Cyclopentenyl cytosine sensitises SK-N-BE(2)c neuroblastoma cells to cladribine

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Introduction

Cyclopentenyl cytosine (CPEC) is an inhibitor of CTP synthetase and possesses anti-tumour activity against neuroblastoma *in vitro* (1, 2). Incubation with CPEC depletes the (deoxy)cytidine nucleotide pools, and causes S-phase accumulation (1, 2). This makes the combination of CPEC with deoxynucleoside analogues attractive for chemotherapy. CPEC proved to be an excellent modifier of the deoxycytidine analogue cytarabine in both neuroblastoma and leukaemic cells (3, 4).

2-Chloro-2'deoxyadenosine or Cladribine (CdA) is an analogue of deoxyadenosine and is used in the treatment of haematological malignancies. However, no anti-tumour activity of CdA against solid tumours has been observed in clinical trials. CdA is an inhibitor of DNA synthesis as well as DNA repair. It is also a potent inhibitor of ribonucleotide reductase, which is the primary source of deoxynucleotides for DNA synthesis (5).

Although CdA is a purine analogue, the first and rate-limiting step in its activation is catalysed by deoxycytidine kinase (dCK). Depletion of dCTP achieved via inhibition of CTP synthetase by CPEC may thus lead to an enhanced uptake and anabolism of CdA.

Methods

CdA metabolism was studied using [³H]CdA and HPLC equipped with online radiochemical detection to measure CdA nucleotides and liquid scintillation to measure the incorporation of [³H]CdA into the DNA. ED_{50} values were determined using modified MTT assays. The experiments were started by the addition of CPEC, after the cells had been allowed to adhere overnight. After 24 hr, the medium containing CPEC was replaced by medium containing radiolabelled CdA. After a 24-hr incubation, the cells were extracted and the nucleotide content was examined. DNA synthesis was studied by measuring incorporation of [¹⁴C]Thymidine into the DNA.

Results

SK-N-BE(2)c cells were insensitive to CdA when in-

cubated during four days with concentrations up to 500 nM CdA (figure 1). Increasing the concentration of CdA to 1.5 µM did not result in cytotoxicity. However, when SK-N-BE(2)c cells were pre-incubated with CPEC (100 or 250 nM) for 24 hr, followed by four days of incubation with CdA sensitivity to CdA was observed, ED_{50} values being 419 ± 125 nM and 70 ± 30 nM CdA after pre-incubation with 100 and 250 nM CPEC, respectively.

As a measure of apoptosis, the in vitro caspase-3 activity in lysates was measured after exposure to CPEC, CdA or in combination. After 3 days of continuous incubation with 500 nM CdA no significant increase of the caspase-3 activity was observed. The caspase-3 activity in SK-N-BE(2)c cells which were incubated with 100 nM CPEC for 24 hr, followed by three days of incubation in drug-free medium was increased 6.4-fold (p<0.01) when compared to untreated controls. The caspase-3 activity in SK-N-BE(2)c cells which were pre-incubated with 100 nM CPEC followed by 3 days of continuous incubation with 500 nM CdA was the same as in cells that had been treated with CPEC only. This indicated that apoptosis may not be the predominant form of cell death as the toxicity of CPEC and CdA combined is greater than the toxicity of the separate drugs.

Preincubation of SK-N-BE(2)c cells for 24 hr with 100 nM CPEC followed by 24 hr incubation with 100 or 250 nM CdA increased the amount of intracellular CdA-nucleotides 35- and 3-fold, respectively, when
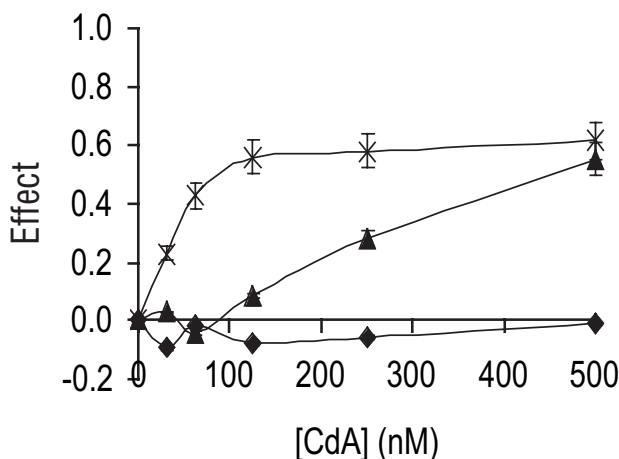

