Antiproliferative and Antitumor Effects of Azacitidine Against the Human Myelodysplastic Syndrome Cell Line SKM-1

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Abstract. *Background: The myelodysplastic syndromes (MDS) are a group of stem cell disorders characterized by dysplasia of one or more hematopoietic cell lineages and a risk of progression to acute myeloid leukemia. The cytidine analog azacitidine (Vidaza), a hypomethylating agent, improves survival in patients with MDS, but its mechanism of action is not well understood. Materials and Methods: The effects of azacitidine on the MDS-derived cell line SKM-1 were investigated by DNA methylation assay, cell proliferation assay, and a subcutaneous xenograft mouse model. Results: Azacitidine and decitabine induced hypomethylation of the tumor suppressor gene cyclin-dependent kinase 4 inhibitor B (CDKN2B) in SKM-1 cells, whereas the deoxycytidine analog cytarabine did not. Azacitidine and decitabine also inhibited SKM-1 cell growth in vitro. In the mouse xenograft model, azacitidine significantly suppressed tumor growth. Conclusion: Inhibition of DNA methyltransferase by azacitidine contributes to its antiproliferative and antitumor effects against SKM-1 cells and may explain its clinical efficacy in MDS.*

The myelodysplastic syndromes (MDS) are a group of clonal stem cell disorders characterized by ineffective hematopoiesis, peripheral blood cytopenia and an increased risk of progression to acute myeloid leukemia (AML). Conventional chemotherapy with cytotoxic agents such as cytarabine is not effective, and, until recently, few treatments have been available.

Studies over the past 15 years (1-3) have demonstrated hypermethylation of several genes in patients with MDS. Methylation of CpG islands in the promoter regions of genes leads to transcriptional inactivation (4) and hypermethylation of tumor suppressor genes such as *CDKN2B*, which codes for cyclin-dependent kinase 4 inhibitor B (also known as multiple tumor suppressor 2 or $p15^{INK4B}$), may play an important role in neoplastic progression (1).

In 2004, the cytidine analog azacitidine (5-azacytidine; marketed as Vidaza by Nippon Shinyaku from 2011 in Japan) was the first drug to be approved by the USA Food and Drug Administration for the treatment of MDS, and it increases median overall survival in higher-risk patients with MDS in comparison to drugs used in conventional care regimens, including the structurally related cytarabine (5). Azacitidine acts by the dual mechanisms of DNA hypomethylation and cytotoxicity. When incorporated into newly synthesized DNA, azacitidine binds irreversibly to DNA methyltransferase and causes DNA hypomethylation (6, 7); and, when incorporated into RNA, it inhibits protein synthesis (8). Azacitidine was synthesized over 40 years ago and was at first considered a conventional cytotoxic agent, but its redevelopment as a low-dose therapeutic agent in the 1990s revealed its activity as a hypomethylating agent in the treatment of MDS (9). However, there have been few *in vitro* studies assessing the hypomethylating and cytotoxic effects of azacitidine and no *in vivo* studies of azacitidine in cell lines derived from patients with MDS, so that the importance of DNA hypomethylation in the mechanism of action of azacitidine in MDS is still unclear. In the present study, we investigated the action of azacitidine *in vitro* and *in vivo* by using a leukemia cell line, SKM-1, derived from a patient with MDS.

Materials and Methods

Reagents and cell lines. Azacitidine was provided by Celgene Corporation (Summit, NJ, USA). Cytarabine and decitabine were purchased from Sigma-Aldrich (Tokyo, Japan). SKM-1, a cell line established from leukemia cells of a 76-year-old Japanese male patient with overt monoblastic leukemia following MDS (10), was obtained from Human Science Research Resource Banks (Osaka, Japan). HL-60 cells (American Type Culture Collection, Manassas, VA, USA) were used as a positive control for the unmethylated *CDKN2B* gene. Both cell lines were maintained in RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37˚C.

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Animals. Five-week-old female NOD/ShiJic-SCID Jcl (NOD/SCID) mice were purchased from Clea Japan (Tokyo, Japan) and used after quarantine and acclimation for a week. The study was conducted in compliance with the Internal Regulations on Animal Experiments at Nippon Shinyaku Co., Ltd., which are based on Law for the Humane Treatment and Management of Animals (Law No. 105, 1 October 1973, as amended on 1 June 2006).

