

Palliative chemotherapy followed by methylation inhibitor in high-risk acute myeloid leukemia: An *in vitro* and clinical study

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Received February 5, 2015; Accepted June 27, 2015

DOI: 10.3892/mco.2015.605

Abstract. Decitabine (5-aza-2'-deoxycytidine; DAC) is a well-tolerated alternative to aggressive chemotherapy for leukemia, which induces differentiation and apoptosis of leukemic cells as a DNA hypomethylating agent. The aim of the present study was to investigate the feasibility of DAC sequentially combined with chemotherapy to reverse drug resistance. HL-60/ADR multidrug-resistant leukemia cells cultured in 96-well plates were pretreated with DAC for 72 h; varying concentrations of aclarinomyacin (ACLA) were then added to the wells, cell proliferation was tested using the Cell Counting Kit-8 assay, and DNA methyltransferase 1 (DNMT1) protein expression was detected by western blot analysis. Furthermore, we analyzed the therapeutic efficacy in 7 patients with high-risk acute myeloid leukemia (AML) receiving induction therapy with DAC sequentially combined with cytarabine, ACLA and granulocyte-colony stimulating factor (CAG regimen). The proliferation inhibition rate of HL-60/ADR cells treated with DAC at concentrations of 0.5 and 1.0 $\mu\text{mol/l}$ sequentially combined with ACLA was significantly higher compared with that with ACLA alone ($P < 0.001$ for both). DNMT1 expression was significantly repressed following treatment with 1.0 $\mu\text{mol/l}$ DAC. Of the 11 patients, 8 (72.7%) received induction therapy with DAC sequentially combined with CAG agents and achieved complete remission (CR) after 2 cycles of treatment; however, 3 (27.3%) patients did not achieve remission. Myelosuppression was observed in all 11 patients and pulmonary infections developed in 9 patients (81.8%) during the course of the study. At the last follow-up, 7 of the 8 patients who achieved CR

remained in remission. The median follow-up was 6 months (range, 3-18 months). Therefore, pretreatment with DAC may increase the sensitivity of HL-60/ADR cells to ACLA via the epigenetic modulation of demethylation and the sequential administration of DAC and CAG regimen appears to be safe and effective for the treatment of patients with high-risk AML.

Introduction

Over the past several decades, improvements in chemotherapeutic agents and supportive care have resulted in significant progress in the treatment of patients with acute myeloid leukemia (AML). However, the treatment options for high-risk AML patients, particularly elderly, relapsed and refractory patients, remain limited. The outcomes of these patients are generally poor due to drug resistance, short durability of response, poor performance status and serious comorbidities following standard dose-intensive therapy (1,2).

It was confirmed that epigenetic changes are crucial for the progression of numerous human neoplasms (3), as they are a universal mechanism of gene inactivation in malignant cells, possibly exceeding mutational events (4). Aberrant DNA methylation in gene promoters has been demonstrated to accompany these epigenetic changes and is also essential for the maintenance of altered gene expression status in malignant cells (5). Over the last few years, a growing number of studies have reported that leukemia is also characterized by high degrees of epigenetic changes (6,7). These observations have led to the renewed interest in therapeutic regimens targeting the aberrant epigenome of cancer cells (8) and DNA methylation inhibitors are expected to be used as antineoplastic agents (3).

Decitabine (5-aza-2'-deoxycytidine; DAC), a DNA hypomethylating agent that induces differentiation and apoptosis of leukemic cells, is a well-tolerated alternative to aggressive chemotherapy. In a study published by Kantarjian *et al* (9), the efficacy and safety of different therapeutic regimens was compared in 485 elderly patients with newly diagnosed AML; the complete remission (CR) rate, including CR with delayed platelet recovery [CRp; platelet (PLT) count $< 100 \times 10^9/l$] was 17.8% with DAC vs. 7.8% with supportive care or cytarabine (Ara-C), without significant differences in safety. However, DAC monotherapy was associated with a relatively

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Key words: decitabine, CAG regimen, HL-60/ADR cells, high-risk acute myeloid leukemia

low rate of CR in AML (10,11). Several groups have attempted to increase the response rate of DAC-based therapy by developing combination treatments (12-14). The aim of the present study was to investigate the effect of DAC sequentially combined with chemotherapeutic drugs in the HL-60/ADR multidrug-resistant leukemia cell line and retrospectively analyze the therapeutic efficacy in 7 high-risk AML patients.

