Induction of Differentiation of Acute Promyelocytic Leukemia Cells by a Cytidine Deaminase-resistant Analogue of 1-β-d-Arabinofuranosylcytosine, 1-(2-Deoxy-2-methylene-β-d-erythro-pentofuranosyl)cytidine

Nozomi Niitsu, Yuki Ishii, Akira Matsuda, and Yoshio Honma

Saitama Cancer Center Research Institute [N. N., Y. Y., H.], Ina-machi, Saitama 362-0806, Japan; First Department of Internal Medicine, Toho University School of Medicine [N. N.], Tokyo, 143-0015 Japan; and the Graduate School of Pharmaceutical Sciences, Hokkaido University [A. M.], Sapporo, 060-0812 Japan

ABSTRACT

Since the establishment of all-trans retinoic acid (ATRA) differentiation therapy, the prognosis of acute promyelocytic leukemia (APL) has improved, and APL has become a curable subtype of acute myelocytic leukemia. Complete remission can be achieved with ATRA alone, but disease-free survival is still too short because of relapse. To overcome this drawback, ATRA has been used in combination with chemotherapeutic agents such as 1-β-d-arabinofuranosylcytosine (araC) and daunorubicin. However, because the APL cell lines NB4 and HT93 is less sensitive to araC than to that of other myeloid leukemia cell lines such as HL-60 and U937, ATRA effectively induced granulocytic differentiation of NB4 and HT93 cells, whereas araC did not, even in a high concentration. A cytidine deaminase-resistant analogue of araC, 1-(2-deoxy-2-methylene-β-d-erythro-pentofuranosyl)cytidine (DMDC), inhibited the growth of NB4 and HT-93 cells and was also effective on HL-60 and U937 cells. The promyelocytic cell lines were induced to differentiate by DMDC and other cytidine deaminase-resistant analogues. Among them, DMDC was the most potent in inducing differentiation and inhibiting the growth of NB4 cells. The ATRA-induced differentiation of NB4 cells was not augmented by araC, whereas combined treatment with ATRA and DMDC had more than additive effects in inducing the differentiation of NB4 cells. Similar results were observed in a primary culture of leukemia cells that had been freshly isolated from APL patients. These results suggest that DMDC may play a role in the treatment of APL.

INTRODUCTION

APL, is characterized by the arrest of granulopoiesis at the promyelocytic stage and is generally associated with a t(15;17) translocation that fuses the PML gene to the RARα gene (1, 2) as well as by coagulopathy and primary fibrinolysis (3). ATRA can induce a high rate of complete remission in patients with APL. It has been well documented that APL cells are eliminated by the differentiating effect of ATRA (4, 5), with a low incidence of the life-threatening coagulopathy and infections that often develop in patients treated with conventional intensive chemotherapy. However, a rapid increase in leukocytes is commonly observed during ATRA therapy, and treatment is often accompanied by retinoic acid syndrome (6). Another drawback of ATRA therapy is the development of resistance, and the duration of remission is relatively short in patients treated with ATRA alone (7, 8). On the other hand, intensive chemotherapy alone achieves long-term survival in approximately 25% to 50% of patients with APL (9). Daunorubicin and araC are the major agents that have been used for conventional intensive chemotherapy to treat AMLs, including APL. Treatment with low concentrations of araC can induce the differentiation of human myeloid leukemia HL-60 cells, but its differentiation-inducing effect has been reported to be weak for NB4 cells, a human APL cell line with t(15;17) and PML-RARα chimera (10, 11). Accordingly, in chemotherapy for APL, araC generally has been used with the expectation that it could induce apoptosis in APL cells. Recently, DMDC, an araC analogue that is resistant to cytidine deaminase, has become available (12–15). It is believed that the nucleoside diphosphates of this agent (DMDCDP) suppress ribonucleotide reductase (16, 17), the key enzyme in the biosynthesis of deoxyribonucleotides, whereas its nucleoside triphosphate (DMDCTP) suppresses DNA polymerase (18). In vivo studies have shown that DMDC suppresses the growth of mouse leukemia cells and human solid tumors for which araC is ineffective (14). In the present study, we found that APL cells were induced to differentiate by cytidine deaminase-resistant araC analogues or araC in combination with THU, an inhibitor of cytidine deaminase, suggesting that APL cells maintain signal transduction systems for araC-induced differentiation.

