Retinoic acid reduces the cytotoxicity of cyclopentenyl cytosine in neuroblastoma cells

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Abstract In this paper, it is demonstrated that all-*trans*, 9-*cis* and 13-*cis* retinoic acid (RA) decreased the sensitivity of SK-N-BE(2)c neuroblastoma cells towards the chemotherapeutic agent cyclopentenyl cytosine (CPEC), a potent inhibitor of cytosine-5′-triphosphate synthetase. Retinoic acid attenuated CPEC-induced apoptosis as reflected by a decreased caspase-3 induction. Retinoic acid decreased the accumulation of CPEC, whereas the salvage of cytidine was strongly increased. Metabolic labeling studies using [^{3}H]uridine showed a strongly decreased biosynthesis of CTP via CTP synthetase. Retinoic acid likely confers resistance of neuroblastoma cells to CPEC in part by slowing down proliferation, and in part by shifting the synthesis of CTP towards the salvage of cytidine, thereby bypassing CTP synthetase. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Neuroblastoma; Cyclopentenyl cytosine; Retinoic acid; Cytosine-5′-triphosphate synthetase; Pyrimidine de novo metabolism; Pyrimidine salvage metabolism

1. Introduction

Neuroblastoma is the most common extra cranial solid tumor of childhood. The tumors are derived from the neural crest and originate where nervous tissue is present. The disease has five distinct prognostic stages, stages 1–4 and 4S. Despite considerable research, the prognosis for patients suffering from stage 4 neuroblastoma, which is characterized by disseminated disease, is still very poor [1]. Stage 4S neuroblastoma, however, is able to differentiate into more mature forms and has a high rate of spontaneous regression [2,3]. This spontaneous differentiation of neuroblastoma cells has triggered a great deal of research focused on the induction of differentiation of neuroblastoma in vivo. Both in vitro and in vivo, neuroblastoma cells can be induced to differentiate by all-*trans* retinoic acid (ATRA) and its conformational isomers 9-*cis* retinoic acid (9-*cis* RA) and 13-*cis* retinoic acid (13-*cis* RA) [4,5]. Clinical trials studying the effects of ATRA and 13-*cis* RA in the treatment of disseminated neuroblastoma indicate a clinical response in some patients [6–11]. Of the retinoic acid isomers 9-*cis* RA has been reported to have the strongest differentiation inducing effect in several cell line systems [12–14]. Furthermore, ATRA has been shown to be isomerized partly to 9-*cis* RA in liver [15,16]. Hence, 9-*cis* RA may be tested in clinical settings as well as ATRA and 13-*cis* RA.

To date, little is known about the effect of differentiating agents on the efficacy of cytostatic nucleoside analogues in neuroblastoma. Results from clinical trials in which patients suffering from leukemia were treated with ATRA and cytarabine, an analog of deoxycytidine, showed a synergistic effect when ATRA and cytarabine were combined [17–19]. In this study, we studied the effect of ATRA, 9-*cis* RA and 13-*cis* RA on the cytotoxicity and metabolism of cyclopentenyl cytosine (CPEC) as well as pyrimidine nucleotide biosynthesis in neuroblastoma cells. CPEC is a pro-drug that in its nucleoside triphosphate form (CPECTP) potently inhibits cytosine-5′-triphosphate (CTP) synthetase, causing depletion of cytidine nucleotides [20]. We have previously shown that CPEC, as single agent, has profound and long lasting cytostatic effects on human neuroblastoma cells in vitro [21]. In this paper we demonstrate that retinoic acid to a large extent attenuates the cytostatic effect of CPEC.

2. Materials and methods

2.1. Chemicals

CPEC (NSC 375575) was obtained from the Developmental Therapeutics Program, NCI (Division of Cancer Treatment, National Cancer Institute, MD, USA). ATRA, 13-*cis* RA, 9-*cis* RA and all nucleotide standards were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). [^{14}C-U]uridine (18.6 GBq/mmol) and [^{14}C-cytosine]cytidine (2.04 GBq/mmol) were obtained from Amersham International (Buckinghamshire, UK). Nonidet P40 was obtained from LKB-produkter AB (Bromma, Sweden). Dulbecco's modified Eagle's medium (DMEM), bovine fetal serum and penicillin/streptomycin/fungizone mix were obtained from BioWhittaker Europe (Verviers, Belgium). L-Glutamine and gentamicin were obtained from Gibco BRL (Paisly, UK). All other chemicals were of analytical grade.