Materials and methods

Reagents. The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Tokyo, Japan). DAC was supplied and formulated by Pharmachemie B.V. (Haarlem, The Netherlands). Aclacinomycin (ACLA) was purchased from Shenzhen Main Luck Pharmaceuticals Inc. (Shenzhen, China). Rabbit monoclonal anti-DNA methyltransferase 1 (DNMT1) antibody (dilution, 1:1,000; cat. no. 5032) and rabbit monoclonal anti-GAPDH antibody (dilution, 1:1,000; cat. no. 5174) and cell lysis buffer were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Polyvinylidene fluoride membranes were purchased from Millipore (Billerica, MA, USA).

Cell culture. The HL-60/ADR human AML cell line, a multidrug-resistant leukemia cell line, was obtained from the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences (Beijing, China). The cells were grown in RPMI-1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies) in plastic tissue culture plates in a humidified atmosphere containing 5% CO₂ at 37°C.

Quantification of cell proliferation using the CCK-8 assay. For the growth inhibition assay, HL-60/ADR cells were cultured at a density of 10⁵ cells/ml and aliquots (100 µl) per well of the cell suspension were dispensed into 96-well plates. At 24 h after plating, DAC was added to the wells at concentrations of 0.5 and 1.0 µM. The plates were incubated in a humidified incubator in 5% CO₂ for 72 h at 37°C. Subsequently, ACLA at varying concentrations was added to the wells. The cell proliferation was determined using the CCK-8 at 24 h after dosing. The plates were then analyzed on an enzyme-linked immunosorbent assay plate reader (Bio-Rad 680; Bio-Rad, Hercules, CA, USA) at 490 nm. All the experiments were performed in triplicate in at least 3 independent experiments.

Western blot analysis. Following treatment with 1 µmol/l DAC for 72 h, the HL-60/ADR cells were harvested and lysed in cell lysis buffer. The proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% skimmed milk and incubated overnight at 4°C with anti-DNMT1 and anti-GAPDH antibodies in Tris-buffered saline [10 mM Tris-HCl (pH 8.0), 150 mM NaCl] with 0.1% Tween-20. Following incubation with peroxidase-conjugated secondary antibodies for 2 h, the blots were developed using enhanced chemiluminescence (Molecular Imager ChemiDoc™ XRS; Bio-Rad).

Patients and treatment protocols. Following approval of the study protocol by the Institutional Review Board of

Nanfang Hospital we retrospectively analyzed 11 high-risk AML patients who were diagnosed according to the World Health Organization criteria (15) between May, 2012 and November, 2014 at the Department of Hematology of Nanfang Hospital. The pretreatment characteristics of the 11 patients are presented in Table I. All the patients received induction therapy with DAC sequentially combined with CAG agents, which consisted of DAC at a dose of 20 mg/m² intravenously over 1 h daily on days 1-3, ACLA 10 mg by intravenous infusion daily on days 4-10 or 13 and Ara-C 25 mg subcutaneously twice daily on days 4-13 or 17. On days 4-13 or 17, granulocyte-colony stimulating factor (G-CSF) 300 µg subcutaneously daily preceded the chemotherapeutic injections by ~4-6 h and was discontinued when the white blood cell count reached >20x10⁹/l. The treatment protocols for patients following achievement of CR were not uniform, due to differences in financial conditions (Table I). Complete blood count, hepatic and renal function tests and electrolyte levels were tested once or twice weekly during the drug administration period and marrow aspirates were monitored prior to and 2 weeks after treatment, to observe the therapeutic efficacy. G-CSF was used during the myelosuppression period and antibiotic therapy was administered as clinically indicated.

Response criteria and side effects. Response was assessed based on the criteria of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia (16). CR required an absolute neutrophil (NEU) count of ≥1x10⁹/l, PLT count of ≥100x10⁹/l, marrow blasts ≤5% and disappearance of all signs and symptoms related to disease. CRp was defined as CR with a PLT count of <100x10⁹/l. Any other response was considered as treatment failure. Neutropenia and thrombocytopenia was defined as NEU count <0.5x10⁹/l and PLT count <20x10⁹/l, respectively. The reported non-hematological side effects included nausea, vomiting, diarrhea, skin rashes, liver or renal dysfunction and fever. The febrile episodes included fever of unknown origin and documented infections. The adverse drug reactions were scored according to the National Cancer Institute Common Toxicity Criteria, version 2.0 (17).