DNA methylation assay. SKM-1 cells were plated in a 60 mm dish at a density of 2×10^6 cells in 3 ml and incubated for 4 h. Serial dilutions of test compounds were then added and the cells were further incubated for three days. Genomic DNA was extracted and treated with sodium bisulfite with the MethylEasy™ Xceed kit (Human Genetic Signatures, Randwick, NSW, Australia) according to the manufacturer's protocol. The *CDKN2B* unmethylated specific primer pair (forward, 5′-TGTGATGTGTTTGTATTTTGTGGTT-3′; reverse, 5′-CCATACAATAACCAAACAACCAA-3′) was purchased from Life Technologies (Carlsbad, CA, USA). The polymerase chain reaction (PCR) products were electrophoresed and the unmethylated *CDKN2B* gene was quantified by scanning and analysis of the bands with Quantity One image analysis software (Bio-Rad Laboratories, Hercules, CA, USA).

Cell proliferation assay. SKM-1 cells were seeded in triplicate at a density of 1,000 cells/well in a 96-well plate. After overnight incubation at 37˚C, serial dilutions of test compounds were added and the cells were further incubated for three days at 37˚C. Cell proliferation was measured by tetrazolium dye assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Nacalai Tesque, Kyoto, Japan). The 50% inhibitory concentration (IC_{50}) values and the 95% confidence intervals (CI) were calculated by fitting the absorbance data to a logistic curve with the program LAP-JSAAE, which was created by the Japanese Society for Biopharmaceutical Statistics. Statistical analyses were performed with SAS version 8.2 (SAS Institute, Cary, NC, USA).

Subcutaneous xenograft mouse model. NOD/SCID mice were injected intraperitoneally with 150 mg/kg cyclophosphamide (Wako Pure Chemical Industries, Kyoto, Japan) on each of two consecutive days to repress residual immunity. The day after the second cyclophosphamide injection, a xenograft was established by subcutaneous injection of 3×10^7 SKM-1 cells into the right flank. On the sixth day after inoculation (day 0), mice were randomized into groups of three according to their tumor volume. The mice were administered azacitidine (2.5 or 5 mg/kg) or vehicle (1% D-mannitol) intravenously once a day from day 1 for seven consecutive days. The dose was set on the basis of toxicity tests with $CD2F_1$ mice in which the maximum tolerated dose of azacitidine was found to be 8.2 mg/kg. Tumors were measured on days 2, 5, 8, 11 and 14 and their volumes (mm^3) were calculated as $(d^2\times D)/2$ (where d is the shortest and D is the longest diameter of the tumor in mm). Tumor volumes are presented as the mean±S.E.M. for each group. Dunnett's test was used to assess the statistical significance of differences between the vehicle-treated and the azacitidine-treated groups with the SAS system, and $p<0.05$ was considered statistically significant.

Results

Hypomethylation of the CDKN2B gene in SKM-1 cells. Unmethylated *CDKN2B* gene was detected in the positive-control HL-60 cells but not in vehicle-treated SKM-1 cells, indicating that the *CDKN2B* gene was fully methylated in SKM-1 cells (Figure 1). Unmethylated *CDKN2B* was slightly detected in SKM-1 cells treated with 0.1 μmol/l azacitidine and clearly detected in cells treated with 0.5 or 1.0 μmol/l azacitidine. When cells were treated with the same concentrations of decitabine, a more potent and specific DNA methylation inhibitor, unmethylated *CDKN2B* was observed at a similarly high band intensity at all three concentrations. The band intensities of unmethylated *CDKN2B* observed for azacitidine at 0.5 and 1 μmol/l were similar to the band intensity observed for decitabine at 0.1 μmol/l. Hypomethylation of *CDKN2B* was not detectably induced by cytarabine at any concentration up to 1 μmol/l.

Inhibition of SKM-1-cell growth. Azacytidine inhibited the growth of SKM-1 cells in a concentration-dependent manner with an IC_{50} value of 0.52 μmol/l (95% CI=0.47 to 0.57 μmol/l), decitabine with an IC_{50} value of 2.0 μmol/l (95% CI=0.92 to 4.4 μmol/l), and cytarabine with an IC_{50} value of 1.3 μmol/l (95% CI=1.0 to 1.6 μmol/l) (Figure 2). At the highest concentration of 30 μmol/l, decitabine did not reduce cell viability below 40%, whereas azacitidine and cytarabine at this concentration reduced viability to about 2% and 10%, respectively.

