Cancer Letters 319 (2012) 23–30

Contents lists available at SciVerse ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Synergistic interaction between cisplatin and gemcitabine in neuroblastoma cell lines and multicellular tumor spheroids

Odette G. Besançon [a], Godelieve A.M. Tytgat [b], Rutger Meinsma [a], René Leen [a], Jerry Hoebink [a], Ganna V. Kalayda [c], Ulrich Jaehde [c], Huib N. Caron [b], André B.P. van Kuilenburg [a,*]

[a] Academic Medical Center, University of Amsterdam, Laboratory Genetic Metabolic Diseases, PO Box 22700, 1100 DE Amsterdam, The Netherlands
[b] Academic Medical Center, University of Amsterdam, Department of Pediatric Oncology/Emma Children's Hospital, PO Box 22700, 1100 DE Amsterdam, The Netherlands
[c] Institute of Pharmacy, Clinical Pharmacy, University of Bonn, An der Immenburg 4, D-53121 Bonn, Germany

ARTICLE INFO

Article history:
Received 4 November 2011
Accepted 7 December 2011

Keywords:
Neuroblastoma
Spheroids
Cisplatin
Gemcitabine
Drug interactions

ABSTRACT

The efficacy and mechanism of action of cisplatin and gemcitabine were investigated in a panel of neuroblastoma cell lines and multicellular tumor spheroids. In neuroblastoma spheroids, the combination of cisplatin and gemcitabine induced a complete cytostasis at clinical relevant concentrations. A synergistic effect was observed when cells were coincubated with both drugs or preincubated with gemcitabine first. These administration sequences resulted in NASS cells in decreased ERCC1 and XPA expression, two key proteins of the NER DNA repair system, and increased platinum adduct formation in DNA. Most of these phenomena were not observed in SJNB8 cells which might explain the lack of synergy between cisplatin and gemcitabine in SJNB8 cells. Our results showed favorable interactions between cisplatin and gemcitabine in 4 out of 5 cell lines. Therefore, we feel that inclusion of gemcitabine into cisplatin-containing regiments might be a promising new strategy for the treatment of neuroblastoma.

Neuroblastoma is the most common solid extra cranial malignancy in children and is responsible for 15% of all childhood cancer deaths. The 5-year survival rates among children with high-risk neuroblastoma have shown only a modest improvement despite an escalation in the intensity of the therapy provided [1], although the addition of anti-GD2 to the treatment is promising [2]. Anticancer agents are rarely used as single agents and effective chemotherapy usually depends on the identification of suitable combinations to treat a specific type of tumor. Therefore, there is an urgent need to improve the efficacy of currently applied chemotherapeutic drugs in the treatment of neuroblastoma by introducing novel combinations of agents which can overcome chemoresistance and increase survival.

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine, dFdC) is a deoxycytidine analog that exerts its anti-tumor activity via multiple mechanisms of action. dFdC undergoes intracellular phosphorylation to the active metabolites dFdC-diphosphate and dFdC-triphosphate, leading to inhibition of ribonucleotide reductase and incorporation of dFdC-triphosphate into DNA and RNA [3]. After

* Corresponding author. Address: Academic Medical Center, Laboratory Genetic Metabolic Diseases, F0-224, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. Tel.: +31 20 5665958; fax: +31 20 6962596.
E-mail address: a.b.vanKuilenburg@amc.uva.nl (A.B.P. van Kuilenburg).

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doi:10.1016/j.canlet.2011.12.016

le is added after which chain elongation stops, rendering the dFdC moiety resistant to excision by DNA exonuclease activity [4]. dFdC has proven anti-tumor activity *in vivo* against human solid tumors [5,6]. Previously, it has been shown that neuroblastoma cells are highly sensitive to dFdC *in vitro* and in animal models [7,8].

Platinum-containing drugs have a broad range of anti-tumor activity in malignant disease and are used to treat many types of cancer [9]. Cisplatin (cis-diamminedichloroplatinum(II), cddp) is a DNA-damaging agent that forms platinum adducts in the DNA. This leads to intrastrand and interstrand crosslinks which may cause alteration in the structure of DNA [10]. Changes in the DNA helix can be recognized and activate DNA repair systems, allowing the cell to repair the damage. It has been suggested that both the nucleotide excision repair (NER) and the mismatch repair (MMR) systems are involved in mediating platinum resistance [11–13]. It was reported before that dFdC could have an inhibiting effect on the expression of key proteins involved in NER and MMR, thereby inhibiting repair of DNA damage caused by cddp [14]. A number of copper transporters have been shown to be involved in the uptake (hCTR1) and efflux (ATP7A and ATP7B) of cddp [15,16]. Modulations in expression levels of these transporters could, therefore, have an effect on the intracellular platinum concentrations and thereby also be involved in mediating platinum resistance.

Favorable interactions have been observed for cddp and dFdC in solid tumors [17,18]. Because of their different mechanism of action

and non-overlapping toxicity, cddp and dFdC might be good candidates for combination therapy for the treatment of neuroblastoma.

In this study, we have performed an in-depth analysis of the effect of cddp and dFdC in a panel of neuroblastoma cell lines as well as in spheroids, which are three dimensional aggregates of cancer cells that, due to their cellular organization, have been shown to resemble *in vivo* tumors with respect to growth rates and sensitivity towards chemotherapeutic drugs.

