation of dFdC cytotoxicity by CPEC in SK-N-BE(2)c cells

Pre-incubation time with 100 nM CPEC (days)	No pre-incubation	1	2	3
ED_{50}(nM)	175 ± 20	34 ± 4*	20 ± 4*	51 ± 24*
LD_{50} (nM)	725 ± 185	160 ± 21*	51 ± 24*	> 500

*$p < 0.001$. The results shown are the mean of 4–6 experiments ± SD

The cytotoxic effects observed appeared to be uncorrelated to the dCK activity. However, the cytotoxic effect of dFdC is not only dependent on dCK activity, but also on the accumulation of dFdC-metabolites beyond dFdCMP, the deactivation of dFdC-metabolites and on the expression level of the target enzymes.

The effect of dFdC was significantly different between *MYCN*-amplified and *MYCN*-single copy cell lines. While the major effect in *MYCN*-single copy cell lines appeared to be cytostasis associated with the induction of differentiation, *MYCN*-amplified cell lines underwent cell death. This phenomenon was also observed in Shep21N cells in which cell-death was the predominant effect when *MYCN* was expressed, but when *MYCN* was not expressed, induction of differentiation was the predominant effect observed. This may be explained by the fact that *MYCN* sensitizes neuroblastoma cells for drug induced apoptosis [12]. The induction of differentiation in *MYCN*-single copy cell lines cannot be explained by the absence of *MYCN* alone, as dFdC also induced differentiation in some *MYCN*-amplified cell lines. It has recently been demonstrated that *MYCN*-overexpressing neuroblastoma cells retain their capacity to undergo differentiation [10]. In general, *MYCN*-single copy neuroblastomas have a relatively more differentiated phenotype than *MYCN*-amplified neuroblastomas. It may be that interference in nucleotide metabolism and DNA synthesis triggers the progression of differentiation in these neuroblastoma cells with a relatively more mature phenotype [14].

Table 4 Deoxycytidine kinase (dCK) activity of neuroblastoma cell lines

MYCN-single copy		*MYCN*-amplified	
Cell line	dCK activity pmol/µg protein/h	Cell line	dCK activity pmol/µg protein/h
SK-N-FI	18.1 ± 0.4	KCNR	29.6 ± 0.8
SK-N-SH	16.3 ± 0.4	NMB	27.7 ± 1.4
SK-N-AS	23.1 ± 1.3	SK-N-BE(2)	23.8 ± 0.8
GI-MEN	14.8 ± 4.3	SK-N-BE(2)c	41.9 ± 1.5
LAN6	42.0 ± 1.3	SJNB6	32.6 ± 1.1
SJNB12	24.1 ± 1.5	AMC106	26.1 ± 0.1
Shep2	17.5 ± 2.0	SJNB8	25.9 ± 0.5
		N206	29.7 ± 0.5
		SJNB10	34.8 ± 3.6

The results shown are the mean of three determinations ± SD

After pre-incubation with CPEC, the efficacy of dFdC was significantly increased in 13 out of 15 cell lines. Metabolic experiments in SK-N-BE(2)c cells demonstrated that the increased sensitivity was caused by highly increased levels of dFdC-metabolites and increased incorporation into the DNA after 1–3 days pre-incubation with CPEC. This increase in dFdC-anabolism declined after 4 days of pre-incubation and was paralleled by a depletion of the UTP and ATP pools and by a reduced cytotoxic effect of dFdC. Because UTP and ATP are the phosphate donors utilized by dCK [7], a decrease in the UTP and ATP pools may cause a decrease of the *in situ* activity of dCK. Moreover, between 1 and 3 days of incubation with 100 nM CPEC, SK-N-BE(2)c cells accumulate in the S-phase of the cell cycle. However, after 4 days of incubation, the cells are arrested in the G_0/G_1-phase of the cell cycle [1]. It is known that the intracellular dCK activity is high in the S-phase of the cell cycle and low in the G_0/G_1-phase of the cell cycle. Therefore, a decreased *in situ* dCK activity due to G_0/G_1-phase accumulation also contributes to the decreased phosphorylation of dFdC [2]. These observations are supported by the fractional effect analysis, which indicated a tendency towards antagonism for prolonged exposure to CPEC or dFdC concentrations greater than 200 nM. The explanation for the antagonism at high dFdC concentrations is the fact that at these concentrations dFdC induced almost 100% toxicity as a single drug. However, the cytotoxic effects of low doses of CPEC and dFdC that are sub optimal in a single-drug setting can dramatically be increased when the drugs are combined at these low doses.