Statistical analysis. The statistical significance of the differences between the proliferation inhibition rate with DAC at two different concentrations sequentially combined with ACLA and the control group was assessed by one-way analysis of variance using the SPSS 13.0 software program (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Response of HL-60/ADR cells treated by DAC sequentially combined with ACLA. To evaluate the effects of sequentially combining DAC and ACLA on HL-60/ADR cell viability, the cells were treated with DAC (0.5 and 1.0 µM for 72 h) and sequentially with different concentrations of ACLA (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 µg/ml for 24 h). The percentages of live/viable cells in the treated plates were measured using the CCK-8 proliferation assay. The growth inhibition rate with

Table I. Data of 11 patients with AML who were ineligible for intensive chemotherapy and received induction therapy with DAC sequentially combined with CAG.

No.	Gender/age	FAB subtype	Cytogenetics	Courses to CR	Duration (days)		Infection site	Therapeutic regimen after CR (x course)	Follow-up	
					PLT <20 G/l	NEU <0.5 G/l			Months	Status
1 ^a	M/43	UN	t(1;7); 7q-/-7	1	23	16	Lung	D+HAGx1, Allo-HSCT	6	CCR
2	F/74	M4	del(7q)/-7; EGR-1/RAR- α deletion; DNMT3A ⁺	1	10	23	Lung	D+CAGx1	4	Relapse
3 ^b	M/48	M2	del(9)	1	8	4	Lung	IAx2, D+CAGx2	6	CCR
4	F/62	M2	Normal karyotype; NPM1 ⁺	2	5	41	Lung	D+HAGx3, IAx3	12	CCR
5	M/62	M5	Normal karyotype	NR	21	0	Unknown origin	HA, IA re-induction	3	Deceased ^c
6	M/60	UN	Normal karyotype; NPM1 ⁺	1	10	23	Lung	D+HAGx2, IAx3, MD-Ara-Cx1	9	CCR
7 ^a	M/57	UN	Normal karyotype; DNMT3A ⁺	1	13	23	Lung	D+HAGx3, IAx3	10	CCR
8	M/60	M5	Normal karyotype; FLT3-ITD ⁺	1	9	20	Lung	D+HAGx2, IAx1	5	CCR
9 ^d	M/43	M2	+8	1	11	26	Lung	D+HAGx2, IAx2, MD-Ara-Cx3	18	CCR
10	F/67	M1	+8,-5 FLT3-ITD ⁺ DNMT3A ⁺	NR	8	19	Lung	-	3	Deceased ^e
11	F/63	M4	-7	NR	12	27	-	-	3	Lost to follow-up

^a AML secondary to myelodysplastic syndrome. ^b This patient used a variety of chemotherapy regimens and received HSCT with relapse twice (refractory AML). ^c Succumbed to intracranial hemorrhage. ^d Severe infection prior to treatment. ^e Succumbed to septic shock. AML, acute myeloid leukemia; DAC, decitabine; FAB, French-American-British; CR, complete remission; CCR, complete cytogenetic remission; PLT, platelet; NEU, neutrophil; M, male; F, female; UN, unclassified; EGR-1, early growth response protein-1; RAR- α , retinoic acid receptor α ; DNMT3A, DNA (cytosine-5)-methyltransferase 3A; NPM1, nucleophosmin; FLT3-ITD, internal tandem duplication of Fms-like tyrosine kinase 3; NR, non-remission; HAG, homoharringtonine + Ara-C + G-CSF; Allo-HSCT, allogeneic hematopoietic stem cell transplantation; CAG, Ara-C + acclinomycin + G-CSF; G-CSF, granulocyte colony-stimulating factor; IA, idarubicin + Ara-C; HA, homoharringtonine + Ara-C; MD, medium dose; Ara-C, cytarabine; D, decitabine.