MATERIALS AND METHODS

Materials. DMDC and FDMDC were synthesized as described previously (1, 7). ATRA, NBT, araC, and 5-fluoro-araC were purchased from Sigma Chemical Co. (St. Louis, MO), and VD3 was from Wako Pure Chemicals (Osaka, Japan). THU was purchased from Calbiochem-Novabiochem Co. (La Jolla, CA). Am80 and 9cRA were obtained from Prof. K. Shudo (University of Tokyo, Tokyo, Japan) and from Biomol Research Laboratories (Plymouth Meeting, PA), respectively. DMDC, FDMDC, and 5-fluoro-araC were dissolved in PBS, and 10 μM stock solutions were prepared and kept at 4°C. [2-14C]araC (2072 MBq/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA).

Cells and Cell Culture. Human myeloid leukemia HL-60, NB4 (19), U937, and HT93 (20) cell lines were cultured in suspension in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2 in air. We confirmed the expression of the PML-RARα gene in NB4 and HT93 cells, but not in HL-60 or U937 cells, by a reverse transcription-PCR technique described previously (2). Peripheral blood from a 48-year-old male, a 46-year-old female, and a 54-year-old male patient with t(15;17)-positive APL was obtained with informed consent at onset before their initial chemotherapy. To purify leukemic cells, heparinized blood cells were mixed with an equal volume of RPMI 1640 and centrifuged on Ficoll-Paque (Pharmacia, Uppsala, Sweden).

Assay of Cell Growth and Differentiation. Cells (5 x 10⁴/ml) were suspended in 2 ml of culture medium and cultured with or without test substances. The costs of culture were covered by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[References: 1–20]

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expression, another myelomonocytic differentiation marker, was also effectively induced by DMDC in the APL cell lines (data not shown). Morphological differentiation of NB4 cells was also induced by DMDC, although mainly myelocytes and metamyelocytes, but not mature neutrophils, were formed.

Induction of Differentiation of NB4 Cells by Cytidine Deaminase-resistant araC Analogues. FDMDC was prepared from 5-fluorouridine, but it should act as a 2′-deoxy-5-fluorocytidine derivative, because DMDC has been reported to not be a substrate of cytidine deaminase from mouse kidney cells (12). We examined the differentiation-inducing effect of FDMDC and 5-fluoro-araC on NB4 cells (Fig. 3). All of the analogues induced NBT reduction of the cells in a concentration-dependent manner. Among them, DMDC was the most potent inducer of NBT reduction in NB4 cells. Similar results were obtained when the expression of CD11b was examined (data not shown). DMDC also had the greatest growth-inhibitory activity in either the presence or the absence of retinoids (Fig. 3 and Table 1).

Synergistic Effect of DMDC with ATRA on Growth Inhibition and the Differentiation of NB4 Cells. ATRA is a potent inducer of the granulocytic differentiation of NB4 cells (19) and has been used successfully to treat APL patients (4, 5). ATRA-induced NBT reduction was scarcely affected by araC in NB4 cells, whereas it was greatly enhanced by DMDC (Fig. 4, B and D). Combined treatment with both ATRA and DMDC for 5 days had more than additive effects in inducing NBT reduction. To determine whether simultaneous treat-