Inhibition of tumor growth in a mouse xenograft model transplanted with SKM-1 cells. Treatment of the xenografted mice with 2.5 or 5 mg/kg azacitidine resulted in significant suppression of SKM-1 tumor growth from days 5 through 14 (Figure 3). Three out of 14 mice in the 5 mg/kg group had died by day 14. On day 7, these three mice weighed 81%, 78% and 80% of the mean body weight of the control group.

Discussion

In the present study, we investigated the effects of azacitidine *in vitro* and *in vivo* on SKM-1, a secondary AML cell line derived from a patient with MDS. SKM-1 cells show karyotype abnormalities with del(9)(q13;q22) and der(17)t(17;?)(p13;?) (11). Chromosomal abnormalities such as del(9q), i(17q) and t(17p) have also been detected in MDS (12).

We found the promoter region of the *CDKN2B* gene to be highly methylated in SKM-1 cells, and azacitidine and decitabine, but not cytarabine, induced hypomethylation of this region of the gene. The hypomethylation of *CDKN2B* induced by azacitidine at concentrations of 0.5 and 1 μmol/l was similar to the maximal hypomethylation induced by decitabine, a known potent hypomethylating agent. Azacitidine inhibited the growth of SKM-1 cells with an IC_{50} value of 0.52 μmol/l, and decitabine partially inhibited their growth. In MDS and secondary AML, hypermethylation occurs in the promoter regions of tumor-suppressor genes, including

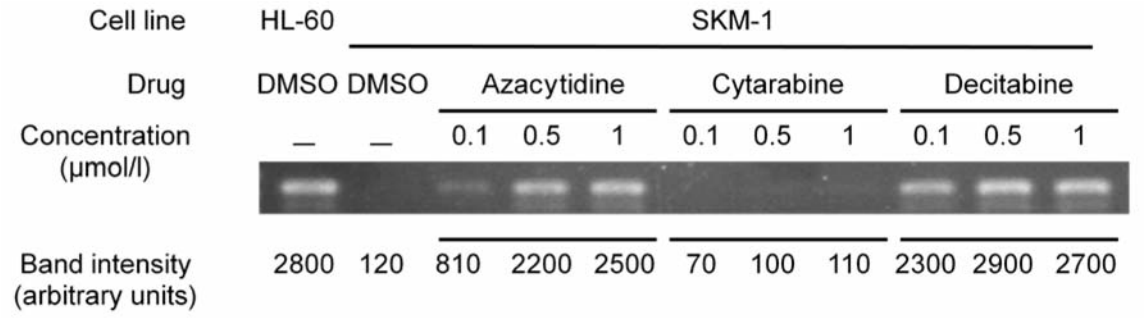

Figure 1. *Effect of azacitidine, decitabine, and cytarabine on the methylation status of the CDKN2B promoter in SKM-1 cells. Cells were cultured for three days in medium containing the indicated concentration of the test compound. Genomic DNA was extracted and treated with sodium bisulfite, and the CDKN2B promoter region was amplified by unmethylated sequence specific PCR. Products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.*

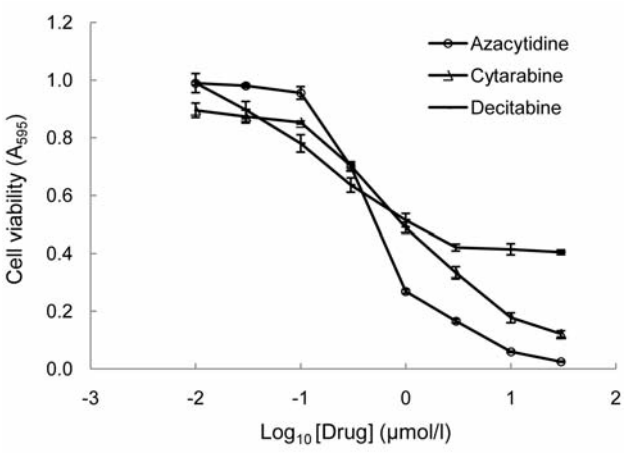

Figure 2. *Effect of azacitidine, decitabine, and cytarabine on the viability of SKM-1 cells. Cell viability was assessed by the tetrazolium dye assay after drug treatment for three days.*