As a result of incubation with CPEC, the dCK-mRNA and protein expression, as well as the dCK activity, increased in SK-N-BE(2)c cells. dCK activity is regulated via feedback inhibition by dCTP [19], while expression of dCK increases in response to inhibition of DNA synthesis and DNA damage [6, 9, 27]. 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, protein and activity. As a result, the increased in situ dCK activity upon incubation with CPEC caused the increased phosphorylation of dFdC in SK-N-BE(2)c cells.

2′,2′-difluorodeoxycytidine-5′-triphosphate and DNA-incorporated dFdC (ratio 2:1) were the predominant metabolites of dFdC, regardless of the presence or absence of CPEC. This is in accordance with the results of Heinemann and colleagues [17]. The fact that no accumulation of dFdCMP was observed indicated that the incorporation of dFdC into DNA is the rate-limiting step in its anabolism once feed-back inhibition of dCK has been relieved. In this respect, dFdC-metabolism differs from AraC-metabolism, of which UMP/CMP kinase is the rate-limiting enzyme once feedback inhibition of dCK is relieved [2]. This is in accordance with

the observation by van Rompay and colleagues who showed that dFdCMP is a better substrate for human UMP/CMP kinase than AraCMP [29].

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 relevance to the treatment of neuroblastoma. We feel that dFdC deserves further development towards a clinical application in the treatment of patients suffering from neuroblastoma.