2. Materials and methods

2.1. Drugs and chemicals

cis-Diamminedichloroplatinum (cddp, Cisplatin) was obtained from Pharmachemie (Haarlem, The Netherlands), 2′,2′-Difluoro-2′-deoxycytidine (dFdC or gemcitabine) was obtained from Eli Lilly (Nieuwegein, The Netherlands). Both were solubilized in phosphate-buffered saline (PBS) to a concentration of 3.3 mM and 1 mM, respectively. Final dilutions of both drugs were made in culture medium. [^{3}H]-2′,2′-difluoro-2′-deoxycytidine 14 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA) and [^{14}C]Thymidine (Thd) (2.04 GBq/mmol) was obtained from Amersham International (Buckinghamshire, UK). All other chemicals were of analytical grade and commercially available.

2.2. Cell culture

Six *MYCN* single copy neuroblastoma cell lines (FISK, NASS, SY5Y, SK-N-SH, GI-M-EN and SHEP2) and six *MYCN* amplified neuroblastoma cell lines (SJNB8, SJNB10, SK-N-BE, NGP, SJNB6 and IMR32.k1) were cultured as described before [19]. Spheroids of NASS and SJNB8 cell lines were prepared as described before [19] and treated with different concentrations cddp, dFdC or coincubated with both drugs for 2 weeks, without changing the culture medium. The experiments were performed in quadruplicate. Spheroid size was monitored weekly by measurement of the cross-sectional area of individual spheroids five-times magnified photos using a microscope.

2.3. Measurement of cell viability and caspase-3 activity

Cells were plated in 96-well plates at a density of 3000–6000 cells per well, depending on the cell line, in a total volume of 100 μl. Cells were allowed to adhere overnight, after which the medium was replaced by medium containing different concentrations of cddp, dFdC or a combination of both drugs. Three different coincubation schedules were used: coincubation with both drugs for 72 h or preincubation with cddp or dFdC for 24 h followed by coincubation of both drugs for the next 48 h. After 24, 48 and 72 h MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was added to the wells containing medium with drugs and the viability of the cells was analysed (Promega, Madison, WI, USA) according to the manufacturer's protocol. Caspase-3 activation was measured by the caspase-glo 3/7 assay (Promega, Madison, WI, USA) according to the manufacturer's protocol.

2.4. Multiple drug effect analysis

Various concentrations of cddp and dFdC were added in a fixed IC_{50}-based molar ratio (cddp:dFdC) of 120:1 for FISK and NASS, and 600:1 for SY5Y, SJNB8 and SJNB10. The mode of interaction (synergy, antagonism or additivity) was determined by the combination index (CI), as described by Chou and Talalay [20] and was calculated for each combination of cddp and dFdC using the Calcusyn computer program (Biosoft, Ferguson, MO).

2.5. Western blotting

Cells (attached and floating) were collected by trypsinization, centrifuged and the pellet was washed twice in ice-cold PBS. Subsequently, cells were lysed in RIPA-buffer (1% w/v NP40 (LKB-Produkter AB), 12.06 mM sodiumdeoxycholate (Sigma), 0.1% w/v SDS (Roche) in PBS) completed with protease inhibitor cocktail (Roche), 0.5 mM NaF and 0.5 mM Na_3VO_4. Protein concentrations were determined using a BCA protein assay [21]. SDS–polyacrylamide gel electrophoresis was performed, essentially as described by Laemmli [22] followed by Western blotting using mouse anti-human poly (ADP-ribose) polymerase (PARP) monoclonal antibody 1:10,000 (Biomol, UK), XPA (sc-56813 clone 12F5) 1:2000, ERCC1 (sc-53281 clone 3H11) 1:200, MLH1 (sc-56159 clone 164C819) 1:1000 and MSH2 (sc-65942 clone 3A2B8C) 1:1000 (Santa Cruz, CA, USA). Antigen–antibody complexes were visualized using horseradish peroxidase-conjugated secondary antibodies. Detection was carried out by ACL (Amersham) on a LAS3000 (Fujifilm). Equal loading was confirmed using β-actin or α-tubulin.

2.6. Extraction and analysis of radiolabeled nucleotides

Cells were seeded in six well plates at a density of 0.5×10^6 cells per well and allowed to adhere overnight. The cells were preincubated with 0.25, 1 or 4 μM cddp for 24 h, after which 50 nM [^{3}H]dFdC and 250 nM [^{14}C]Thymidine (Thd) were added to the wells. After 3 h of incubation, the cells were extracted and analyzed as described previously [23].

2.7. Platinum–DNA adduct measurement

Cells were seeded in 75 cm^2 flasks at a confluency of approximately 20% and allowed to adhere overnight. Medium was replaced by medium containing different concentrations of cddp or dFdC or the coincubations as described above. After 24 hrs, cddp or dFdC was added to the cells preincubated with dFdC or cddp, respectively, and incubated for 48 h. Subsequently, cells were harvested by trypsinization and DNA was isolated using Wizard Genomic DNA Purification (Promega) according to the manufacturer's protocol. DNA platination was measured using a previously published method adapted for tumor cells [16].

2.8. Quantitative reverse transcription-PCR

Cells were seeded in six well plates at approximately 20% confluency and allowed to adhere overnight. Medium was replaced by medium containing cddp and/or dFdC, using the coincubation schedules as described above. *ATP7A*, *ATP7B* and *CTR1* mRNA levels were measured using a quantitative PCR (qPCR) method of detection of relative amounts of first-strand cDNA. cDNA was generated from mRNA isolated using Trizol (Invitrogen). qPCR was performed on a Roche LightCycler® 480 machine and data was analyzed by using LinReg [24]. The forward and reverse primers for ATP7A, ATP7B and CTR1 were: ATP7A, 5′-GAGAAAAGGTCGGACTGCTG-3′ (forward) and 5′-TGCCAACCTGAGAAGCAATAG-3′ (reverse); ATP7B 5′-TACCCATTGCAGCAGGTGTC-3′ (forward) and 5′-ACTTGAGCTGCAGGGATGAG-3′ (reverse); CTR1 5′-AGCTGGAGAAATGGCTGGAG-3′ (forward) and 5′-AGGTGAGGAAAGCTCAGCATC-3′ (reverse) [16]. Three independent sample sets were analyzed, with experiments in each sample set being performed in triplicate.