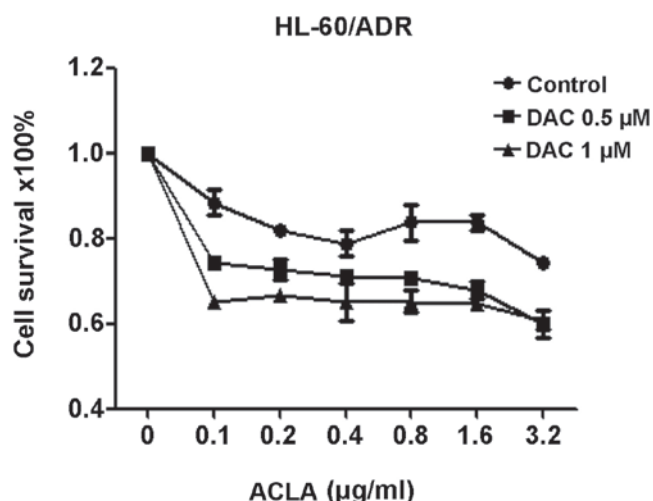


Figure 1. Sensitivity of HL-60/ADR cells to ACLA following pretreatment with DAC for 72 h. ACLA, aclinomycin; DAC, decitabine.

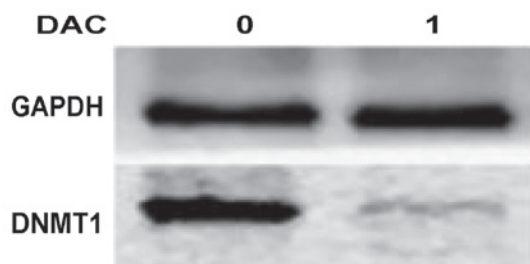


Figure 2. DNMT1 protein expression in HL-60/ADR cells after 72 h of treatment with 1.0 $\mu\text{mol/l}$ DAC, and in the control group. DNMT1, DNA methyltransferase 1; DAC, decitabine.

DAC at the two different concentrations sequentially combined with ACLA was significantly higher compared with that in the control group ($P < 0.001$ for both, Fig. 1). The data revealed that the proliferation of HL-60/ADR cells was significantly inhibited when combining DAC and ACLA sequentially, compared with the treatment groups using ACLA alone.

DNMT1 protein expression in HL-60/ADR cells. DNMT1 protein expression was analyzed in HL-60/ADR cells following treatment with 1.0 $\mu\text{mol/l}$ DAC for 72 h. The western blot analysis demonstrated that DNMT1 expression was significantly repressed following treatment with 1.0 $\mu\text{mol/l}$ DAC in HL-60/ADR cells (Fig. 2).

Response to treatment. The therapeutic effects in the 11 patients are detailed in Table I. The median follow-up time was 6 months (range, 3-18 months) and the median course of consolidation chemotherapy after CR was 5 months (range, 1-7 months). Of the 11 patients, 7 (63.6%) achieved CR after one treatment cycle and 1 patient (9.1%) achieved CR after two cycles. Of the 8 patients achieving CR, 1 patient underwent allogeneic hematopoietic stem cell transplantation after one cycle of consolidation therapy with DAC sequentially combined with HAG. One patient discontinued therapy after one cycle of consolidation therapy and did not receive any subsequent chemotherapy until he relapsed 4 months later, at which time re-induction with the

original induction regimen was unsuccessful (non-remission; NR). A total of 6 patients (5 who achieved CR after one cycle and 1 who achieved CR after two cycles) received a total of 3-6 courses of consolidation therapy with DAC combined with CAG or homoharringtonine + Ara-C + G-CSF (HAG) and IA [idarubicin (IDA) + Ara-C] regimens. The patients remained in remission at the last follow-up. Two patients exhibited NR and succumbed to leukemia-related intracranial hemorrhage and septic shock. One patient was discharged from the hospital due to therapy discontinuation.

Side effects. Myelosuppression was observed in all the patients (Table I). The median duration of neutropenia was 23 days (range, 0-41 days) and of thrombocytopenia 10 days (range, 5-23 days). During the myelosuppression period, a febrile episode of unknown origin was reported in 1 patient and pulmonary infections developed in 9 patients, but the symptoms were controlled following treatment with hematopoietic stimulating factor and symptomatic antibiotic support treatment. A total of 3 patients developed nausea and vomiting. Extramedullary toxicity was generally mild, without grade ≥ 2 hepatic or renal dysfunction. Of the 8 patients who achieved CR, 6 patients receiving consolidation therapy with sequential combination of DAC and CAG or HAG were analyzable for myelosuppression. The median time to NEU $< 0.5 \times 10^9/l$ was 8 days (range, 0-10.3 days) and to PLT $< 20 \times 10^9/l$ 6 days (range, 0-7 days). The duration of myelosuppression during consolidation therapy was shorter compared with that during the induction period.