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References

1. Bierau J, van Gennip AH, Helleman J, van Kuilenburg ABP (2001) The cytostatic and differentiation inducing effect of cyclopentenyl cytosine on human neuroblastoma cell lines. Biochem Pharmacol 62:1099–1105
2. Bierau J, van Gennip AH, Leen R, Helleman J, Caron HN, van Kuilenburg ABP (2003) Cyclopentenyl cytosine primes SK-N-BE(2)c neuroblastoma cells for cytarabine toxicity. Int J Cancer 103:387–392
3. Bierau J, Leen R, van Gennip AH, Caron HN, van Kuilenburg ABP (2004) Determination of the deoxycytidine kinase activity in cell homogenates with a non-radiochemical assay using reversed-phase high performance liquid chromatography. Identification of a novel metabolite of 2-chlorodeoxyadenosine. J Chrom B 805:339–346
4. Boon K, Caron HN, van Asperen R, Valentijn L, Hermus MC, van Sluis P, Roobeek I, Weis PA, Voûte PA, Schwab M, Versteeg R (2001) N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. EMBO J 20:1383–1893
5. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM. (1984) Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science 224:1121–1124
6. Castro Kreder N, van Bree C, Peters GJ, Loves WJ, Haveman J (2002) Enhanced levels of deoxycytidine kinase and thymidine kinase 1 and 2 after pulsed low dose rate irradiation as an adaptive response to radiation. Oncol Rep 9:141–144
7. Cheng YC, Domin B, Lee LS (1997) Human deoxycytidine kinase. Purification and characterization of the cytoplasmic and mitochondrial isozymes derived from blast cells of acute myelocytic leukemia patients. Biochim Biophys Acta 481:481–492
8. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–55
9. Csapo Z, Sasvari-Szekely M, Spasokoukotskaja T, Talianidis I, Eriksson S, Staub M (2001) Activation of deoxycytidine kinase by inhibition of DNA synthesis in human lymphocytes. Biochem Pharmacol 61:191–197
10. Edsjö A, Nilsson H, Vandesompele J, Karlsson J, Pattyn F, Culp LA, Speleman F, Påhlman S (2004) Neuroblastoma cells with overexpressed *MYCN* retain their capacity to undergo neuronal differentiation. Lab Invest 84:406–417
11. Ford H Jr, Cooney DA, Ahluwalia GS, Hao Z, Rommel ME, Hicks L, Dobyns KA, Tomaszewski JE, Johns DG (1991) Cellular pharmacology of cyclopentenyl cytosine in Molt-4 lymphoblasts. Cancer Res 51:3733–3740
12. Fulda S, Lutz W, Schwab M, Debatin KM (1999) MycN sensitizes neuroblastoma cells for drug-induced apoptosis. Oncogene 18:1479–1486
13. Grovas A, Fremgen A, Rauck A, Ruymann FB, Hutchinson CL, Winchester DP, Menck HR (1987) The National Cancer Data Base report on patterns of childhood cancers in the United States. Cancer 80:2321–2332
14. Hatse S, De Clerq E, Balzarini J (1999) Role of antimetabolites of purine and pyrimidine nucleotide metabolism in tumor cell differentiation. Biochem Pharmacol 58:539–555
15. Heinemann V, Xu YZ, Chubb S, Sen A, Hertel LW, Grindey GB, Plunkett W (1990) Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2′,2′-difluorodeoxycytidine. Mol Pharmacol 38:567–572
16. Heinemann V, Schulz L, Issels RD, Plunkett W (1995) Gemcitabine: a modulator of intracellular nucleotide and deoxynucleotide metabolism. Semin Oncol 22:11–18
17. Heinemann V, Hertel LW, Grindey GB, Plunkett W (1998) Comparison of the cellular pharmacokinetics and toxicity of 2′, 2′- difluorodeoxycytidine and 1-beta-D-arabinofuranosylcytosine. Cancer Res 48:4024–4031
18. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W (1991) Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. Cancer Res 51:6110–6117
19. Ives DH, Durham JP (1970) Deoxycytidine kinase. 3. Kinetics and allosteric regulation of the calf thymus enzyme. J Biol Chem 245:2285–2294
20. Kang GJ, Cooney DA, Moyer JD, Kelley JA, Kim HY, Marquez VE, Johns DG (1989) Cyclopentenylcytosine triphosphate. Formation and inhibition of CTP synthetase. J Biol Chem 264:713–718
21. King RS (1996) Gemcitabine. New first-line therapy for pancreatic cancer. Cancer Pract 4:353–354
22. Lutz W, Stohr M, Schurmann J, Wenzel A, Lohr A, Schwab M. (1996) Conditional expression of N-myc in human neuroblastoma cells increases expression of alpha-prothymosin and ornithine decarboxylase and accelerates progression into S-phase early after mitogenic stimulation of quiescent cells. Oncogene 3:803–812
23. Manegold C, Drings P, von Pawel J, Ricci S, Dornoff W, van Walree N, ten Bokkel HW, Chemaissani A, Stahel P, Bergman G, Wagenius C, Sederholm C, Mattson K, Liipo K, Kellokumpu-Lehtinen P (1997) A randomized study of gemcitabine monotherapy versus etoposide/cisplatin in the treatment of locally advanced or metastatic non-small cell lung cancer. Semin Oncol 24:S8
24. Norris MD, Burkhart CA, Marshall GM, Weiss WA, Haber M (2000) Expression of N-myc and MRP genes and their relationship to N-myc gene dosage and tumor formation in a murine neuroblastoma model. Med Pediatr Oncol 35:585–589
25. Peters GJ, van der Wilt CL, van Moorsel CJ, Kroep JR, Bergman AM, Ackland SP (2000) Basis for effective combination cancer chemotherapy with antimetabolites. Pharmacol Ther 87:227–253
26. Ruiz van Haperen V, Veerman G, Vermorken JB, Peters GJ (1993) 2′,2′-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. Biochem Pharmacol 46:762–766
27. Sasvari-Szekely M, Spasokoukotskaja T, Szoke M, Csapo Z, Turi A, Szanto I, Eriksson S, Staub M (1998) Activation of deoxycytidine kinase during inhibition of DNA synthesis by 2-chloro-2′-deoxyadenosine (Cladribine) in human lymphocytes. Biochem Pharmacol 56:1175–1179
28. Slingerland RJ, van Gennip AH, Bodlaender JM, Voûte PA, van Kuilenburg ABP (1995) Cyclopentenyl cytosine and neuroblastoma SK-N-BE(2)-C cell line cells. Eur J Cancer 31A:627–631
29. van Rompay AR, Johansson M, Karlsson A (1999) Phosphorylation of deoxycytidine analog monophosphates by UMP-CMP kinase: molecular characterization of the human enzyme. Mol Pharmacol 56:562–569

30. Verschuur AC, van Gennip AH, Leen R, Muller EJ, Elzinga L, Voûte PA, van Kuilenburg ABP (2000) Cyclopentenyl cytosine inhibits cytidine triphosphate synthetase in paediatric acute non-lymphocytic leukaemia: a promising target for chemotherapy. Eur J Cancer 36:627–635

31. Verschuur AC, van Gennip AH, Leen R, Muller EJ, Elzinga L, Voûte PA, van Kuilenburg ABP (2000) Cyclopentenyl cytosine inhibits cytidine triphosphate synthetase in paediatric acute non-lymphocytic leukaemia: a promising target for chemotherapy. Eur J Cancer 36:627–635