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Antiproliferative effects and DNA hypomethylation by 5-aza-2′-deoxycytidine in human neuroblastoma cell lines

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5-Aza-2′-deoxycytidine (5-AZA-CdR) is an inhibitor of DNA methylation, and its antileukemic activity has been shown in preclinical and clinical studies. This paper describes the ability of 5-AZA-CdR to inhibit DNA methylation, DNA synthesis and cell growth in several human neuroblastoma cell lines. The stability of cell growth inhibition was ascertained, as well as the ability of the metabolite thymidine to enhance the antiproliferative effect of 5-AZA-CdR. The activity of phosphorylating enzyme deoxycytidine kinase (dCK) was correlated to different levels of sensitivity in several cell lines. The results obtained indicate that 5-AZA-CdR may be an agent for the chemotherapy of neuroblastoma.

Key words: 5-Aza-2′-deoxycytidine, cell proliferation, DNA methylation, human neuroblastoma.

Introduction

Several lines of evidence have related the control of gene expression in higher organisms to the methylation of cytosine in DNA. Specific patterns of DNA methylation have been described which play a role in selective gene silencing and thus act as a mechanism to regulate cell differentiation; typically in fact, methylated genes are not transcribed whereas unmethylated genes are.[1]

5-Aza-2′-deoxycytidine (5-AZA-CdR) is a deoxycytidine analog, which can be used as a substrate by all enzymes [deoxycytidine kinase (dCK), deoxycytosine monophosphates kinase, etc.], leading to the formation of the deoxynucleotide triphosphate pool;[2] it can thus readily replace deoxycytidine and become incorporated into DNA, where it primarily inhibits the methylation process[3] by inhibition of DNA methyltransferase.[4] The postulated role of DNA methylation in the regulation of gene expression[5] suggests that 5-AZA-CdR is a drug of potential interest. It has been reported that this compound is capable of stimulating the expression of differentiated properties in cells of different phenotypes.[6,7] Following the observations of Creusot *et al.*[8] on Friend erythroleukemic cells, it has been reported that 5-AZA-CdR also induces differentiation of human leukemic cells[9] and is very effective as an inhibitory agent of leukemic cell growth.[10,11] Moreover, the different mechanism of action of 5-AZA-CdR, with respect to other nucleoside analogs commonly used as antineoplastic agents, has raised considerable interest due to its possible use for the clinical treatment of leukemia. Since the methylation pattern is transmitted to daughter cells in cell division, any effects arising from its alteration can be expected to be long-lasting.[12] Clinical trials in patients with acute leukemia have so far produced promising results along this line.[13]

Similarly, in murine and human neuroblastoma lines, 5-AZA-CdR has been described as being more effective in preventing cell proliferation as compared with cytosine arabinoside (ARA-C).[7,14]

Human neuroblastoma is a highly malignant tumor of the nervous system; it usually manifests itself in early childhood. The tumor is characterized by the presence of immature and rapidly proliferating cells. The expression of several specific neuronal markers can be stimulated in neuroblastoma cell lines by several agents (e.g. cAMP, prostaglandin, nucleoside analogs, retinoic acid, specific factors) which usually also block cell proliferation in a reversible way.[15]

Therefore, neuroblastoma cells appear to respond to a number of factors capable of modulating their genomic expression and their differentiated state. These properties are shared by C1300 mouse and human neuroblastomas.[15,16]

Another common feature of human neuroblast-

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omas is their poor responsiveness to currently available chemotherapeutic agents. The occasional spontaneous regression of highly immature neuroblastoma to more differentiated benign ganglioneuroma,[17] together with the ability of several neuroblastoma clones to undergo differentiation *in vitro*, have suggested the use of differentiating agents for possible chemotherapeutic strategies.

On the basis of these considerations, we have undertaken a study to examine the response of several human neuroblastoma lines to 5-AZA-CdR exposure.