2.2. Cell culture

The SK-N-BE(2)c neuroblastoma cell line was obtained from the American Type Culture Collection. The cells were routinely cultured

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Abbreviations: CTP, cytosine-5′-triphosphate; CPEC, cyclopentenyl cytosine; CPECTP, cyclopentenyl cytosine-5′-triphosphate; ATRA, all-*trans* retinoic acid; 9-*cis* RA, 9-*cis* retinoic acid; 13-*cis* RA, 13-*cis* retinoic acid; UDPG, UDP-galactose+UDP-glucose; UDPNAG, UDP-*N*-acetylgalactosamine+UDP-*N*-acetylglucosamine

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in DMEM, supplemented with 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, 0.2 mg/ml gentamicin, 0.25 μg/ml fungizone and 10% v/v bovine fetal serum at 37°C in humidified air with 5% CO_2. The cells were maintained in 75 cm^2 loosely capped culture flasks (Co-star Corp.) and maintained in logarithmic growth phase. SK-N-BE(2)c was used up to passage 45. Differentiated cells were obtained by incubation with ATRA (100 nM for 6 days or 10 μM for 2 days, resulting in the same degree of differentiation). Cell cultures were consistently free of mycoplasma (tested with Mycoplasma PCR ELISA, Boehringer Mannheim, Mannheim, Germany).

2.3. Proliferation and differentiation

Cells were seeded into 6-well plates (Corning Co-star) at a density of $0.25–0.50\times10^6$ cells per well, and allowed to adhere overnight. The experiments were started by replacing the medium with medium containing the appropriate retinoic acid isomer in 0.1% ethanol. As a control, the proliferation of cells in medium containing only 0.1% ethanol was also measured. Adherent cells were harvested by trypsinization and cell numbers were determined after solubilizing the cells in isoton II containing 2.7×10^{-7}% (v/v) Triton X-100 and 2.7×10^{-3}% (w/v) saponin. The nuclei were counted with a Coulter Counter Z 1000 with a 100 μM orifice (Coulter Electronics Ltd, Buckinghamshire, UK). The viability of adherent cells was more than 99%, as determined by exclusion of trypan blue.

To assess the degree of differentiation induced by retinoic acid, 400 cells per culture per day 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.

2.4. Assay for drug sensitivity

Cells were plated in 24-well plates at a density of 30×10^3 cells per well in a total volume of 0.5 ml. After overnight adherence, the medium was changed for medium containing CPEC and/or retinoic acid. In the case of pre-incubation with retinoic acid, cells were incubated for 2 days with 100 nM retinoic acid, and subsequently harvested and cultured in medium without retinoic acid in 24-well plates, as described above. After the incubation time, the number of viable cells was measured using the MTT assay as described previously [22] and IC_{50} values were determined.

2.5. Nucleotide extraction and analysis

Cells were seeded in 6-well plates at a density of $0.25–1\times10^6$ cells per well. After overnight adherence, the cells were incubated with 100 nM ATRA for 6 days or with 10 μM ATRA for 2 days. After incubation with ATRA, the medium was changed with fresh medium and subsequently CPEC was added to the culture medium. Control cells, which were not pre-treated with ATRA, were incubated with CPEC after overnight adherence. After 6 h incubation with CPEC, the cells were washed once with phosphate-buffered saline (PBS) and extracted with perchloric acid and analyzed, as described previously [21].

2.6. Extraction and analysis of radiolabeled nucleotides

Cells were seeded and pre-treated as described in Section 2.5. After incubation with ATRA, the medium containing ATRA was removed and replaced by medium spiked with 1.8 μM [^{14}C]cytidine or 0.36 μM [^{14}C]uridine. The final concentrations (radiolabeled+endogenous nucleosides) of cytidine and uridine in the medium were 2.0 μM and 1.8 μM, respectively. As a consequence, the specific activity of the label was corrected for the presence of the endogenous amounts of cytidine and uridine. After 3 h, the cells were extracted with 0.4 M perchloric acid and nucleotide profiles were determined by ion-exchange high performance liquid chromatography (HPLC), as described previously [21]. Radioactivity was detected online with a Radiomatic 525TR Flow Scintillation Analyzer with a 500 μl TR-LSC cell (Packard Instrument, Meriden, CT, USA) using Ultima Flo AP (Packard, Dowers Grove, IL, USA) at an effluent to scintillation fluid ratio of 1:1.