2.9. Statistical analyses

Data were expressed as mean values ± SD, and statistical analyses were performed by using GraphPad Prism (version 5.01).

3. Results

3.1. Effect of cddp and dFdC on viability and apoptosis in neuroblastoma cells

The IC_{50} values for cddp and dFdC as a monodrug after 72 h were determined in a panel of neuroblastoma cell lines growing in monolayers, consisting of six *MYCN* single copy and six *MYCN* amplified cell lines (Table 1). There was a tendency for *MYCN* amplified cell lines to be more sensitive for dFdC compared to *MYCN* single copy cell lines ($P = 0.1$). A difference in sensitivity towards cddp between *MYCN* single copy and *MYCN* amplified cell lines was not observed. Furthermore, cddp administered as a monodrug or in combination with dFdC resulted in an increased caspase-3 activity, reflecting the induction of apoptosis (Fig. 1), except for SJNB10. Treatment of cells with dFdC for 24 h showed an increased caspase-3 activity in SY5Y cells only, but not in other cell lines tested. NASS and SJNB8 cells exposed to cddp or dFdC for 72 h showed PARP cleavage, a marker of apoptosis (Fig. 2).

3.2. Synergism between cddp and dFdC dependent on administration sequence

In 4 out of 5 cell lines an additive/synergistic effect for the drug combination cddp–dFdC was observed, depending on the administration sequence of the drugs (Fig. 3). In the NASS cell line a synergistic effect was observed for the administration sequence preincubation with dFdC followed by a coincubation with cddp and dFdC for 72 h and coincubation with both drugs for 72 h. In contrast, an antagonistic effect was observed when NASS cells were preincubated with cddp prior to the administration of dFdC. In SJNB8 cells an antagonistic effect of all combinations was

Table 1
IC_{50} concentrations ± SD of all cell lines.

Cell lines *MYCN* single copy	cddp (μM)	dFdC (nM)	Cell lines *MYCN* amplified	cddp (μM)	dFdC (nM)
FISK	2.1 ± 0.9	13.7 ± 6.9	SJNB8	6.0 ± 3.7	4.2 ± 1.4
NASS	3.2 ± 2.0	22.3 ± 2.3	SJNB10	2.3 ± 0.7	2.9 ± 0.5
SY5Y	1.2 ± 0.4	4.6 ± 1.7	SK-N-BE	2.3 ± 1.4	29.9 ± 7.7
SK-N-SH	1.4 ± 0.3	28.5 ± 5.1	NGP	1.9 ± 0.7	5.1 ± 1.7
GI-M-EN	2.7 ± 1.2	6.2 ± 0.6	SJNB6	3.8 ± 2.6	8.0 ± 7.4
SHEP2	8.4 ± 1.2	28.0 ± 2.0	IMR32.k1	0.8 ± 0.7	4.6 ± 1.5

Cells were treated with cddp or dFdC for 72 h.

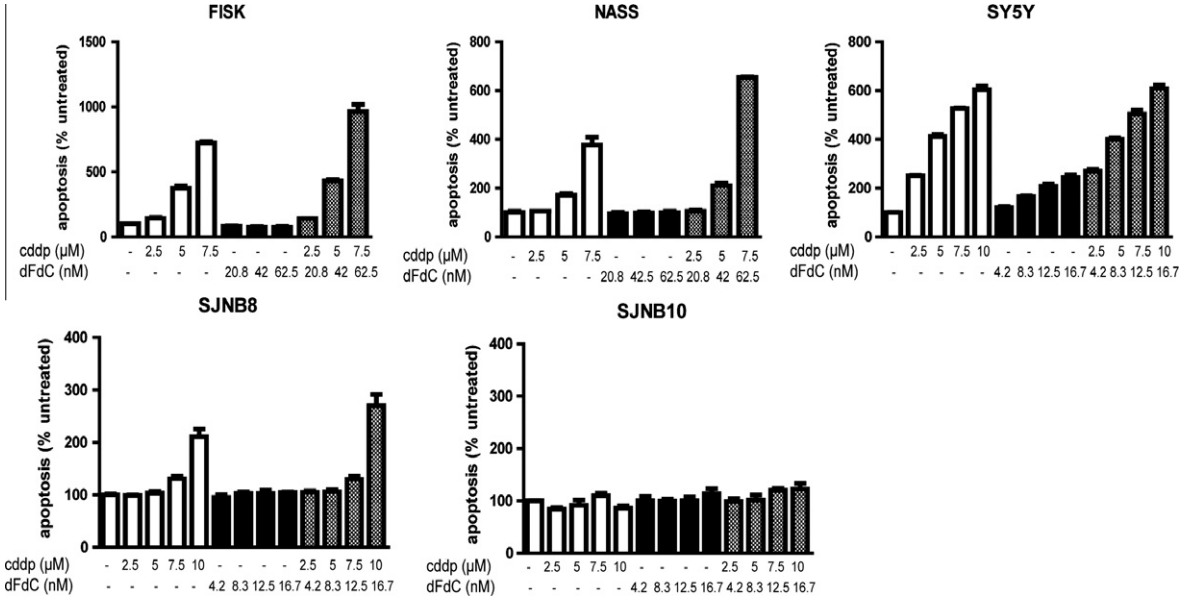

Fig. 1. The effect of cddp and dFdC on apoptosis. Cells were treated with different concentrations of cddp, dFdC or the combination of both administrated at the same time for 24 h. Caspase-3 activity was measured and depicted as percentage compared to the untreated control. Each bar represents the mean ± SD of three experiments.