Materials and methods

Materials

5-AZA-CdR (Pharmachemie, Haarlem, The Netherlands) dissolved in 10 mM potassium-phosphate buffer, pH 7.0, was sterilized by filtration through a 0.22 μm filter (Millipore, Bedford, MA) and stored until use for a maximum of 2 months at −80 °C. The drug was added to the culture medium at a final concentration ranging between 0.008 and 5.0 μM for 24 h. Thymidine (TdR) (Sigma, St Louis, MO) was dissolved in the same buffer; stock solutions were sterilized as described above and stored at −20 °C until use. [Methyl-^{3}H]TdR (41 Ci/mmol), [6-^{3}H]uridine (23 Ci/mmol) and deoxy[5-^{3}H]cytidine (22.5 Ci/mmol) were obtained from Amersham International (Amersham, UK). All other chemicals were of the highest grade available.

Cell culture

Several human neuroblastoma lines were used in these studies. CHP-100, CHP-134,[18] SK-N-BE,[19] LA-N-1[20] and IMR-32[21] (American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) supplemented with penicillin (50 IU/ml), streptomycin (50 μg/ml) and 10% (v/v) heat-inactivated (56 °C for 30 min) fetal calf serum (FCS) (all obtained from Flow Laboratories). SK-N-MC[22] (ATCC) was cultured in Eagle's minimum essential medium supplemented with 10 ml/l of (100 times) non-essential amino acids, 10% (v/v) FCS, penicillin and streptomycin as described above. All cell lines were grown at 37 °C in a humidified 5% CO_2 incubator.

Cell proliferation assays

Cell counts were performed as described previously.[7,14] Cells (1×10^5) were plated in 60 mm culture dishes (Falcon, Lincoln Park, NJ) containing 5 ml complete medium, and the drug was added to the medium 24 h later. At 24, 48 and 72 h later the cells were removed with 0.25% trypsin (Flow Laboratories) in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS), pH 7.4, and counted with a Burker hemocytometer.

DNA synthesis measurements

Cells (2×10^4/well) were seeded in 24-well culture plates (Costar, Cambridge, MA) containing 13 mm round cover slips (Lux Thermanox 5413; Miles, Naperville, IN) and treated with the drug as above. At different intervals, cells were pulsed with 0.25 μCi/well [methyl-^{3}H]TdR. Following 4 h incubation with labeled TdR, the cells on the cover slips were washed twice with PBS and DNA precipitated with cold 10% (w/v) trichloroacetic acid (TCA), and repeatedly washed with cold 1% (w/v) TCA. The cover slips were then transferred into counting vials and cells solubilized with Lumasolve (Lumac/3M, Schesberg, The Netherlands) for 20 min at 50 °C. Incorporated radioactivity was determined by counting in a liquid scintillation counter (Model LS 8100 Beckman Liquid Scintillation System).

Cloning efficiency

The viability of the neuroblastoma cells after exposure to 5-AZA-CdR was determined by cloning efficiency.

The cells, plated at low density (1000–4000 in 60 mm culture dishes), were treated with the drug 24 h after plating. Between 8 and 10 days later the colonies were fixed and stained with 10% Giemsa in 20% (v/v) methanol for 10 min. Colonies containing more than 200 cells were counted with a magnifying transillumination lens. The cytotoxicity of the drug was expressed as a percentage of survival relative to the untreated control.

dCK assay

The cells, grown in normal conditions, were harvested, centrifuged and resuspended in 5 mM Tris-HCl pH 7.4. Cell lysis was obtained with a sequence of fast freeze–thaws, the homogenates were centrifuged, and the recovered supernatants assayed for dCK as described by Vesely *et al.*[23] The reaction was carried out at 37 °C using deoxy[5-^{3}H]-cytidine as substrate. At appropriate points in time, aliquots were removed and filtered through DEAE 81 filters (Whatman, Maidstone, UK). After being washed and dried, the radioactivity on the filters was measured in a Liquid Scintillation Counter. The activity of the enzyme was expressed as pmol of product formed/10 min/100 μg of protein.

Protein concentration of the homogenates was determined according to the Bradford method,[24] using bovine serum albumin as standard.