Figure 1. Dose-effect curves of CdA with and without 24 hrs pre-incubation with CPEC determined in SK-N-BE(2)c cells. The data shown are the mean of 4 experiments ± SD. ♦: no CPEC, ▲: 100 nM CPEC, x: 250 nM CPEC.

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Table 1. Modulation of CdA metabolism by pre-incubation with 100 nM CPEC and its effect on DNA synthesis. The results are the mean of three experiments ± SD and are expressed in fmol/µg protein. N.D.: not detected. * p<0.01.

	100 nM [^{3}H]CdA		250 nM [^{3}H]CdA	
	CdA only	CPEC pre-incubation	CdA only	CPEC pre-incubation
[^{3}H]CdAMP	0.13 ± 0.02	3.42 ± 0.43*	1.93 ± 0.28	7.34 ± 0.49*
[^{3}H]CdADP	N.D.	0.32 ± 0.08	0.42 ± 0.02	0.86 ± 0.10*
[^{3}H]CdATP	N.D.	0.74 ± 0.08	0.68 ± 0.05	1.46 ± 0.16*
[^{3}H]CdA in DNA	4.26 ± 0.68	16.1 ± 1.5	41.6 ± 3.1	45.9 ± 2.3
DNA synthesis	100 %	45 %*	240 %*	5%*

compared to cells that had not been preincubated with CPEC. CdAMP was the major metabolite to accumulate (table 1), and may contribute to the observed toxicity. The accumulation of CdAMP indicates that when feedback inhibition on dCK has been suspended, UMP/CMP-kinase becomes the rate-limiting enzyme in the anabolism of CdA.

After preincubation with CPEC the incorporation of [^{3}H]CdA into the DNA increased 4-fold when the cells were incubated with 100 nM CdA for 24 hr (table 1). However, when the cells were incubated with 250 nM CdA, the incorporation of [^{3}H]CdA into the DNA did not increase. Apparently the incorporation of CdA is maximal at 250 nM CdA and cannot be increased further by pre-incubation with CPEC.

Untreated SK-N-BE(2)c cells incorporated 0.22 ± 0.02 pmol [^{14}C]Thymidine/hr into their DNA. DNA synthesis was inhibited by 45% by 100 nM CPEC, unaffected by 100 nM CdA and inhibited by 55 % (p<0.01) by 100 nM CPEC and 100 nM CdA combined. The combination of 100 nM CPEC and 250 nM CdA inhibited DNA synthesis > 95 % (p <0.01), while 250 nM CdA itself did not inhibit DNA synthesis, in fact the incorporation of thymidine into the DNA increased 2.4-fold (p<0.01). It has been shown previously that this increase in thymidine incorporation is not caused by an increased rate of DNA synthesis, but rather an increase in S-phase cells synthesizing DNA (6). The fact that the combination of CPEC and CdA show such profound toxicity towards SK-N-BE(2)c cells may be explained by the following. Both CPEC and CdA cause a depletion of deoxynucleotide pools. CPEC indirectly causes depletion of dCTP pools and CdA is an inhibitor of ribonucleotide reductase. Moreover, CdA causes the erroneous incorporation of deoxynucleotides into DNA (7). These effects combined may cause a metabolic catastrophe, which is fatal to SK-N-BE(2)c cells.

Conclusion

Our results demonstrate that the cytotoxic effects of the deoxyadenosine analogue CdA may be enhanced by inhibition of CTP synthetase using CPEC. While the overall intracellular levels of CdA metabolites are increased, CdAMP is the major metabolite to accumulate upon pre-incubation with CPEC. CdAMP might contribute to the observed toxicity in SK-N-BE(2)c cells. However, a catastrophic imbalance of DNA-precursors may very well be the main cause of the combined toxicities of CPEC and CdA.

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