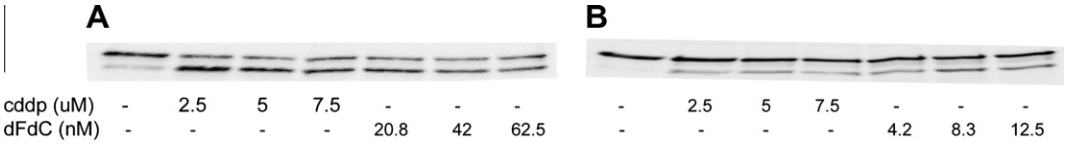

Fig. 2. PARP cleavage. NASS (A) and SJNB8 (B) neuroblastoma cells treated with different concentrations of cddp and dFdC for 72 h followed by analysis of PARP.

observed, independent of the administration sequence of the two drugs. In the remaining three cell lines, an additive to synergistic effect was observed in case the cells were preincubated with dFdC followed by the administration of cddp.

3.3. Effect of cddp and dFdC on neuroblastoma spheroids

The sensitivity for cddp and dFdC in SJNB8 spheroids was comparable to the sensitivity observed in cells growing in monolayer. In contrast, NASS spheroids showed approximately a 10-fold increased sensitivity for dFdC, and a comparable IC_{50} value for cddp. Cytostasis was observed in spheroids treated with high concentrations of the combination cddp–dFdC (Fig. 4), which was more profound in NASS spheroids compared to SJNB8 spheroids.

3.4. Effect of cddp and dFdC on proteins involved in the NER and MMR system

A downregulation of the expression of XPA and ERCC1, two key proteins of the NER pathway, was observed in the NASS cell line exposed to cddp and dFdC as a monodrug or in combination with cddp. The most profound effect was seen when cells were preincubated with dFdC or coincubated with both drugs. This effect was not, or to a lesser extent, observed in SJNB8 cells (Fig. 5). No differences in the MLH1 and MSH2 expression were detected (data not shown).

3.5. The dFdC and thymidine incorporation into DNA

To study the growth-inhibiting effect of cddp and dFdC, [^{14}C]Thd and [^{3}H]dFdC incorporation into DNA was measured. Pretreatment of NASS and SJNB8 cells with cddp decreased the [^{14}C]Thd incorporation into DNA in a concentration-dependent manner. The effect of cddp on the maximal reduction of [^{14}C]Thd incorporation was more profound for NASS cells then for SJNB8, ~90% and ~60%, respectively. On the contrary, cddp pretreated cells showed a slightly increased [^{3}H]dFdC incorporation in newly synthesized DNA compared to cells treated with [^{3}H]dFdC only (Fig. 6) The ratio [^{3}H]dFdC incorporation/[^{14}C]Thd incorporation, reflecting the amount of incorporated dFdC in newly synthesized DNA, was 1.8-fold higher in NASS cells compared to SJNB8 (Table 2).

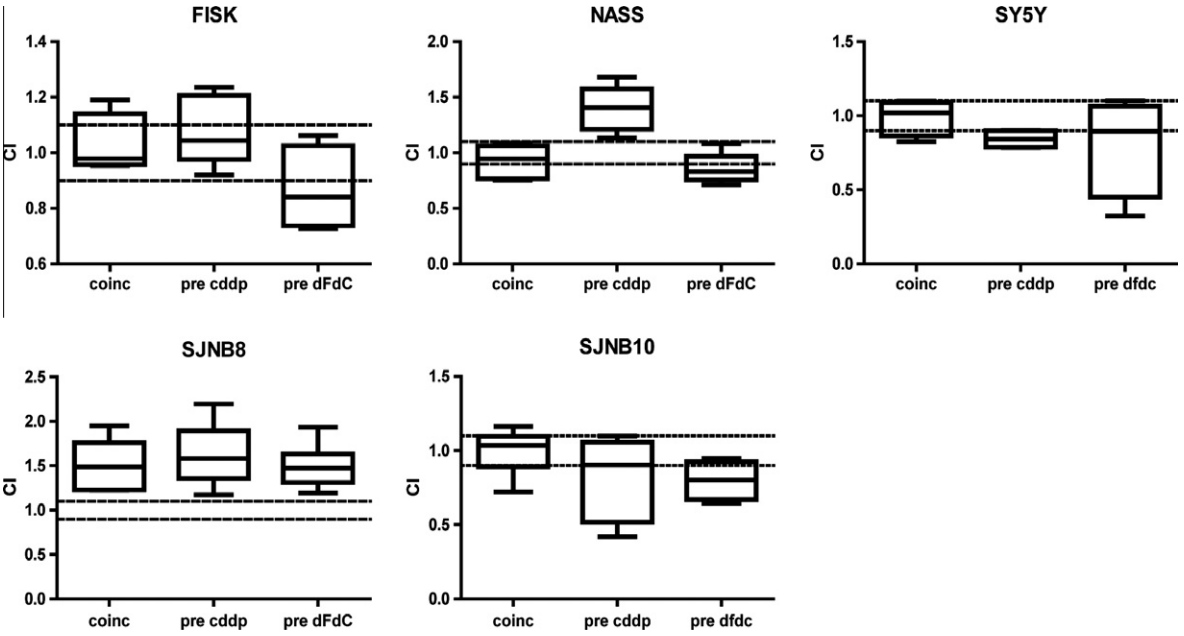

Fig. 3. Box plots of the combination index (CI) values of cddp and dFdC in five neuroblastoma cell lines. The top, bottom and line through the middle of a box correspond to the 75th percentile, 25th percentile and 50th percentile, respectively. The whiskers on the bottom extend from the lowest value and top to the highest value. An average CI was calculated from data points with FA (fraction affected) 0.6, 0.75 and 0.9. CI > 1.1, antagonism; 0.9–1.1, additive effect; <0.9, synergistic effect. The results shown are the mean ± SD of at least four independent experiments.