The acid precipitate was taken up in 300 μl of 0.2 M NaOH and precipitated again by adding an equal volume of 1.2 M perchloric acid. The protein and DNA containing fraction was obtained by centrifugation and dissolved in a final volume of 200 μl 0.2 M NaOH. An aliquot of the dissolved pellet was mixed with scintillation fluid and the radioactivity was measured on a β-counter. The protein content was determined using bicinchonic acid solution containing 0.1% $CuSO_4$, as described previously [23].

2.7. CTP synthetase assay and calculation of the flux through CTP synthetase

The CTP synthetase activities in crude homogenates were determined as described previously [21,24].

From the distribution of the radiolabel after labeling with [^{14}C]uridine the flux through CTP synthetase could be calculated. The flux through CTP synthetase was calculated using the following ratio [^{14}C]CTP/([^{14}C]UDPG+[^{14}C]UDPNAG+[^{14}C]UTP+[^{14}C]CTP).

2.8. Apoptosis

Apoptosis was detected by measuring caspase-3 activity using the caspase-3 colorimetric assay of R&D Systems (Oxon, UK). The assay was performed according to the manufacturer's manual, except that after lysis of the cells (2×10^6 cells per 50 μl), the cell lysates were centrifuged for 5 min at $18\,620\times g$ at 4°C. The caspase-3 activity is expressed relative to that observed in untreated control cells. The caspase-3 activities of the various cultures that had been treated with CPEC, ATRA or the combination were analyzed with the *t*-test for paired samples, using the MS Excel software package. A *P*-value <0.05 was considered to be statistically significant.

3. Results

3.1. The effect of retinoic acid on the proliferation and induction of differentiation of SK-N-BE(2)c cells

All three retinoic acid isomers inhibited the proliferation of SK-N-BE(2)c neuroblastoma cells in a concentration-depen-

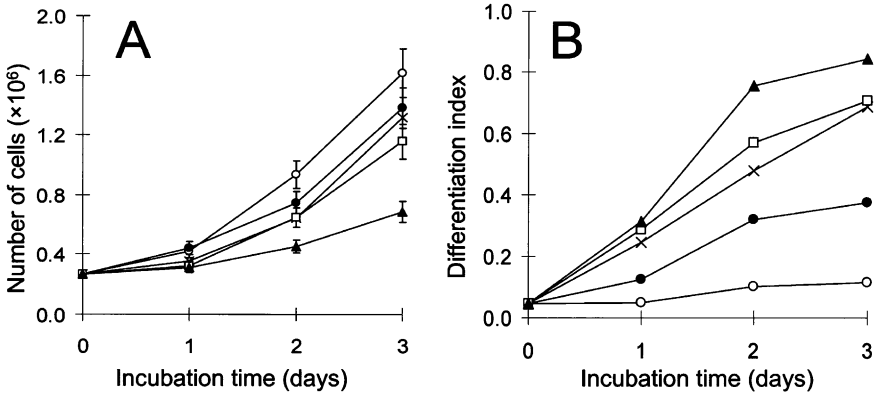

Fig. 1. The effect of ATRA on the proliferation and differentiation of SK-N-BE(2)c cells. A: The effect of ATRA on proliferation. The results shown are the mean of three experiments ± S.D. B: The induction of differentiation by ATRA. The differentiation index was assessed by microscopically examination of 400 cells per culture per day. ○ 0.1% ethanol; ● 1 nM ATRA; × 10 nM ATRA; □ 100 nM ATRA; ▲ 1 μM ATRA.

