Activation of Expression of p15, p73 and E-Cadherin in Leukemic Cells by Different Concentrations of 5-Aza-2’-Deoxycytidine (Decitabine)

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Abstract. Background: Inactivation of genes that suppress neoplasia by aberrant DNA methylation is a key event that occurs during the development of leukemia. The inhibitor of DNA methylation, 5-aza-2’-deoxycytidine (5aza), which can re-activate these genes, is under clinical investigation for therapy of leukemia. The objective of this study was to determine the concentrations of 5aza that will re-activate target silent genes in human leukemic cell lines. Materials and Methods: RT-PCR was used to evaluate the effect of concentrations of 1 to 100 ng/ml of 5aza on the re-activation of p15 and p73 in KG1a myeloid leukemic cells and E-cadherin in HL-60 myeloid leukemic cells. The effect of 5aza on inhibition of growth, DNA synthesis and colony formation in these cell lines was also investigated. Results: The extent of activation of the target genes was dependent on the concentration of 5aza. For p15, pronounced activation was observed at 10 ng/ml or greater. For p73 and E-cadherin significant activation was observed at 100 ng/ml of 5aza. Maximal inhibition of growth, DNA synthesis and colony formation occurred at 100 ng/ml. Conclusion: The in vitro antineoplastic and gene re-activation activity of 5aza is dependent on the concentration of this analog. These data may be helpful in the design of optimal dose-schedules of 5aza for the clinical therapy of leukemia.

The silencing of the expression of genes that suppress neoplasia by an epigenetic event, such as aberrant DNA methylation, can play an important role during the development of malignant disease (1). The cyclin kinase inhibitor gene, p15CKN2B, the p53 gene homologue, p73, and the cell adhesion gene, E-cadherin, have been reported to be silenced by DNA methylation in leukemia (2-4). 5-aza-2’-deoxycytidine (5aza), a potent inhibitor of DNA methylation, can re-activate the expression of many genes silenced by promoter hypermethylation (5). In phase I-II studies 5aza was shown to be an active agent for the therapy of hematological malignancies (6-9). Several clinical trials on 5aza are currently underway in patients with leukemia. Preclinical studies of this analog can provide data that can be helpful in the design of optimal dose-schedules of 5aza for clinical trials (10).

Materials and Methods

Materials. 5aza (Dacogen, Decitabine) was obtained from Pharmachemie (Haarlem, The Netherlands). Stocks were made in phosphate-buffered saline pH 7.4 and stored at -70°C. HL-60 and KG1a human myeloid leukemic cells were obtained from ATCC, Manassas, Virginia, USA. Both cell lines were cultured in RPMI 1640 medium (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Wisent, St-Bruno, Quebec, Canada). The cells were incubated at 37°C in a 5% CO2 atmosphere.

Drug treatment and in vitro assays. For KG1a, different concentrations of 5aza were added to the medium once at time 0. For HL-60 leukemic cells 5aza was added to the medium at time 0, 24 and 48h. For the growth inhibition assay, cells were counted at 72h using a model ZM Coulter counter. For clonogenic assay, an aliquot of 100 cells of HL-60 was placed in 2 ml of 3% agar medium containing 20% serum. After 15 days incubation at 37°C in 5% CO2 the number of colonies (>500 cells) was counted. The rate of DNA synthesis was measured by the incorporation of radioactive thymidine into DNA. At the end of drug treatment, the cells were centrifuged and suspended in 2 ml of medium containing 5% agar medium containing 20% serum. After 15 days incubation at 37°C in 5% CO2 the number of colonies (>500 cells) was counted. The rate of DNA synthesis was measured by the incorporation of radioactive thymidine into DNA. At the end of drug treatment, the cells were centrifuged and washed in 2 ml of medium containing 5% dialyzed serum and 0.5 μCi [3H] thymidine (6.7 Ci/mmol, ICN, Irvine, CA, USA) and incubated at 37°C for an additional 6h. The cells were placed on GF/C glass fiber filters (2.4 cm diameter), washed with cold 0.9% NaCl, 5% cold
trichloroacetic acid and ethanol. The filters containing the DNA were then dried, placed in scintillation liquid and the radioactivity measured using Beckman LS 6000IC scintillation counter.