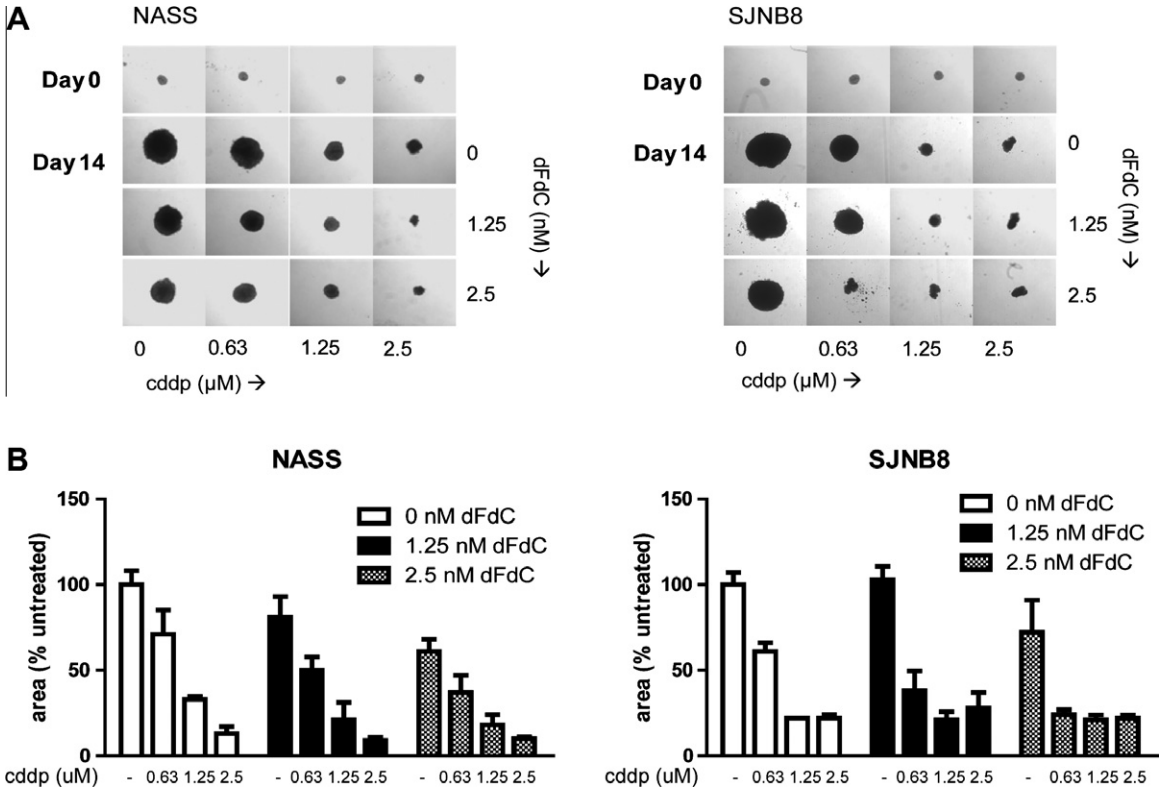

Fig. 4. The effect of cddp and dFdC on spheroid growth. Spheroids were treated with cddp or dFdC, administered as monodrug or as a combination of both and photographed weekly to monitor the increase of the area. Panel A shows NASS and SJNB8 spheroids treated with cddp and/or dFdC. Panel B shows the calculated area of spheroids treated with different concentrations cddp and/or dFdC for 14 days, depicted as percentage compared to the untreated control. Each bar represents the mean area ± SD of four experiments.

3.6. DNA platination

After exposure of NASS and SJNB8 cells to increasing concentrations of cddp, a dose-dependent increase of DNA platination was observed. In NASS cells, preincubation with 42 nM dFdC followed by 5 μM cddp as well as coincubation with both drugs resulted in an increase of 45% ($P < 0.05$) and 84% ($P < 0.05$), respectively in platinum adduct formation in DNA compared to cells exposed to