### RESULTS

#### Effects of araC and DMDC on the Growth and Differentiation of Human Myeloid Leukemia-resistant Cell Lines. We examined the effect of araC and its deaminase-resistant analogue, DMDC, on the growth and differentiation of APL cell lines (NB4 and HT93) and other myeloid leukemia cell lines (U937 and HL-60). Both araC and DMDC suppressed the growth of these cells in a concentration-dependent manner. The IC$_{50}$ of araC was 40.6 ± 5.2 nM for HL-60 cells, 32.2 ± 4.6 nM for U937 cells, 158.2 ± 12.6 nM for NB4 cells, and 154.6 ± 20.2 nM for HT93 cells. The respective IC$_{50}$ values of DMDC were 4.6 ± 0.8, 3.4 ± 0.8, 7.5 ± 1.1, and 7.3 ± 0.6 nM. These results indicate that NB4 and HT93 cells are less sensitive to araC than HL-60 and U937 cells with respect to growth inhibition. Fig. 2 shows that araC induced NBT reduction of HL-60 and U937 cells, whereas even in a high concentration, it did not induce NBT reduction of NB4 and HT93 cells, as in previous reports (10). On the other hand, DMDC did induce NBT reduction of NB4 and HT93 cells, with dose-response curves similar to those of HL-60 and U937 cells (Fig. 2). CD11b

### Statistical Evaluation. Statistical analyses were performed using an unpaired two-tailed Student’s $t$ test.
Table 1 Effects of the combination of deoxycytidine analogs and retinoids on growth inhibition of NB4 cells

Cells were treated with various concentrations of deoxycytidine analogs in the absence or presence of 4 nM retinoic acid for 5 days and then IC_{50} values were determined from the means of triplicate data.

<table>
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<tr>
<th>Treatment</th>
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<th>+ ATRA</th>
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* ND, not determined.

Fig. 4. Effect of AraC analogues on the growth inhibition and NBT reduction of NB4 cells in the presence of ATRA. Cells were treated with various concentrations of AraC (A and B), DMDC (C and D), or FDMDC (E and F) with 0 ( ), 4 ( ), or 40 nM ( ) ATRA for 5 days. Values are means ± SD of three separate experiments.

Fig. 5. Enhancement by DMDC of ATRA-induced NBT-reducing activity in NB4 cells. A, cells were incubated with various concentrations of DMDC in the absence ( ) or presence of 4 ( ) or 40 ( ) nM ATRA for 5 days. B, cells were incubated with various concentrations of DMDC for 2 days. The cells were washed with fresh medium and reincubated without ( ) or with 4 ( ) or 40 ( ) nM ATRA for 5 days. C, cells were cultured without ( ) or with 4 ( ) or 40 ( ) nM ATRA for 2 days and then reincubated with various concentrations of DMDC for 5 days. Means ± SD of three determinations.
Am80 and 4.4 nM DMDC alone, but the activity increased to 8.2 (A 560 / 10^7 cells) when the cells were treated with both 4 nM Am80 and 4.4 nM DMDC for 5 days, indicating that the combined effect was more than additive. Similar results were obtained when the cells were treated with DMDC plus 9cRA or FDMDC plus ATRA (data not shown). These results suggest that retinoids and deaminase-resistant analogues can co-operate in inducing differentiation and inhibiting the growth of APL cells.

Effect of DMDC on ATRA-resistant NB4 Cells. The effect of DMDC was investigated on ATRA-resistant NB4 cells that failed to respond to ATRA with regard to growth inhibition or differentiation. DMDC dose-dependently increased the NBT-reducing activity in both the parent and the ATRA-resistant cells (Fig. 7). DMDC more than additively increased the NBT-reducing activity of the resistant cells when it was combined with 40–400 nM ATRA (data not shown). These results suggest that retinoids and deaminase-resistant analogues can co-operate in inducing differentiation and inhibiting the growth of APL cells.

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Effect of ATRA in Combination with DMDC or araC on NBT Reduction of APL Cells in Primary Culture. Peripheral blood mononuclear cells (>80% blasts and promyelocytes) were isolated from three APL patients. These leukemia cells responded to ATRA in a concentration-dependent manner with respect to the induction of NBT reduction, as described previously (30–32). DMDC, but not araC, significantly induced NBT reduction in all three cases (Fig. 8). Although DMDC and ATRA cooperatively enhanced NBT reduction, the combination of ATRA and araC was not effective in cases 1 and 2 (Fig. 8), as with the APL cell lines.