DNA methylation measurements

One day after plating, the cells (9×10^6) in the complete medium were exposed for 9 h to 5-AZA-CdR at indicated concentrations, washed free of the drug and then incubated with 60 μCi [6-^{3}H]uridine for 18 h. At the end of the incubation period, the cells were collected and lysed in a buffer containing 10 mM Tris, 1 mM EDTA, pH 8.0, and 1% sodium dodecyl sulfate (SDS). The lysates were centrifuged overnight through a solution of 4.5 M CsCl with 2 mM ethidium bromide[25] at 50 000 r.p.m. in a Beckman VTi-65 rotor and the DNA was recovered from the fluorescent band. After removal of ethidium bromide, DNA was dialyzed overnight against 10 mM Tris, 1 mM EDTA, pH 7.0, and precipitated with cold ethanol.

The DNA was digested in 70% (v/v) perchloric acid at 60 °C for 16 h. After neutralization with 10 N KOH, the supernatant was analyzed by HPLC using a MicroPak MCH-5-n-cap column (0.46 × 150 mm) (Varian Associates, Sunnyvale, CA), with 100 mM sodium formate, pH 4.2, as the mobile buffer. The 5-methylcytosine (MC) formed during labelling was quantified from the counts present in the methylcytosine and cytosine (C) peaks using the formula:

$$\%\ \text{DNA methylcytosine} = \frac{\text{c.p.m. in MC peak}}{\text{c.p.m. in C + MC peaks}} \times 100$$

Results

Cell proliferation assays

Six human neuroblastoma lines were used in this study. The action of 5-AZA-CdR on cell growth was evaluated using different approaches. Figure 1 shows the results of the experiments comparing growth curves of the cell line SK-N-MC after a 24 h treatment with increasing concentrations of the drug (0.05–1 μM). The result of this treatment is a dose-dependent inhibition of cell proliferation. The inhibition gradually increases over the time period considered; it already appears at the end of the period of exposure to the drug (24 h) and becomes progressively more prominent as control cells continue to increase in number.

Similar dose- and time-dependent responses to 5-AZA-CdR treatment were observed in all the cell lines tested in this study; however, the level of sensitivity to the drug varied considerably among them. Figure 2 shows a comparison of the extent of growth inhibition for the lines tested following a 24 h exposure to the drug and an additional 48 h culture time. It can be observed that a 0.5 μM concentration induces a growth inhibition ranging from 30% for IMR-32 to 90% for SK-N-MC, with intermediate values for other lines. Very similar results were obtained when the effect of 5-AZA-CdR on DNA synthesis was taken into consideration. Table 1 shows the inhibition of the DNA synthesis in two lines which appear more responsive

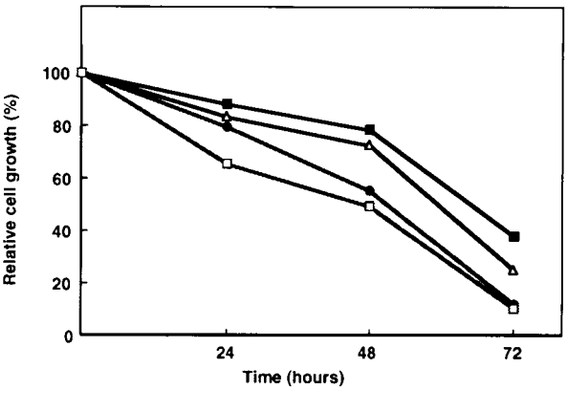

Figure 1. Inhibition of SK-N-MC cell proliferation by 5-AZA-CdR at different concentrations, shown as percentage of cell count with respect to the control. The drug was added to the medium at 24 h, and cells counted 24, 48 and 72 h later. Values represent the average of duplicate cultures from four independent experiments with SD ranging between 3 and 13%. ■, 0.05 μM; △, 0.1 μM; ●, 0.5 μM; □, 1.0 μM.

