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New retinoids and arsenic compounds for the treatment of refractory acute promyelocytic leukemia: clinical and basic studies for the next generation

Abstract All-trans retinoic acid (ATRA) is a potent differentiation drug for acute promyelocytic leukemia (APL) and is now incorporated into first-line therapy. However, ATRA resistance has become a major clinical problem. This limitation has prompted the development of alternative agents with desirable pharmacologic properties. We describe (1) our recent clinical trial using the new synthetic retinoid Am80 to overcome acquired resistance to ATRA and (2) basic in vitro effects of arsenic trioxide, a possible alternative to ATRA, on APL cells. A total of 19 APL patients who had relapsed after ATRA-induced complete remissions (CRs) received 6 mg/m^2 Am80 p.o. daily until CR; 11 (58%) patients achieved a CR between days 20 and 58 (median day 37). The in vitro sensitivity to Am80, based on PML immunostaining, correlated well with the clinical effect in all patients tested. All three patients whose blasts were sensitive to Am80 in vitro despite a poor response to ATRA achieved CRs. Thus, Am80 might be an effective compound for the treatment of refractory APL and is a promising alternative retinoid. Since arsenic compounds have reportedly induced CRs in APL patients in China, we studied the in vitro effect of arsenic and other metal ions on myeloid leukemia cell lines. The effects of arsenic were limited mainly to APL cells, and the arsenic concentration was critical for the APL cell line NB4: 1 mM As^{3+} induced time-dependent apoptosis, whereas 0.1 mM As^{3+} allowed partial NB4 cell differentiation. Arsenic trioxide was equally effective when used on ATRA-resistant NB4 cells. Among the clinical leukemia samples tested, the in vitro cytotoxic effects of As^{3+} were observed selectively in APL cells, regardless of their ATRA sensitivity. These data suggest that APL cells are sensitive to As^{3+} and that As^{3+} acts on APL cells via a different pathway to ATRA.

Key words APL · ATRA · ATRA resistance · Am80 · As_2O_3

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Introduction

All-trans retinoic acid (ATRA) is a potent differentiating agent for acute promyelocytic leukemia (APL) [1, 8, 10]. Clinical studies have demonstrated high rates of complete remission (CR), even in patients with chemotherapy-resistant APL. Furthermore, a combination of ATRA and standard chemotherapy to treat previously untreated APL has produced higher CR rates and longer survival [13]. However, clinical resistance to ATRA may develop and become a serious problem for differentiation-inducing ATRA therapy [28, 29]. The features of clinical resistance in APL patients include (1) relapse rates are high after ATRA treatment alone, (2) ATRA is frequently ineffective in patients who have relapsed after ATRA-induced CRs, (3) clinical resistance is not necessarily consistent with in vitro resistance, and (4) ATRA resistance is relative rather than absolute in vitro and in vivo. Since ATRA efficacy in APL patients depends on the presence of specific retinoic acid (RA) receptors (RARs), their affinity for ATRA, and their intranuclear concentration, several factors may be involved in clinical ATRA resistance (Table 1).

One possible mechanism of ATRA resistance is molecular alteration of ligand receptors. Several groups have isolated ATRA-resistant HL-60 cell lines with a point mutation at codon 271 or codon 411 of RARa [4, 16, 17, 24]. These mutations produce an RARa with a truncated

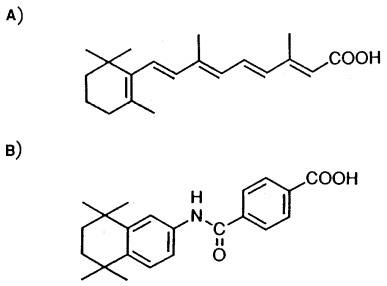

Fig. 1A, B Structures of A ATRA and B Am80

ligand-binding domain and decreased ligand affinity or decreased retinoid X receptor-RAR heterodimer formation. However, HL-60 cells do not carry t(15;17), i.e., PML-RARa chimeric protein; therefore, the differentiation pathways activated by RA and the resistance mechanisms may be different from those of NB4 cells.

