Pergamon

Biochemical Pharmacology, Vol. 50, No. 2, pp. 277–279, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00024-0

Short Communication

THE EFFECT OF CYCLOPENTENYL CYTOSINE ON HUMAN SK-N-BE(2)-C NEUROBLASTOMA CELLS

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(*Received* 29 *July* 1994; *accepted* 23 *December* 1994)

Abstract—Human neuroblastoma SK-N-BE(2)-C cell-line cells were cultured in the presence of various concentrations of cyclopentenyl cytosine (CPEC). In the absence of cytidine, the IC_{50} value of CPEC for SK-N-BE(2)-C cells was 100 nM after 72 hr drug exposure. The IC_{20} value was 1 μM after 24 hr of exposure to CPEC in the presence of 10 μM cytidine, whereas in the absence of cytidine, CPEC at 1 μM resulted in an IC_{40} value after 24 hr. Therefore, cytidine partially prevented the cytostatic effect of CPEC. Cells cultured with 1 μM CPEC for 72 hr were enriched by approximately 410% with mono- and oligonucleosomes in comparison with cells cultured without CPEC. This enrichment was partially prevented with 10 μM deoxycytidine and completely prevented with 10 μM cytidine.

Key words: CPEC; cyclopentenyl cytosine; apoptosis; CTP-synthetase; neuroblastoma; SK-N-BE(2)-C

Mature neuronal cells no longer proliferate and therefore do not need large amounts of nucleotides. In contrast, cancer cells such as neuroblastoma cells exhibit a high need for nucleotides, as their metabolism is accelerated to facilitate cell proliferation. In many types of tumour cells increased activities of 'key'-enzymes of the nucleotide pathways, such as CTP-synthetase and inosine-monophosphate-dehydrogenase, have been observed [1–4]. The regulation of CTP-synthetase is not only critical for maintaining the level of CTP but for the level of dCTP as well, since the latter is the only deoxyribonucleoside triphosphate which does not regulate its own synthesis at the level of ribonucleoside diphosphate reductase [5].

A chemotherapeutic strategy based on reducing the overactivity of these 'key'-enzymes may induce an imbalance in the cell-cycle 'machinery', cause a reduction of the proliferative potential and initiate apoptosis [6]. CPEC† (NSC 375575), a carbocyclic analogue of cytidine in which the ribofuranose moiety is replaced by a cyclopentenyl ring, has an antineoplastic activity against various tumours [7]. Recently, phase I clinical trials with this agent have been initiated. The effect of CPEC on neuroblastoma cells has not been studied so far.

Materials and Methods

Cell line. SK-N-BE(2)-C human neuroblastoma cell-line cells were cultured in DMEM (Gibco Laboratories, Paisley, U.K.) supplemented with 2 mM L-glutamine (Flow Laboratories, Irvine, U.K.), 50 IU/mL penicillin, 50 μg/mL streptomycin (Imperial, U.K.), 10% (v/v) FCS (Gibco Laboratories) and propagated continuously [2]. These SK-N-BE(2)-C cells were originally isolated from a patient who had undergone a chemotherapeutic treatment and had a 150-fold amplified *N-myc* gene [8]. For experiments, cells were cultured in a chemically-defined medium consisting of DMEM supplemented with 0.5% (w/w) highly purified human serum albumin (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany), 50 IU/mL penicillin, 50 μg/mL streptomycin (Imperial, U.K.), 3 μM uridine, 4 μM hypoxanthine, 2 mM L-glutamine (Flow Laboratories) and other components as prescribed by Bottenstein and Sato [9]. The viability of the cells prior to the start of experiments was greater than 95% as determined with the trypan-blue exclusion method.

Determination of drug sensitivity. Cells (2×10^3 cells or dilutions thereof) were plated into 96-well microtitre plates and incubated with CPEC (NSC 375575, Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, NC, U.S.A.) at 37° for 24 hr or 72 hr. A modified (MTT) assay (Boehringer Mannheim, GmbH Biochemica) was used to determine the sensitivity of SK-N-BE(2)-C neuroblastoma cells to CPEC [10]. Additionally, cells were counted under a phase-contrast microscope and/or quantified by measuring protein concentrations [11].

Quantitative determination of apoptosis. Cells were plated as described above and after 72 hr exposure to CPEC were washed gently with PBS. Apoptosis was assessed by detection of core histones and DNA-fragments (mono- and oligonucleosomes) with a sandwich ELISA (Boehringer Mannheim GmbH Biochemica) [12]. Apoptosis was also morphologically confirmed by the presence of cells showing apoptotic bodies and/or zeiosis.

Nucleotide determination. Cells were cultured with 1 μM CPEC for 24 hr and subsequently nucleotides were extracted [2]. Analysis of the ribonucleotides was performed by anion-exchange HPLC [7]. The protein content of the remaining cell pellet was determined [2, 11].

Results and Discussion

Cell-survival percentages for non-synchronized SK-N-BE(2)-C cells exposed for 72 hr to various CPEC

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† Abbreviations: CPEC, cyclopentenyl cytosine; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide.