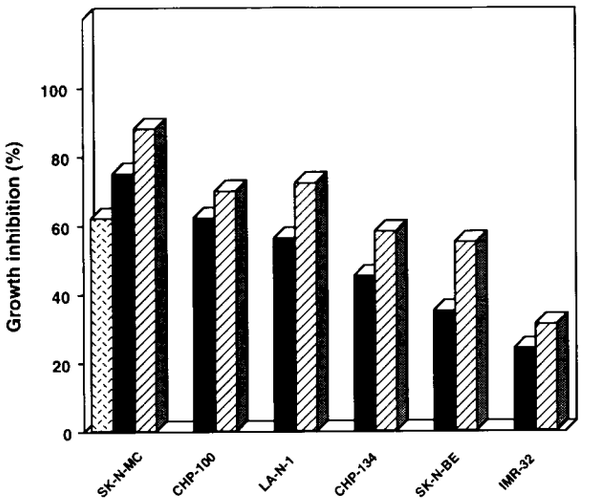

Figure 2. Growth inhibition of 5-AZA-CdR on six human neuroblastoma cell lines. Cells were exposed to the micromolar drug concentrations as indicated for 24 h and counted 72 h after the addition of the drug. The values shown represent the percentage of growth inhibition with respect to the untreated cultures. Each histogram represents the mean of at least three independent experiments with SD ranging between 5 and 10%. ⊡, 0.05 μM; ■, 0.1 μM; ▨, 0.5 μM.

Table 1. Effect of 5-AZA-CdR on DNA synthesis in CHP-100 and SK-N-MC

Cell line	Concentration (μM)	Inhibition (%)		
		24 h	48 h	72 h
CHP-100	0.1	24 ± 15	41 ± 30	47 ± 12
	0.5	30 ± 2	50 ± 14	77 ± 2
	1.0	27 ± 15	57 ± 14	84 ± 5
SK-N-MC	0.05	ND[a]	33 ± 19	57 ± 21
	0.1	19 ± 12	38 ± 25	70 ± 16
	0.5	19 ± 10	54 ± 19	84 ± 14
	1.0	31 ± 14	53 ± 18	84 ± 15

Cells (2×10^4/well) were plated in Costar multi-well trays and treated with the different concentrations of the drug. At 24, 48 and 72 h, 0.25 μCi of [methyl-^{3}H]TdR was added per well and the incorporated radioactivity determined 4 h later. Inhibition is expressed as percentage of the control values. The reported values represent the average of quadruplicate cultures from four independent experiments ±SD.
[a] ND = not determined.

to treatment. The levels of inhibition are comparable to those shown in Figure 2.

Cloning efficiency

Cell viability following a 24 h exposure to 5-AZA-CdR was estimated as colony forming

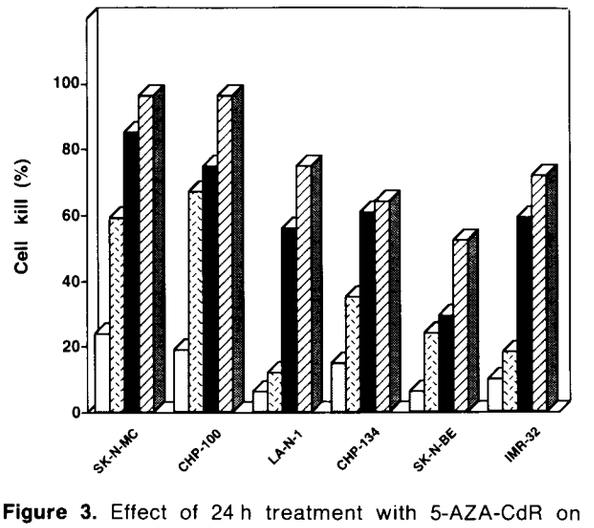

Figure 3. Effect of 24 h treatment with 5-AZA-CdR on colony formation by six human cell lines. Drug concentrations indicated are micromolar. Cell kill was calculated from the number of surviving colonies as a percentage of colonies in the control. Values represent the average of duplicate cultures from four independent experiments with SD ranging between 5 and 12%. □, 0.01 μM; ⊡, 0.05 μM; ■, 0.1 μM; ▨, 0.5 μM.

ability. Figure 3 shows the dose-dependent cell kill for all the cell lines examined. At a concentration as low as 0.05 μM 5-AZA-CdR, an inhibition of cloning efficiency was observed which ranged between 14 and 68% for the various cell lines. SK-N-MC and CHP-100 appeared to be the most sensitive cell lines also in this assay.