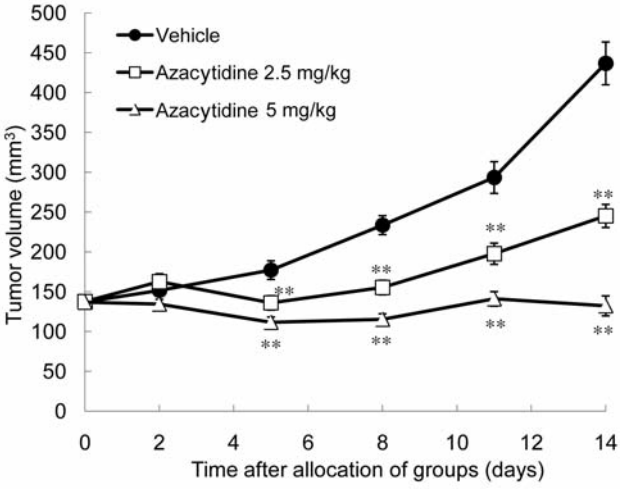

Figure 3. *Effect of azacitidine on tumor volume in a mouse xenograft model transplanted with SKM-1 cells. Day 0, allocation of groups; days 1-7, intravenous administration once a day. **p<0.01 versus vehicle-treated group (Dunnett's multiple-comparison test). Tumor volume is expressed as the mean±S.E.M. (n=14 except for the 5 mg/kg group on day 14, for which n=11).*

CDKN2B, and this induces gene silencing. Hypermethylation of *CDKN2B* is also associated with MDS progression (1). Although we did not examine other genes in the present study, the antiproliferative activity of azacitidine and decitabine against SKM-1 cells may result in the re-expression of silenced genes such as *CDKN2B* and *PI-PLCβ1*, which codes for phosphoinositide phospholipase Cβ1 (13, 14), and this would be expected to reduce cell viability and promote cellular differentiation.

The difference in the extent of maximum growth inhibition by these compounds is in line with the findings of Hollenbach *et al*. (8), who investigated human AML cell lines, including KG-1a, THP-1, OCI-AML3 and HL-60. Decitabine is a deoxyribonucleoside that is incorporated specifically into DNA, preventing its methylation. Azacytidine, on the other hand, is a ribonucleoside that is largely incorporated into RNA, leading to the inhibition of protein synthesis. However, azacitidine can also be incorporated into DNA, presumably after conversion into 5-aza-2′-deoxycytidine by ribonucleotide reductase (15), and this leads to DNA hypomethylation. In the present study, the plateau in cytotoxicity exhibited by decitabine may be explained by the fact that its effects were limited to DNA hypomethylation. Although azacitidine and cytarabine are both cytidine derivatives with antiproliferative activity against SKM-1 cells, they had different effects on the methylation status of *CDKN2B* in SKM-1 cells. Cytarabine is a conventional cytotoxic agent that acts mainly by inhibiting DNA synthesis (16), but it is not a hypomethylating agent. A dual mechanism of action of azacitidine may explain why it is the most effective nucleotide analog antitumor drug for the treatment of MDS, increasing median overall survival in high-

risk MDS patients to more than 24 months from the 15 months obtained with conventional care regimens, including cytarabine therapy (5).

To evaluate the antitumor effect of azacitidine *in vivo*, we tested it in a mouse xenograft model transplanted with SKM-1 cells. Treatment with azacitidine resulted in a significant suppression of tumor growth. On the basis of the C_{max} observed for azacitidine in a previous study (17), its C_{max} in the present study can be estimated to be 0.43-0.86 μmol/l for the 2.5 mg/kg dose. Azacytidine inhibited the growth of SKM-1 cells *in vitro*, with an IC_{50} value of 0.52 μmol/l, and induced hypomethylation at concentrations of 0.1-1.0 μmol/l. Thus, the *in vivo* effects of azacitidine were observed at estimated concentrations similar to those that produced its *in vitro* effects.

In conclusion, azacitidine had an antiproliferative effect on the MDS-derived leukemia cell line SKM-1 and an antitumor effect in a mouse xenograft model transplanted with SKM-1 cells at concentrations that induced hypomethylation of the *CDKN2B* gene *in vitro* in that cell line. These results are consistent with a mechanism in which inhibition of DNA methyltransferase by azacitidine contributes to its antiproliferative and antitumor effects against MDS cells and its clinical efficacy in patients with MDS.

Conflict of Interest

The Authors are employees of Nippon Shinyaku Co., Ltd.

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