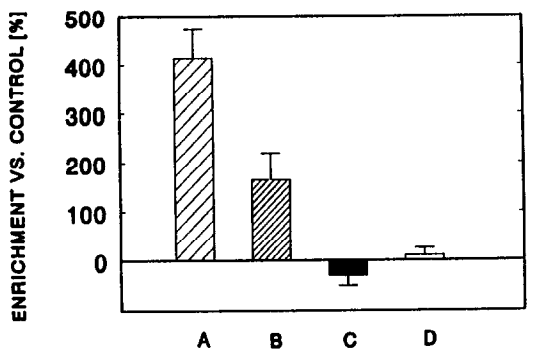

Fig. 1. The relative enrichment of SK-N-BE(2)-C cells with mono- and oligonucleosomes after 72 hr exposure to 1 μM CPEC compared to control cells (bar A), with 1 μM CPEC and 10 μM deoxycytidine (bar B), with 1 μM CPEC and 10 μM cytidine (bar C) and with 1 μM CPEC and 10 μM deoxycytidine and 10 μM cytidine (bar D). The mono- and oligonucleosomes were colorimetrically quantified (N = 3) in a sandwich ELISA using monoclonal antibodies directed against H2A, H2B, H3 and H4 core-histone-associated DNA-fragments and an anti-DNA peroxidase monoclonal antibody [11]. The absorbance value (A_{405}–A_{490}) of the control = 0.32.

concentrations were determined in comparison to control cells with an MTT-assay. The IC_{50} values of CPEC determined under these conditions was approximately 100 nM. After 24 hr exposure to 1 μM CPEC, CPE-CTP was present in a concentration of 3.3 ± 0.5 pmol/μg protein (mean ± SD, N = 3). The CTP pool was 3.8 ± 1.0 pmol/μg protein (mean ± SD, N = 3) in untreated SK-N-BE(2)-C cells and was almost completely depleted in the CPEC-treated cells. The UTP-pool size remained unchanged under these conditions. The cytostatic effect of CPEC in neuroblastoma cells is therefore, as in other cell-types [7], mediated by a depletion of the CTP pool and an increase of the CPE-CTP pool.

Incubation of non-synchronized SK-N-BE(2)-C cells for one day with 1 μM CPEC resulted in 60% of the number of cells of the control (IC_{40}). After an incubation period of 3 days approximately 30% of the cells were present compared to control cells, representing presumably mainly G(0)/G(1)-phase cells. These latter cells were enriched by 410% (Fig. 1) with mono- and oligonucleosomes and showed further markers of apoptosis such as apoptotic bodies and zeiosis (morphologically comprising approximately 110 cells per 300 cells). Co-incubation of 1 μM CPEC with 10 μM deoxycytidine resulted in a two-fold increase of cells at the end of the third day of this incubation compared to CPEC alone. With CPEC and deoxycytidine, viability increased from 50 to 67% and the percentual enrichment with mono- and oligonucleosomes per cell (Fig. 1) was partially prevented from 410 to 165% of the controls (approximately 70 cells per 300 cells showed morphologic features of apoptosis). Therefore, the cytostatic and the cytotoxic action of CPEC in these neuroblastoma cells is not only mediated by an inhibition of RNA-elongation and consequently of protein synthesis but also by a disturbance of DNA-synthesis.

An incubation period of three days with CPEC (1 μM) supplemented with cytidine (10 μM) resulted in 80–85% of these cells when compared to the control cells (this IC_{20} value had already been observed at the end of the first day) with a concomitant rise in the viability of the cells from 50% with CPEC alone to 91% in the presence of CPEC and cytidine. This partial reversal of the cytostatic effect of CPEC with cytidine is in line with the competitive manner by which uridine, cytidine and CPEC are metabolized by the same uridine/cytidine kinase [13]. IC_{50} values of CPEC obtained for cells cultured in a medium containing serum should therefore be interpreted with care since the levels of pyrimidine-nucleosides do show considerable variations between different batches of FCS [14, 15]. With CPEC and cytidine, no increase in the amount of mono- and oligonucleosomes/cell was observed compared to control cells.

The mechanism of apoptosis itself as well as all the specific metabolic alterations imposed by CPEC leading to apoptosis are still unknown. The shortage of dCTP caused by CPEC may, without the incorporation of CPEC, have led to erroneous DNA-strand formation by misincorporation of other deoxyribonucleotides into DNA and subsequently in an abortive mitosis resulting in apoptosis [16]. This is in agreement with our result that deoxycytidine partially prevented this process. Our results suggest that apoptosis initiated by CPEC is at least partially caused by a disturbed DNA-synthesis. In the presence of a sufficient amount of dCTP, apoptosis was still observed. This apoptosis is still CTP-dependent. This latter process is presumably mediated by an inhibited RNA-elongation and as a consequence an inhibited protein synthesis. Therefore, another mechanism for the initiation of apoptosis by CPEC must exist. This mechanism could be related to conflicting signals concerning the cell-cycle traverse, already suggested as a possible cause of apoptosis [17]. The levels of some cell-cycle proteins might decrease more rapidly than others, depending on the turnover rate of these proteins.

Acknowledgement—This study was supported by a grant from the Foundation for Pediatric Cancer Research S.K.K. no. 90-02.

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