We tested the possibility of increasing the 5-AZA-CdR effect with a synergistic mechanism, treating the cells with both 5-AZA-CdR and a natural compound such as TdR. The TdR concentration was chosen because it was not toxic for cells. Figure 4 shows an experiment of colony formation ability on SK-N-MC cells treated with different concentrations of 5-AZA-CdR in the presence of 2 μM TdR. The inhibition of cloning efficiency was referred to culture controls grown without or in the presence of 2 μM TdR. An increase in cell kill was observed, which was more prominent at the lowest concentrations of the drug (about four times more at 0.008 μM 5-AZA-CdR).

DNA methylation measurements

Since 5-AZA-CdR does not directly alter DNA synthesis and cell cycle progression,[26] but primarily inhibits DNA methyltransferase reaction, the conversion of DNA cytosine to methylcytosine in the presence of the drug has been measured. An

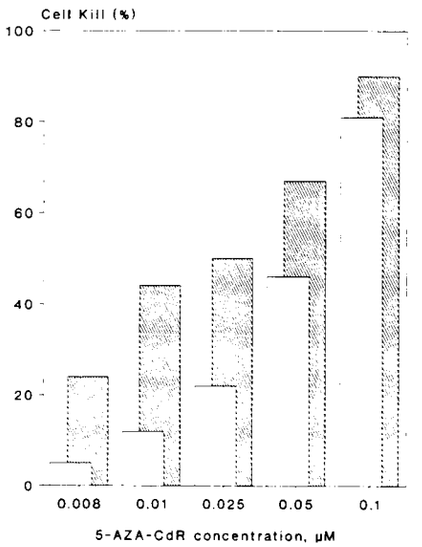

Figure 4. Synergistic effect of 5-AZA-CdR and TdR on colony formation by SK-N-MC. Cells were exposed simultaneously for 24 h to both compounds. TdR concentration was 2 μM. The reported values are the mean of three experiments. □, 5-AZA-CdR; ▒, 5-AZA-CdR + TdR.

exposure time of 9 h to different concentrations of 5-AZA-CdR was followed by an 18 h incubation with [6-^{3}H]uridine, in order to label pyrimidine bases, which were then separated by HPLC. The radioactivity present in each peak was measured and the extent of methylation calculated from the ratio MC/C. As shown in Table 2, a significant inhibition of DNA methylation is observed in all cell lines although quantitative differences are evident. It is interesting to note that the lowest hypomethylation is found in IMR-32, which is also the line showing the lowest cell growth inhibition.

Table 2. Effect of 5-AZA-CdR on DNA methylation

Cell line	Inhibition (%)		
	0.1 μM	1.0 μM	5.0 μM
IMR-32	<1	<1	12
SK-N-BE	<1	21	ND[a]
SK-N-MC	ND[a]	28	ND[a]
LA-N-1	<5	31	ND[a]
CHP-134	31	33	ND[a]
CHP-100	24	47	ND[a]

Six human neuroblastoma cell lines were treated with different concentrations of 5-AZA-CdR. Cells were labeled with 60 μCi [6-^{3}H]uridine and DNA was extracted. After hydrolysis C. MC and T were separated by HPLC and radioactivity measured. Inhibition is expressed as percentage of the control values. The values reported represent the average of experiments carried out in duplicate for each cell line.
[a] ND. not determined

Table 3. Activity of dCK

Cell line	Specific activity
SK-N-MC	31.2 ± 5.4
IMR-32	9.7 ± 2.5
CHP-100	15.5 ± 1.7
LA-N-1	17.4 ± 3.7

Enzyme activity was expressed as pmol of dCMP formed in 10 min/100 μg of protein. Values represent the average of three experiments ±SD

dCK assay

In order to clarify the basis for the different sensitivity levels to 5-AZA-CdR in the various cell lines, the activity of the enzyme dCK was measured in a representative group of cell lines. Table 3 reports the specific activity of the enzyme from the cell lines considered. The level of activity seemed to correlate positively with the drug effectiveness. In the SK-N-MC cell line which displayed the higher drug response, the activity was about three times higher compared with IMR-32.