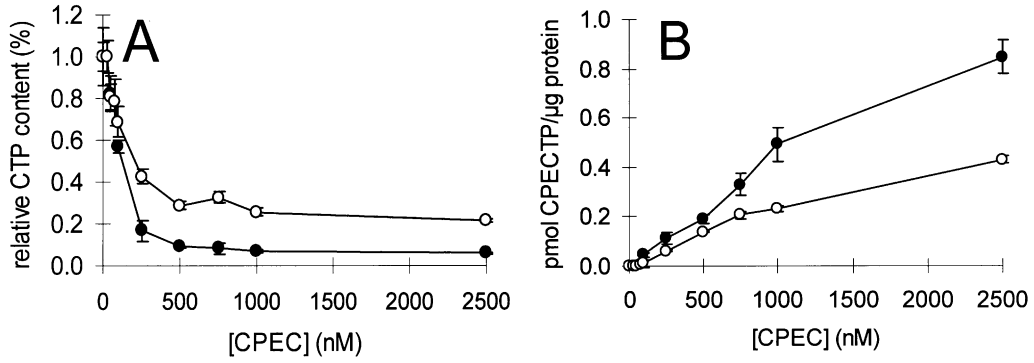

Fig. 2. CTP depletion and CPECTP accumulation before and after treatment of SK-N-BE(2)c cells with ATRA. Cells were incubated with CPEC for 6 h and subsequently extracted and the intracellular CTP content (A) and CPECTP content (B) were determined. ● control cells; ○ cells treated for 6 days with 100 nM ATRA. The values shown are the mean of three experiments ± S.D. The CTP content of control cells was 3.6 ± 0.5 pmol CTP/µg protein and of ATRA-treated cells was 2.8 ± 0.1 pmol CTP/µg protein.

dent manner (Fig. 1A). The results obtained with ATRA were also representative for the results obtained with 9-*cis* and 13-*cis* RA. Proliferation was not strongly inhibited by retinoic acid concentrations up to 100 nM ATRA, but was inhibited strongly by 1 µM ATRA.

Retinoic acid had a strong differentiation inducing effect on SK-N-BE(2)c cells (Fig. 1B). After 3 days of incubation with 1 nM ATRA 35% of the cell population was differentiated. However, when SK-N-BE(2)c cells were incubated with 10–1000 nM ATRA for 3 days 65–80% of the cell population was differentiated. Apparently, differentiation did not exclude proliferation.

3.2. The effect of retinoic acid on CPEC cytotoxicity

Treatment of SK-N-BE(2)c cells with retinoic acid followed by incubation with CPEC attenuated the cytostatic effect of CPEC when compared to cells treated with CPEC only. After pre-treatment of SK-N-BE(2)c cells with ATRA, 9-*cis* RA or 13-*cis* RA, the IC_{50} values were approximately 5 times higher than SK-N-BE(2)c cells treated for 4 days with CPEC only (50 ± 2 nM) (Table 1). The inhibitory effect of retinoic acid on the cytostatic effect of CPEC was even greater when SK-N-BE(2)c cells were co-incubated with CPEC and retinoic acid. In that case, the IC_{50} values were at least 8–20-fold higher when compared to SK-N-BE(2)c cells treated with merely CPEC (Table 1).

Table 1
The effect of retinoic acid on CPEC cytotoxicity

	Pre-treatment IC_{50} (nM)	Co-treatment IC_{50} (nM)
Normal medium	50 ± 2	50 ± 2
ATRA	237 ± 55	400–1000
9-*cis* RA	253 ± 45	> 1000
13-*cis* RA	258 ± 58	> 1000

To assess the effect of pre-treatment, cells were cultured in the presence of 100 nM ATRA, 9-*cis* RA or 13-*cis* and subsequently plated in 24-well plates in the absence of retinoic acid. After overnight adherence the medium was changed for medium containing CPEC and the cells were incubated for 4 days. To assess the effect of co-incubation, cells were plated in 24-well plates and after overnight adherence, the medium was changed for medium containing CPEC and 100 nM retinoic acid and the cells were incubated for 4 days. IC_{50} values (± S.D., $n = 3$) were determined using the MTT assay.