Enhancing Effect of THU on the araC-induced Growth Inhibition and Differentiation of NB4 and HT93 Cells. A cytidine deaminase inhibitor, THU, was applied in combination with araC to various
myelomonocytic leukemia cell lines to evaluate the ineffectiveness of araC in inhibiting cell growth and inducing differentiation in APL cells because of the high activity of cytidine deaminase: araC is inactivated when it is broken down by cytidine deaminase to form araU (33), whereas DMDC is resistant to cytidine deaminase. Treatment with THU alone hardly affected, whereas THU greatly enhanced, araC-induced growth inhibition. NBT reduction was induced by araC and THU, but not by araC or THU alone in NB4 and HT93 cells (Fig. 9). On the other hand, THU did not essentially affect the growth or differentiation of HL-60 and U937 cells in either the presence or the absence of araC (Fig. 9). These results suggest that the ineffectiveness of araC in APL cells may be attributable to a higher cytidine deaminase activity than in other AML cells.

**Uptake and Metabolism of araC in APL Cells.** APL (NB4 and HT93) and non-APL (U937 and HL-60) cells were incubated with [14C]araC at 37°C or 4°C for various times up to 180 min. At 37°C, uptake of araC by all of the cells increased steadily with incubation up to 90 min, but the cellular uptake of araC was scarcely observed at 4°C in these cells. There was no significant difference in uptake among the cell lines (data not shown). However, the amount of araC in the culture medium of the APL cells was much greater than that of non-APL cells when incubation was longer than 24 h, suggesting that araC metabolism in APL cells is slower than that in non-APL cells (data not shown). The extracts of these cells incubated with [14C]araC at 37°C for 120 min were subjected to TLC (Fig. 10). Synthesis of the active products, araC nucleotides, in APL cells was significantly lower than that in non-APL cells in both the presence and absence of THU. The major catabolic product, araU, was detected at a much higher amount in the extracts of APL cells, and treatment with THU significantly reduced the amounts of araU. We determined the deaminase activity in crude extracts of these cells, but there was no significant difference in this activity between APL and non-APL cells. These results suggest that araC metabolism in APL cells is lower than that in other AML cells, and the effect of THU on araC metabolism in APL cells was compatible with the effect of THU on the growth inhibition and differentiation of araC-treated APL cells (Fig. 9).

**Synergistic Effects of DMDC with VD3 or ATRA on the Growth and Differentiation of U937 and HL-60 Cells.** DMDC inhibited the proliferation of human monoblastic leukemia U937 cells with an IC50 of 3.4 ± 0.8 nM, and this concentration of DMDC slightly reduced NBT reduction and CD11b expression (Fig. 11). VD3 is a promising inducer of differentiation in the treatment of some types of leukemia and cancer (34). VD3 induces the differentiation of several human myelomonocytic leukemia cells (35) and prolongs the survival of mice inoculated with myeloid leukemia cells (36, 37). Therefore, we examined the effects of DMDC and araC on the differentiation of U937 cells induced by VD3. VD3 at 3 nM did significantly inhibit the proliferation of U937 cells, but the combination with DMDC caused more than additive suppression of cell growth (data not shown). VD3 at 0.3 nM hardly induced the NBT-reducing activity in U937 cells, but DMDC-induced NBT reduction was effectively enhanced by 0.3 nM VD3, and the enhancing effect was statistically significant (Fig. 11C). However, araC-induced NBT reduction was not affected by 0.3 or 3 nM VD3, although araC alone did not induce NBT reduction (Fig. 11A). The expression of CD11b was also greatly enhanced by combined treatment with DMDC and VD3 (Fig. 11D), but not by araC and VD3 (Fig. 11B). Morphologically, DMDC enhanced the monocytic differentiation of U937 cells induced by suboptimal concentrations of VD3 (data not shown).

The differentiation-inducing effect of DMDC or FDMDC in the presence of clinically applicable concentrations of VD3 or ATRA was also examined in HL-60 cells (Table 2). The NBT-reducing activity of cells treated with 3 nM VD3 and 2.4 nM DMDC were 1.4 and 2.7 (A560/10^7 cells), respectively. The combined effect of VD3 and DMDC was more than additive with respect to the induction of NBT reduction of HL-60 cells (4.6 A560/10^7 cells). In contrast, the combination of VD3 and FDMDC had merely additive effects (data not shown). Similar results were obtained in the induction of other differentiation-associated phenotypes, such as lysozyme production, expression of CD11b, and morphological changes (Table 2). ATRA at clinically applicable concentrations and these drugs also cooperated in inducing the differentiation of HL-60 cells (Table 2). These results
suggest that DMDC may be superior to araC in inducing differentiation in certain APL and non-APL leukemia cells when combined with ATRA or VD3.