Expression of differentiation markers

On the basis of previous observations on murine neuroblastoma,[7] the capability of 5-AZA-CdR to induce expression of the neuronal phenotype was also considered. In SK-N-BE cells drug exposure causes a moderate increase in cell size and neurite outgrowth. In several cases, a slight increase (about 20–25%) of acetylcholinesterase activity was observed after 5-AZA-CdR treatment. However, no significant differences in treated or control cells were detected for the expression of other neuronal markers such as 68K neurofilament, VGF8 protein and secreted tissue plasminogen activator (data not shown).

Discussion

5-AZA-CdR is an interesting antileukemic agent and, because of its specific hypomethylating effect on DNA, a potential differentiation inducer.

Neuroblastoma sometimes undergoes spontaneous differentiation accompanied by a benign evolution.[17] Cell lines derived from various human neuroblastomas appear to be an appropriate experimental system to test the effectiveness of 5-AZA-CdR on neuroblastoma growth.

The results presented confirm that the drug was

effective on several human neuroblastoma cell lines isolated from different biopsies and exhibiting different properties in culture, as far as cell proliferation, morphological and biochemical differentiation are concerned.

The growth inhibition produced by 5-AZA-CdR on all cell lines was time- and dose-dependent (Figures 1 and 2). Although the drug sensitivity level varied among the various cell lines, cell proliferation was significantly inhibited in all cases studied. The higher inhibition during longer culture times, although drug presence in the culture was limited to the initial 24 h, indicates that 5-AZA-CdR effects on cell proliferation are rather stable; it is also in agreement with the known effect of 5-AZA-CdR which acts primarily as an inhibitor of DNA methylation. The data reported in Table 2 show that DNA hypomethylation is evident after a 9 h exposure to the drug before significant inhibition of DNA synthesis and cellular proliferation can be detected.

As for inhibition of growth, the level of the DNA hypomethylation after 5-AZA-CdR treatment also varied in the cell lines tested. Although a direct correlation between the extent of hypomethylation and growth inhibition cannot be clearly established, it is interesting to note that the cell line showing the lowest inhibition of DNA methylation (IMR-32) is also the lowest inhibited in cell growth and in cloning efficiency.

In order to explain the different levels of cell sensitivity to the drug, the level of the enzyme dCK, which converts 5-AZA-CdR to 5-AZA-dCMP, was measured. In fact, 5-AZA-CdR phosphorylation to nucleotides by specific kinases is the rate-limiting step for its incorporation into DNA.

Indeed, our results indicate a correlation between the activity of dCK (Table 3) and the level of growth inhibition (Figure 2) and cytotoxicity (Figure 3). In fact, the rate of dCK activity was about three times higher for SK-N-MC, which is 10 times more sensitive with respect to IMR-32, with intermediate values for other lines.

In addition, the stability of drug action was evaluated by assaying its effects on colony forming ability. A very low drug concentration caused a clear inhibition of cloning efficiency. A low recovery from growth inhibition was expected due to the inheritability of the methylation pattern, which means the effect of the drug is independent from its continuous administration. These results suggest that continuous administration of 5-AZA-CdR is not necessary to obtain a stable inhibition of cell proliferation.

Moreover, restriction of cloning efficiency is thought to reflect a reduction of tumorigenity.

5-AZA-CdR incorporation into DNA depends on its utilization by the complex metabolic pathways which regulate the synthesis of nucleotide triphosphates. It is thus conceivable that natural cellular metabolites modulate 5-AZA-CdR incorporation and may possibly enhance its effects. In a previous paper we reported that TdR could increase the cytotoxic action of 5-AZA-CdR on human leukemic cells.[2] In neuroblastoma cells the synergistic effect of dTR and 5-AZA-CdR on cell survival was also observed (Figure 4).

The results presented indicate that TdR acts as a biochemical modulator for 5-AZA-dCTP incorporation into DNA. The mechanism of potentiation of 5-AZA-CdR anabolism is most likely due to an inhibition of the enzyme dCMP deaminase which converts 5-AZA-dCMP to 5-AZA-dUMP, a very unstable compound.[10]

The limited time of drug exposure necessary to obtain a marked growth inhibition, its irreversibility and the existence of potentiation of effects by natural metabolites such as TdR are some of the factors which suggest that 5-AZA-CdR merits further consideration as a possible chemotherapeutic agent for the treatment of neuroblastoma.

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