3.3. The effects of retinoic acid on CTP depletion and CPECTP accumulation

The CTP depletion caused by incubating SK-N-BE(2)c cells with CPEC was less in cells pre-treated for 6 days with 100 nM ATRA than in control cells (Fig. 2A). After 6 h of incubation with 1 µM CPEC, the CTP concentration of ATRA-treated cells was 26% of that of untreated cells whereas the CTP content of control cells had dropped to 7% of the initial value. Control cells accumulated more CPECTP than cells that had been pre-incubated with 100 nM ATRA for 6 days (Fig. 2B). After 6 h of incubation with 2.5 µM CPEC, control SK-N-BE(2)c cells had accumulated twice the amount of CPECTP when compared to SK-N-BE(2)c cells that had been pre-treated with 100 nM ATRA for 6 days.

3.4. The effect of retinoic acid on pyrimidine metabolism and ribonucleotide triphosphate levels

The biosynthesis of CTP via CTP synthetase was less in SK-N-BE(2)c cells that had been treated with ATRA than in untreated control cells. The CTP synthetase activity in crude cell homogenates of cells treated with 100 nM ATRA for 6 days decreased from 16.6 ± 2.1 pmol CTP/mg protein/h in control cells to 9.4 ± 0.7 pmol CTP/mg protein/h in ATRA-treated cells. The same trend was observed with higher concentrations of ATRA and shorter incubation periods (data not shown). On the other hand, the salvage of pyrimidine nucleosides was increased (Fig. 3). Both the accumulation of cytosine nucleotides and incorporation into DNA of radiolabeled cytidine increased 2-fold after treatment of SK-N-BE(2)c cells for 2 days with 10 µM ATRA. When radiolabeled uridine was used instead of radiolabeled cytidine, the accumulation of metabolites and the incorporation of label into DNA were, respectively, 1.6- and 1.4-fold higher in ATRA-treated cells than in control cells. After 3 h incubation with 1.8 µM [^{14}C]uridine control cells contained 0.183 ± 0.010 pmol {[^{14}C]UDPG+[^{14}C]UDPNAG}/µg protein, 0.226 ± 0.020 pmol [^{14}C]UTP/µg protein and 0.0668 ± 0.080 pmol [^{14}C]CTP/µg protein. SK-N-BE(2)c cells that had been treated with 10 µM ATRA for 2 days prior to the labeling with 1.8 µM [^{14}C]uridine contained 0.277 ± 0.020 pmol {[^{14}C]UDPG+[^{14}C]UDPNAG}/µg protein, 0.415 ± 0.036 pmol [^{14}C]UTP/µg protein and 0.070 ± 0.005 pmol [^{14}C]CTP/µg protein. Thus, the flux through CTP synthetase, calculated as described in Section 2, was 0.14 in control cells and 0.09 in

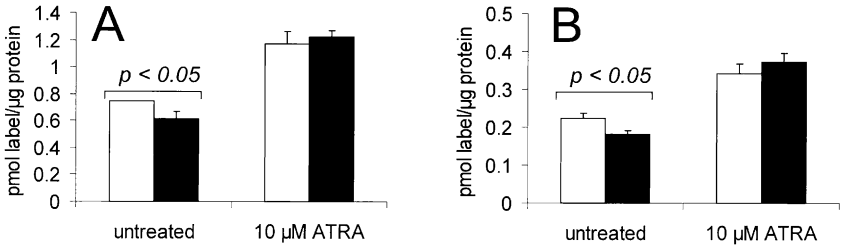

Fig. 3. The effect of ATRA on pyrimidine salvage. Cells were pre-treated with 10 μM ATRA for 2 days after which they were incubated with 2.0 μM [^{14}C]cytidine or 1.8 μM [^{14}C]uridine for 3 h. The total amount of radioactive metabolites was measured (A), as well as the incorporation of label into DNA (B). In untreated control cells, the salvage and the incorporation of metabolites into DNA of uridine was significantly higher than that of cytidine ($P < 0.05$), after incubation with ATRA, the difference between uridine and cytidine salvage was not statistically significant. The results shown are the mean of three experiments ± S.D. When no S.D. bar is visible, then S.D. < 0.05 pmol radiolabel/μg protein. White bars: [^{14}C]uridine, black bars [^{14}C]cytidine.

cells that had been treated with 10 μM ATRA for 2 days. Uridine and cytidine mono- and diphosphates were only present in trace amounts. The contribution of CTP synthetase to the biosynthesis of CTP was, therefore, decreased by 36% in SK-N-BE(2)c cells treated with ATRA when compared to untreated control cells.