**DISCUSSION**

Myeloid leukemia cells can be induced to undergo cell differentiation into mature granulocytes and macrophages by various differentiation-inducers. Almost all of the inducers of cell differentiation are difficult to use clinically because of their in vivo instability, potent adverse reactions, or intensely unpleasant odor. The inducer ATRA has been used clinically in the treatment of APL. On the basis of its ability to achieve high complete remission rates, ATRA has an established role in differentiation induction therapy. However, the response is frequently transient, and relapse of APL is sometimes associated with the acquisition of resistance to ATRA (7, 8). To prevent such relapse, ATRA is now used together with certain chemotherapeutic agents such as daunorubicin and araC, and consolidation chemotherapy is administered after complete remission is achieved with ATRA. However, the utility of omitting araC from the treatment of diagnosed APL has been reported (9, 38). Although araC is one of the most effective drugs for the treatment of adult AML, its usefulness is limited by several drawbacks, including its short half-life in plasma (which is attributable in part to rapid deamination to chemotherapeutically inactive araU by the action of cytidine deaminase), development of drug resistance, and ineffectiveness on solid tumors (39, 40). araC is known to induce the differentiation of some myeloid leukemia cells, and other inhibitors of nucleotide metabolism also induce the differentiation of myeloid leukemia cells (41, 42). This indicates that some inhibitors of nucleotide metabolism may induce leukemia cells to differentiate and may enhance the differentiation induced by other compounds more effectively than other types of anticancer drugs. In some myeloid leukemia cell lines, araC combined with ATRA is known to be more effective than araC alone in inducing differentiation. In the present study, araC did not significantly enhance the ATRA-induced differentiation of APL cells in primary culture, as in NB4 and HT93 cells. If an anticancer agent were available that not only inhibited the proliferation of APL cells, but also induced their differentiation, it would be very useful for the treatment of APL.

DMDC induced granulocytic differentiation of APL cells, but >10 nM DMDC significantly induced apoptosis of the cells, indicating that the antiproliferative effect of DMDC may stem from a combination of differentiation and apoptosis. It is difficult to distinguish between inhibition of proliferation attributable to differentiation induction and that attributable to apoptosis in the DMDC-treated cells. However, DMDC significantly enhanced G1 accumulation of the ATRA-pretreated cells without appreciable apoptosis, although DMDC alone induced the apoptosis as well as the differentiation. These results suggest that ATRA prevents the apoptosis of the DMDC-treated cells and promotes the DMDC-induced differentiation.
DMDC has a structure similar to 2'-deoxycytidine, except that it has a methylene group in place of the 2'-Hs in 2'-deoxycytidine. Whereas araC is promptly inactivated as a result of deamination by cytidine deaminase, DMDC is resistant to this enzyme (12). It has the most potent cytoidal effect on cells in the S phase, based on the blockade of DNA synthesis by the inhibition of DNA polymerase (12). DMDC was administered by the same procedure as that used clinically. The incorporation of araC in DNA chains in a manner similar to that of araC (18). The incorporation into DNA might be associated with induction of the differentiation, because some DNA-reacting agents can induce differentiation of leukemia cells (37, 41). Unlike araC, DMDC does not exert feedback inhibition on deoxycytidine kinase (15). A Phase I trial of DMDC is currently underway in patients with cancer of the lung, esophagus, stomach, colon, and large intestine (43). In the present study, we found that DMDC and FDMDC could induce the differentiation of HL-60 cells, and these agents alone also induced NB4 and HT93 cells; their differentiation-inducing activity was markedly increased by combination with ATRA. In NB4 cells, when ATRA was combined with DMDC, the NBT-reducing activity was increased 2.7-fold compared with that using ATRA alone. Similarly, the activity was increased 2.1-fold when ATRA was combined with FDMDC. When 200 mg/m² of DMDC was administered by the same procedure as that used clinically, Cmax was 0.82 ± 0.05 µg/ml. When the dose was increased to the maximum tolerated dose of 400 mg/m², Cmax was 0.47 ± 0.34 µg/ml. When the drug was administered repeatedly at low doses, Cmax was 0.17–0.22 µg/ml (43). The doses used in the present study correspond to a blood concentration of 0.05 µg/ml or less, suggesting that this therapy can be clinically applicable to APL and other AMLs.