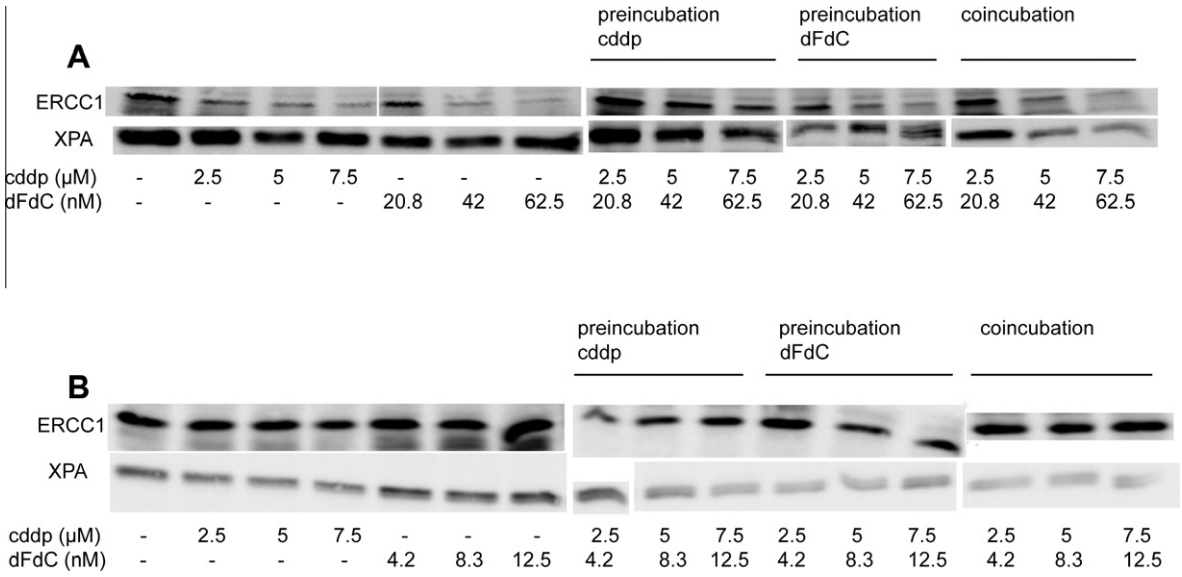

Fig. 5. Immunoblot analysis of ERCC1 and XPA associated with cddp resistance. Blots show the changes in ERCC1 and XPA expression after treatment of (A) NASS neuroblastoma cells and (B) SJNB8 neuroblastoma cells. Cells were treated for 72 h with different concentrations of cddp and dFdC and different administration sequences.

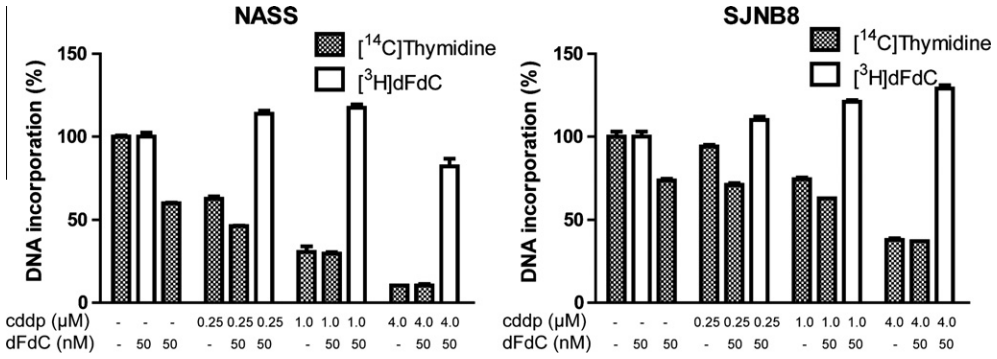

Fig. 6. The effect of dFdC and cddp on DNA synthesis. Cells were pretreated with 0, 0.25, 1 or 4 μM cddp for 21 h after which they were exposed to 250 nM [^{14}C]Thd or 50 nM [^{3}H]dFdC for 3 h. The amount of [^{14}C]Thd incorporated into DNA in untreated NASS and SJNB8 cells was 555 ± 4 fmol/μg protein and 584 ± 20 fmol/μg protein, respectively. The amount of [^{3}H]dFdC incorporated in NASS and SJNB8 cells, without prior incubation of cddp, was 1.7 ± 0.04 fmol/μg and 1.5 ± 0.05 fmol/μg protein, respectively. The amount [^{14}C]Thd incorporation was given as a percentage of untreated control cells. The incorporation of [^{3}H]dFdC in the DNA was given as a percentage of cells treated with [^{3}H]dFdC only. The results shown are the mean ± SD of three independent experiments.

cddp only (Fig. 7). A preincubation of NASS cells with 7.5 μM cddp decreased platinum adduct formation in DNA by approximately 22% ($P = 0.19$). In contrast, in SJNB8 cells, irrespective of the administration sequence used, the combination of cddp and dFdC resulted in a decreased formation of platinum adducts in DNA compared to cells treated with cddp only. Thus, the levels of platinum adduct formation in DNA are in line with the degree of toxicity observed after treatment of NASS and SJNB8 cells with the combination cddp–dFdC.

Table 2
Ratio of [^{3}H]dFdC incorporation/[^{14}C]Thd incorporation.

Cell line	0 μM	0.25 μM	1 μM	4 μM
Concentration cddp (μM)				
NASS	100	150	240	480
SJNB8	100	110	140	260

Ratio of [^{3}H]dFdC incorporation/[^{14}C]Thd incorporation in NASS and SJNB8 cell lines exposed to an increasing cddp concentration. The [^{3}H]dFdC incorporation/[^{14}C]Thd incorporation ratio of cells exposed to 50 nM dFdC was set to 100%. Cells were pretreated with 0, 0.25, 1 or 4 μM cddp for 21 h after which they were exposed to 250 nM [^{14}C]Thd or 50 nM [^{3}H]dFdC for 3 h.

3.7. Gene expression analysis of CTR1, ATP7A and ATP7B

Exposure of neuroblastoma cells to dFdC as a monodrug or in combination with cddp resulted in a downregulation of the expression of the copper efflux transporter *ATP7A* in NASS cells ($P < 0.05$) which was not observed in SJNB8 cells (Fig. 8). An upregulation of the expression of the copper uptake transporter *hCTR* was observed in NASS cells preincubated with dFdC ($P < 0.05$) which was also not observed in SJNB8 cells. NASS cells pretreated with cddp resulted in a decreased expression of the copper efflux transporter *ATP7B.*

4. Discussion

The favorable interactions which have been observed for cddp and dFdC in some solid tumors prompted us to explore the effect of the interaction between cddp and dFdC in a panel of neuroblastoma cell lines. Due to the heterogeneity of neuroblastoma, we have investigated the effectiveness of this combination in a panel of five neuroblastoma cell lines.