Discussion

Although DAC has been found to be clinically effective as a single agent in patients with AML, the overall response rate (ORR) is relatively low, reportedly ranging between 8.5 and 26% (10,16). In order to improve the curative effect without increasing the toxicity, a therapeutic regimen using DAC combined with other antileukemic drugs has entered the stage of experiments *in vitro* and clinical studies. A number of hypomethylating combination trials for AML are underway and include the use of all trans-retinoic acid, Ara-C, IDA, daunorubicin (DNR), ACLA, thalidomide and homoharringtonine (12,19-21). In our study, treatment with DAC prior to ACLA inhibited the proliferation of cultured HL-60/ADR multidrug-resistant cells.

Li *et al* (12) reported that the sequential combination of DAC and IDA induced synergistic cell death in U937 cells and AML cells isolated from AML patients, while tumor growth inhibition with this sequential combination was found to be higher compared with single-agent treatment or controls *in vivo*. However, other anthracyclines, including DNR and ACLA, did not exert a synergistic effect when sequentially combined with DAC, which was not consistent with our results. Sequential treatment with DAC and Ara-C was found by Leonard *et al* (19) to be more effective in reducing tumor burden compared with treatment with Ara-C alone in xenograft models of childhood AML.

Clinically, several data also investigated the strategy of hypomethylating agent-based combinations using DAC. A study by Zhang *et al* (22) compared the clinical efficacy and

adverse reactions between low-dose DAC combined with CAG and CAG alone in intermediate- to high-risk myelodysplastic syndrome, and found that the CR rate was higher (75%) in patients treated by the combination regimen compared with CAG alone (50%), although the difference was not statistically significant. Jing *et al* (23) found that DAC combined with modified CAG regimen for relapsed and refractory AML patients with AML1-ETO⁺ displayed higher CR rate and fewer side effects. Benton *et al* (24) conducted a phase I study of patients with relapsed/refractory acute lymphocytic leukemia treated with DAC alone or in combination with the hyper-CVAD regimen; the ORR (including CR, CRp and marrow CR) was 21% in the DAC alone group and 56% in the combination regimen group, and certain patients, who had previously developed disease progression on Hyper-CVAD alone, achieved a CR when DAC was added. In the present study, we presented a retrospective analysis of a single-institution experience with the therapeutic efficacy of DAC sequentially combined with CAG in newly diagnosed patients with AML who were considered unfit for intensive chemotherapy. All the patients were monitored after treatment for the development of thrombocytopenia, neutropenia and infection, which were controllable. There was no reported drug-related mortality. The results were consistent with those reported by Bhatnagar *et al* (25).

These findings suggest that DAC sequentially combined with chemotherapy may enhance the antileukemic effect *in vitro*, as well as in clinical trials. Our results also confirmed that pretreatment with DAC may act as a potential sensitizer to ACLA, thereby improving its curative efficacy via the epigenetic modulation of demethylation in HL-60/ADR cells and AML patients.

Multiple mechanisms may be involved in this phenomenon. Aberrant expression of DNMTs, which promote DNA methylation, is recognized as a key factor in the onset of cancer and drug resistance (26,27). DNMT1 is crucial for the maintenance of the methylation landscape due to its ability to recognize hemimethylated DNA and conserve methylation during somatic cellular division (27). High levels of DNMT1 expression have been reported in cancer patients who are not responsive to chemotherapy (26). DAC incorporated into DNA covalently binds to DNMT1, leading to the reduction of available DNMT1 protein in cells, which in turn results in DNA demethylation and expression of methylation-silenced genes (28,29). In the present study, we demonstrated that DAC decreased DNMT1 protein expression levels, which was consistent with previous findings.

In conclusion, we retrospectively reported a single-institution experience using DAC sequentially combined with CAG in newly diagnosed high-risk AML patients. These findings suggest clinical potential in the sequential administration of DAC and CAG regimen for the treatment of elderly and relapsed/refractory AML patients, or patients with secondary AML. However, due to the limited number of cases, large-scale multicenter studies are required to confirm our results.

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