**Isolation of RNA and RT-PCR analysis.** After drug treatment the cells were harvested and total RNA and cDNA prepared as previously described (11). PCR was performed using HotStar Taq DNA polymerase (Qiagen, Mississauga, Ontario, Canada) and specific primers. The primers for 18S ribosomal RNA (GenBank accession no. X03205) were sense 5'-TCG AGT GTG TTC GGC CCT GCT CCT A- 3', antisense 5'-CTG CTG CCT TCC TTG GAT GTG GTA- 3' which amplify a 110 bp product. For p15 (GenBank accession no. NM_004360) the primers were sense 5'- CGG AGA CCC TGC CAC TCT CA- 3', antisense 5'- AGG CAT CGC GCA GGT GTA- 3' and amplify a 113 bp product. For p73 (GenBank accession no. NM_005427) the primers were sense 5'- GCA CCA GTG TTC AGC ACC TCT GGA- 3', antisense 5'- GAA TCC GTT CGC CCC ACC AC- 3' and amplify a 116 bp product. For E-cadherin (GenBank accession no. NM_000546) sense 5'-CAC AGG AGT CAT CAG TGT GGT CAC- 3', antisense 5'-CAC CTT GAA GGT CAG CAG CTT G- 3' amplify a 92 bp product.

The PCR amplifications were done under the following conditions: 95°C for 15 min followed by: for 18S ribosomal RNA: 94°C for 45 sec, 60°C for 30 sec, 72°C for 30 sec for 5 cycles followed by 15 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec and a single cycle of 10 min at 72°C and 10 min at 10°C. The same program was used for p15 except the initial annealing temperature was 62°C followed by 35 cycles with annealing temperature of 61°C. A similar program was used for p73 except the initial annealing temperature was 62°C followed by 35 cycles with annealing temperature of 60°C. For each gene, the number of cycles was terminated during the exponential phase of DNA amplification.

The PCR products were electrophoresed on 2% agarose gel and detected by ethidium bromide staining. For quantitative detection of gene expression we used 18S ribosomal RNA as the internal standard. The amount of cDNA to use as template for each gene was determined so that it would amplify the identical amount of 18S ribosomal RNA (±20%) during exponential phase. We measured the absolute concentration of amplified DNA by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). This instrument determines the size and amount of DNA using capillary electrophoresis and detection by fluorescence with high precision. The relative amount of amplified DNA for each specific gene to the amount of amplified 18S RNA DNA was quantified for each sample.
Discussion

The re-activation of genes that suppress neoplasia by the potent inhibitor of DNA methylation, 5AZA, a cytosine nucleoside analog, is a novel mechanism of action. Optimization of its administration will help realize its full potential since the antineoplastic activity of 5AZA is very dose-schedule-dependent due to its S-phase specificity and short in vivo half-life (6). In addition, therapy with 5AZA can produce severe hematopoietic toxicity, its major side-effect (13).

The objective of this study was to find the in vitro concentrations that produce a potent antineoplastic effect on human leukemic cell lines and to correlate these data with the concentrations required to re-activate genes that suppress leukemogenesis. Using 5AZA in the range of 1 to 100 ng/ml, we observed that both the inhibition of growth, DNA synthesis and colony formation increased with the concentration of this analog (Tables I and II). It is interesting to note that 100 ng/ml 5AZA, which produced a 100% loss of clonogenicity for HL-60 leukemic cells, also leads to the most pronounced reactivation of the expression of p15, p73 and E-cadherin. (Figures 1-3). This correlation suggests that one mechanism by which 5AZA produces an irreversible loss of clonogenicity is related to its re-activation of genes that suppress leukemogenesis. We do not know which are the most important genes involved in this process. It is possible that the re-activation of other genes that were not investigated in this study are also required to block colony formation by leukemic cells.

In an attempt to translate these data into clinical application, it is tempting to speculate that the minimal plasma concentration of 5AZA should be in the range of 100 ng/ml. In clinical trials on patients with leukemia, remissions were observed when 5AZA was infused at a rate of 30 mg/m²/h, which produced plasma levels in the range of 200 ng/ml.
to 400 ng/ml (6,13). Lower doses of 5AZA were used to treat patients with myelodysplastic syndrome (9). In these patients 5AZA was infused at a rate of 1.67 mg/m²/h (9), which by pharmacokinetic calculations should have produced a plasma level in the range of 10 ng/ml. This concentration of 5AZA showed significant re-activation of p15 in leukemic cell lines, although to a lesser extent than seen at 100 ng/ml (Figure 1).

The low dose therapy of myelodysplastic syndrome with 5AZA produced demethylation and protein expression of p15 in some patients (14).

Future investigations on the analysis of gene re-activation by 5AZA in patients with leukemia and correlation with its response should also provide data that will assist in the design of the optimal dose-schedule for this analog.

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References


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