Both *MYCN* amplified and *MYCN* single copy neuroblastoma cell lines proved to be highly sensitive towards dFdC with IC_{50} values in

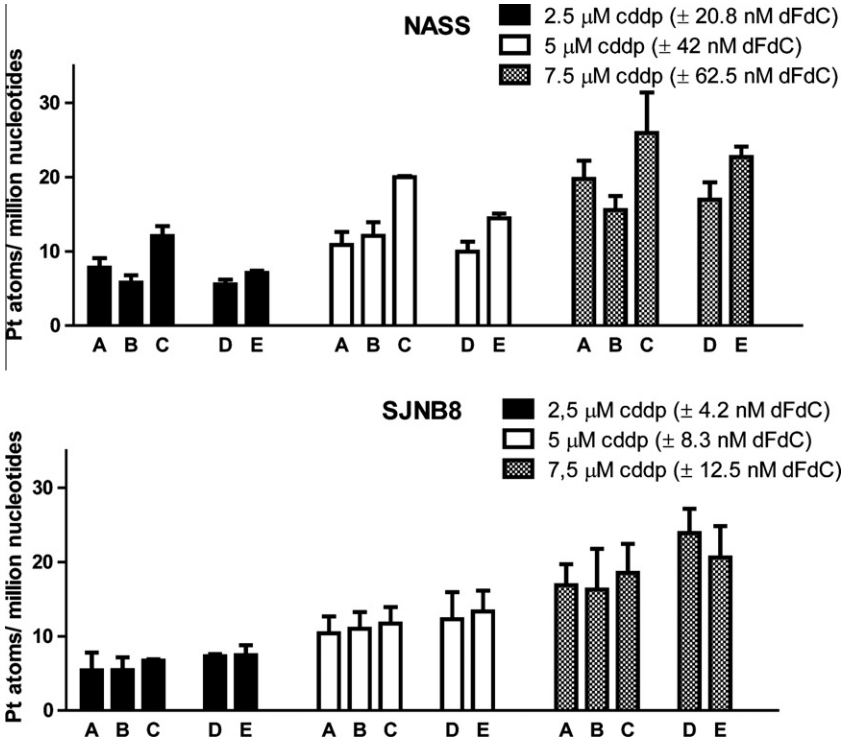

Fig. 7. Platinum content in DNA. The amount of platinum adduct formation in DNA was determined after (A) incubation with cddp alone for 72 h, (B) preincubation for 24 h with cddp followed by 48 h coincubation with dFdC, (C) 72 h of coincubation with cddp and dFdC, (D) incubation with cddp alone for 48 h and after (E) preincubation for 24 h with dFdC followed by 48 h coincubation with cddp. Administration sequences (A–C) were exposed to cddp for 72 h. Since administration sequence (E) had an exposure for 48 h to cddp, this should be compared to (D). Platinum adducts are depicted as adducts per million nucleotides in NASS and SJNB8 cells. The results shown are the mean ± SD of three independent experiments.

the low nanomolar range. *MYCN* amplified cell lines tended to be more sensitive for dFdC compared to *MYCN* single copy cell lines, which has been described before [7,25]. dFdC is a pro-drug that has to be activated by phosphorylation to be therapeutically effective. In particularly, the incorporation of dFdC into DNA has profound effects on DNA synthesis. This phenomenon might explain the observation that only minimal toxicity was observed after 24 h whereas profound apoptosis was detected after 72 h of incubation with dFdC. The high sensitivity of neuroblastoma cells towards dFdC might be due to the high activity of the anabolic enzyme deoxycytidine kinase and the low activity of the inactivating enzyme cytidine deaminase in neuroblastoma [8].

In neuroblastoma spheroids, cddp and dFdC induced a complete cytostasis at clinical relevant concentrations (2.5 μM and 1.25 nM, respectively). Peak plasma levels (PPL) for cddp between 6 and 12 μM were measured in neuroblastoma patients after initial chemotherapy [26]. PPL for dFdC in neuroblastoma patients are not known but a study performed in adult patients with refractory solid cancer, PPL between 2 and 512 μM, depending on the dose administered, were reached [27].

It has been shown that overexpression of NER genes is associated with cddp resistance in ovarian, glioma, bladder and lung cancer cells [28]. The most profound inhibition of the ERCC1 and XPA, two proteins which play a key role in the NER system, was observed after exposure of NASS cells to dFdC, cddp, cells preincubated for 24 h with dFdC, or cddp and dFdC administrated at the same time. These phenomena were not observed in SJNB8 cells, which could explain the synergistic effect and antagonistic effect between cddp and dFdC in NASS and SJNB8 cells, respectively.

In addition, an increase in the formation of DNA platinum adducts was observed when NASS cells were exposed simultaneously to dFdC and cddp or when cells were preincubated with dFdC followed by the addition of cddp. This observation is in accordance with results of previous studies obtained in human ovarian cancer cell lines [14,29,30]. The increase in platinum adducts in DNA could be related to the inhibition of the NER system by dFdC, which has been described before, thereby inhibiting the removal and repair of the platinum adducts [31].

It has been reported that the copper uptake transporter *hCTR1* and copper efflux transporters *ATP7A* and *ATP7B* are involved in cddp sensitivity or resistance [15,16,32]. Our results showed a down regulating effect of dFdC on *ATP7A* and *ATP7B* and an upregulating effect of *hCTR* gene expression in NASS cells only. This phenomenon might also contribute to the observed synergistic toxicity of cddp and dFdC based treatment in NASS cells. It has to be mentioned that the expression of the *ATP7B* transporter is ~50 times lower compared to *ATP7A*.