Previous reports show that treatment with some nonretinoid inducers after pretreatment with ATRA is more potent than simultaneous treatment with ATRA and some nonretinoid inducers in inducing the differentiation of NB4 cells (10, 21). Our data support that initial treatment with ATRA with subsequent exposure to DMDC is more effective than pretreatment or concomitant treatment with DMDC. Recently, it has been suggested that ATRA causes the PML oncogenic domain of APL cells to approach that in normal cells with respect to number and size. During this process, the PML-RARα protein undergoes ATRA-dependent catabolism by the action of proteasome and, hence, is removed from the nucleus (44, 45). Consequently, after the administration of ATRA, the cells become sensitive to nonretinoid inducers in the course of the normalization of PML oncogenic domain.

DMDC inhibited the growth and induced the differentiation of ATRA-resistant NB4 cells, suggesting that DMDC may be a useful therapeutic agent against ATRA-resistant APL. In primary cultures of leukemia cells isolated from three APL patients, araC did not induce differentiation, whereas DMDC was able to do so. Furthermore, the NBT-reducing activity was increased by 2- to 4-fold when cells were treated with the combination of DMDC and ATRA, compared with the increase achieved by ATRA alone. These results support the clinical usefulness of this combination therapy.

The mechanism by which DMDC inhibits growth and induces differentiation was studied in NB4 cells. Expression of PML-RARα mRNA was not affected by treatment with DMDC in NB4 cells. There is a significant difference in araC stability between APL (NB4 and HT93) and non-APL (HL-60 and U937) cultures. DMDC has been reported to be effective for tumors with a high cytidine deaminase activity (46). Although growth inhibition was slightly augmented when THU, a cytidine deaminase inhibitor, was used in combination with DMDC (data not shown), the combination of araC and THU far more strongly inhibited the growth of NB4 and HT93 cells. On the other hand, THU hardly enhanced araC-induced growth inhibition in HL-60 and U937 cells, although THU significantly enhanced araC nucleotide formation in these cells. Similar results were obtained with regard to the induction of NBT-reducing activity. It is not completely clear why THU is less effective in enhancing araC-induced differentiation in HL-60 and U937 cells. There was no significant difference in total cellular cytidine deaminase activity between APL and non-APL cells. Additional experimentation is necessary to explain fully the differentiation-inducing effects of araC and DMDC.

The combination of araC and THU has been administrated to some patients with AML. However, no useful therapeutic effects were noted in previous clinical trials (47, 48). Intracellular accumulation of DMDC was proportional to the concentration of DMDC used in treatment of human SK-MEL-28 melanoma cells, whereas that of araC was not (15). Feedback inhibition of araC phosphorylation by an araC pool accumulating in the cells is likely responsible for the plateau in araC accumulation, but DMDC inhibits deoxycytidine kinase activity more weakly than does araC (15). The lipophilicity of DMDC was 3-fold greater than that of araC, a factor that may contribute to the cellular penetration of DMDC by passive diffusion. The differences in metabolism between DMDC and araC would explain their different antitumor activities in vivo. The present preclinical study indicates that DMDC may be preferentially effective against AML cells, suggesting that this therapy is a possible alternative to araC for the treatment of APL.

REFERENCES

Induction of Differentiation of Acute Promyelocytic Leukemia Cells by a Cytidine Deaminase-resistant Analogue of 1- \( \beta \)-d-Arabinofuranosylcytosine, 1-(2-Deoxy-2-methylene-\( \beta \)-d-erythro-pentofuranosyl)cytidine

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