No major changes in the total pools of UTP, CTP, ATP and GTP were observed in cells that were treated with ATRA (0.1–10 μM) for 2–6 days when compared to untreated cells.

3.5. The effect of retinoic acid on CPEC-induced apoptosis

When SK-N-BE(2)c cells were incubated with 250 nM CPEC for 3 days, a 2-fold increase in the caspase-3 activity was observed as compared to untreated controls, reflecting the induction of apoptosis. Culturing the cells in the presence of 100 nM ATRA for 3 days did not induce caspase-3 activity. Combined incubation of 100 nM ATRA and 250 nM CPEC resulted in less caspase-3 activity (1.6-fold when compared to controls) than incubation with 250 nM CPEC alone ($P = 0.03$). Pre-treatment with 100 nM ATRA for 2 days, followed by 3 days of incubation with 250 nM CPEC resulted in 1.9-fold increase in caspase-3 activity when compared to controls.

4. Discussion

We have previously shown that CPEC as a single agent has profound and long lasting cytostatic effects on human neuroblastoma cells in vitro [21]. In this paper, we demonstrate that retinoic acid largely attenuates the cytostatic effect of CPEC. This is best illustrated by the 5–20-fold increase in the IC_{50} value for CPEC upon either co- or pre-incubation with retinoic acid.

Because all three isomers of retinoic acid had the same effect on the toxicity of CPEC, ATRA was used as the representative compound in the metabolic studies. To some extent, the acquired resistance towards CPEC might be due to decreased proliferation caused by retinoic acid. When proliferation slows down or is arrested, de novo nucleotide metabolism is reduced and salvage metabolism upregulated [25]. In such a situation, a drug targeted at de novo synthesis is likely to become less cytotoxic.

Our metabolic studies demonstrated that cells pre-treated with retinoic acid showed a decreased accumulation of CPECTP, the effective compound that inhibits CTP synthetase [20], when compared to untreated control cells. A decreased uptake of CPEC would be in line with the fact that a decreased expression of the nucleoside transporters, by which CPEC is taken up by the cell, was observed in differentiated neuroblastoma cells [26,27].

Although the accumulation of CPECTP was less in SK-N-BE(2)c cells treated with ATRA, the depletion of CTP caused by CPEC was still profound. Therefore, the decreased accumulation of CPECTP was probably not the sole cause of the resistance towards CPEC induced by retinoic acid. The flux through CTP synthetase of radiolabeled uridine was reduced by 36% in ATRA-treated cells when compared to control cells. This observation is in line with the fact that the CTP synthetase activity in cell homogenates was 43% less in SK-N-BE(2)c cells that had been treated with ATRA than in untreated SK-N-BE(2)c cells. The preferential salvage of cytidine over uridine in ATRA-treated cells might allow the cells to bypass at least partly the inhibition of CTP synthetase by CPECTP. Previous studies have also shown a decreased contribution of CTP synthetase in the biosynthesis of CTP in differentiated neuroblastoma and gastric cancer cells as compared to their more primitive counterparts [28,29]. Thus, a less significant role for the synthesis of CTP via CTP synthetase in ATRA-treated cells and a relatively increased utilization of cytidine nucleotides may contribute to the decreased sensitivity towards CPEC.

The resistance induced by retinoic acid towards CPEC-induced apoptosis may partially be explained by induction of differentiation, which is paralleled by decreased induction of apoptosis in ATRA-treated cells [30,31]. ATRA has been reported to inhibit the response to apoptotic stimuli in neuroblastoma by several mechanisms such as suppression of the cell death pathway by altered Bcl-2 expression and altered trafficking of the p53 protein [30–33].

In conclusion, we have shown that retinoic acid largely blocks the cytostatic and apoptotic effects of CPEC in SK-N-BE(2)c neuroblastoma cells. Furthermore, retinoic acid increased the salvage of uridine and cytidine and decreased the contribution of CTP synthetase to the intracellular CTP pool. Our findings may have clinical implications as they demonstrate that the sequence of administration of drugs in a multidrug schedule plays a pivotal role. At present, retinoic acid is being used for the treatment of patients suffering from neuroblastoma. In case of a relapse after treatment with retinoic acid neuroblastoma cells might have become resistant to other anti-metabolites than CPEC as well.

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