Exposing NASS or SJNB8 neuroblastoma cells to cddp resulted in a profound inhibition of DNA synthesis. The inhibiting effect of cddp on DNA synthesis when cells are pre-exposed to cddp could be a possible explanation for the tendency for this administration sequence to be antagonistic, since dFdC needs to be incorporated into DNA before it can exerts its cytotoxic effect. dFdC-triphosphate itself competes with the natural substrate dCTP for incorporation into DNA [4]. Furthermore, dFdC-diphosphate inhibits ribonucleotide reductase, leading to depletion of the intracellular dCTP pools and facilitating incorporation of dFdC-triphosphate into DNA [33]. Furthermore, cddp has been shown to alter deoxynucleotide pools and this mechanism could be a conceivable explanation as to why a slightly increased incorporation of dFdC into newly synthesized DNA was observed in cddp pretreated cells [34].

Previously, the efficacy and tolerability of dFdC administrated as a monodrug in pretreated pediatric patients with refractory solid tumors, including neuroblastoma, was determined. Although there was a good tolerability there is probably no general relevance in using dFdC as a monodrug, since no objective responses were

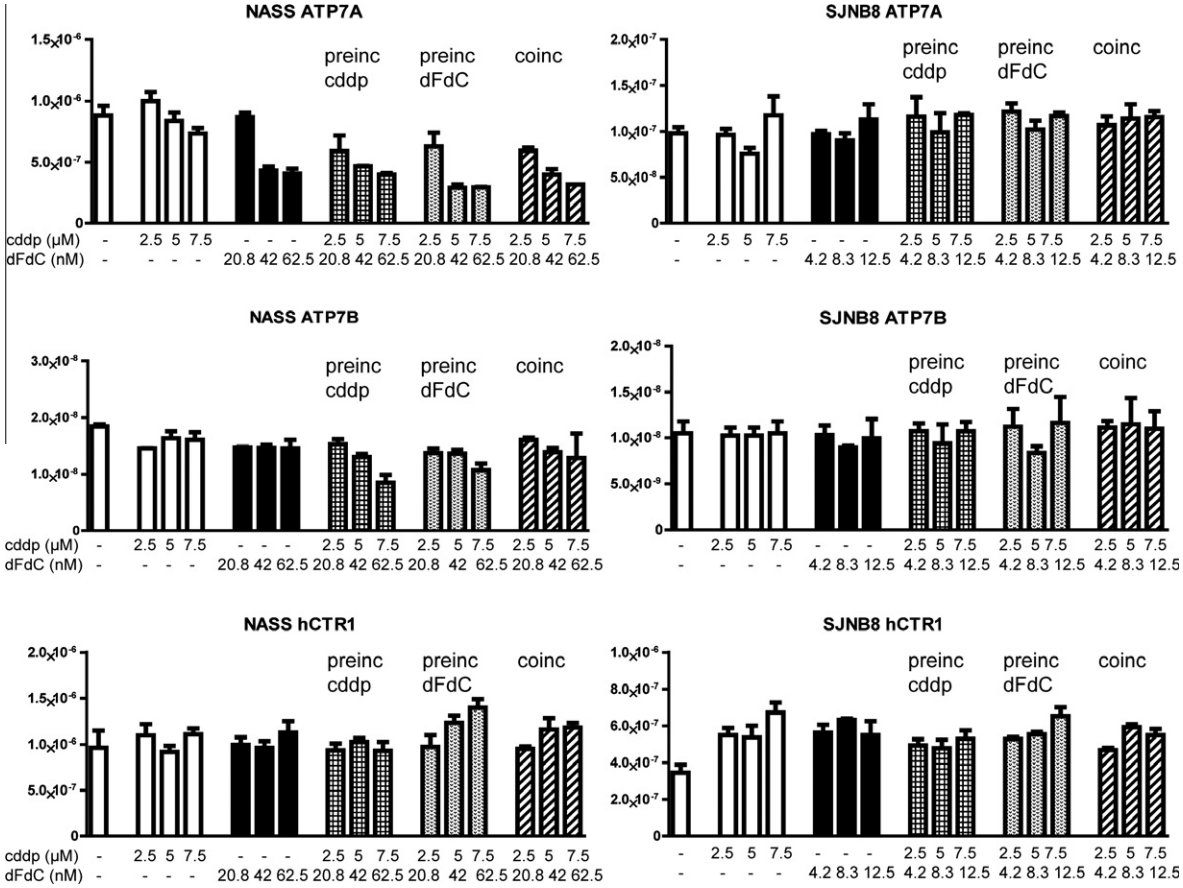

Fig. 8. Quantitative PCR analysis of *ATP7A*, *ATP7B* and *hCTR1* mRNA. The amount of mRNA was determined after incubation with cddp or dFdC alone, after preincubation for 24 h with cddp followed by 48 h coincubation with dFdC (preinc cddp), after preincubation for 24 h with dFdC followed by 48 h coincubation with cddp (preinc dFdC) or after 72 h coincubation of cddp and dFdC (coinc). The molar ratio of cddp: dFdC was 120:1 for NASS cells and 600:1 for SJNB8 cells. The results shown are the mean ± SD of three independent experiments.

observed [35]. In a recent study, a dFdC–oxaliplatin combination was administrated consecutively on the same day in a bi-weekly schedule. This combination has an acceptable safety profile with limited activity in children with relapsed or refractory solid tumors [36]. However, our *in vitro* results provide evidence that synergism between cddp and dFdC was dependent on the administration sequence used and is more likely to occur when neuroblastoma cells are exposed to dFdC prior to the administration of cddp.

In summary, favorable interactions between cddp and dFdC could be achieved in 4 out of 5 neuroblastoma cell lines due to the inhibition of the NER system by dFdC, a decreased expression of ATP7A and increased formation of DNA platinum adducts. Therefore, we feel that inclusion of dFdC into cddp-containing regiments might be a promising new strategy for the treatment of neuroblastoma.

Acknowledgements

This work was supported by a Grant from the Stichting Kinderen Kankervrij (KIKA) of The Netherlands. The study sponsor had no involvement in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

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