Recently we established a new RA-resistant subclone of the APL cell line NB4, designated NB4/RA, and determined that it carries a point mutation in the ligand-binding domain of PML-RARa (Kitamura et al., unpublished results). Although generation of mutations in ligand receptor-encoding DNA is a plausible explanation for ATRA resistance, RARa and PML-RARa mutations have not been reported in clinical APL samples ([19], unpublished data). Extra- and intracellular pharmacological alterations of ATRA may be more important in clinical resistance. Increased expression of proteins involved in ATRA metabolism, such as cytoplasmic RA-binding proteins (CRABPs) and cytochrome P-450, has been reported in APL samples resistant to ATRA [3, 15, 21]. Therapeutic strategies to overcome ATRA resistance are now being developed: the combination of interferon-a or 9-cis RA with ATRA; the addition of cytochrome P-450 enzyme inhibitors, i.e., ketoconazole and clotrimazole; and the use of new synthetic retinoids with better efficacy.

ATRA has certain undesirable pharmacologic properties [20, 27] that might be overcome using other retinoids that modulate effective intranuclear RA concentrations or compounds that act via a pathway other than that used by ATRA. We report herein on a clinical trial of a new synthetic retinoid, Am80, in APL patients who had relapsed after ATRA-induced CRs, and on the in vitro effect of arsenic trioxide (As_2O_3), an agent with a mechanism of action different from that of ATRA, on APL cells.

New synthetic retinoid Am80

Am80 {4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl] benzoic acid; Fig. 1} is a stable retinobenzoic derivative of RA synthesized by Shudo et al. (University of Tokyo, Tokyo, Japan) and has several advantages over ATRA, including (1) little, if any, binding to RARg; (2) differentiation activity against NB4 and HL60 cells that is severalfold greater than that of ATRA; (3) low affinity for CRABPs; and (4) light, heat, and oxidation stability [7, 9, 12].

Table 1 Mechanisms of ATRA resistance

1. Generation of genetic mutations in ligand receptors, i.e., RARa, PML-RARa:
   a. Truncated RARa in ATRA-resistant HL-60
   b. Clinical samples?
2. Extra- and intracellular pharmacokinetic alterations of ATRA:
   a. Increased expression of CRABPs (sequestration)
   b. Increased catabolism in the P-450 pathway
   c. Acceleration of RA oxidation in lipid hydroperoxides

Table 2 Am80 treatment of APL that relapsed after ATRA-induced CR

Clinical outcome	Number of patients (%)	Time since prior ATRA therapy Months (median)
CR	11 (58)	12–45 (23)
Partial response	1 (5)	2–30 (17)
Failure	7 (37)	

The Japanese Ministry of Health and Welfare (MHW) Leukemia Study Group conducted a pilot study of Am80 therapy in APL patients who had relapsed after ATRA-induced CRs [26]. A total of 19 patients aged 21–76 years (median 48 years) with myeloblast + promyelocyte levels in bone marrow of 14–96% (median 64%), WBCs of 400-3900/ml (median 1300/ml), and time since prior ATRA therapy of 2–45 months (median 21 months) who had given written, informed consent received 6 mg/m^2 Am80 p.o. daily until CR. In all, 11 (58%) patients achieved a CR between days 20 and 58 (median 37 days), and remissions occurred due to a differentiation process identical to that seen with ATRA therapy. There was no correlation between the effect of Am80 and the time since prior ATRA therapy (Table 2). Adverse effects were minimal but included hyperlipidemia, skin and lip dryness, and headache. These effects were controllable and resolved when treatment was terminated.

We and other investigstors have previously reported that the PML-RARa-immunostaining pattern in APL cells is highly specific and that RA restores microgranular localization in nuclei and cytoplasm to the normal speckled nuclear pattern within 24 h, leading to subsequent morphologic differentiation [5, 6, 22, 30, 31]. On the basis of these findings, we tried to predict the sensitivity of APL cells to ATRA and Am80 by examining whether the PML-immunostaining pattern was restored by RA in vitro. After incubation of leukemia cells for 48 h in the presence of 0.01 or 1 mM ATRA or Am80, restorative effects were examined using PML immunostaining. Table 3 summarizes the clinical characteristics and the in vitro response of APL cells from the patients tested. The degree of restoration induced by Am80 was closely associated with the clinical response. The sample from patient 1 did not show relocalization with either concentration of ATRA or Am80; however, that from patient 2 responded to Am80 but not to ATRA and showed the normal localization pattern after

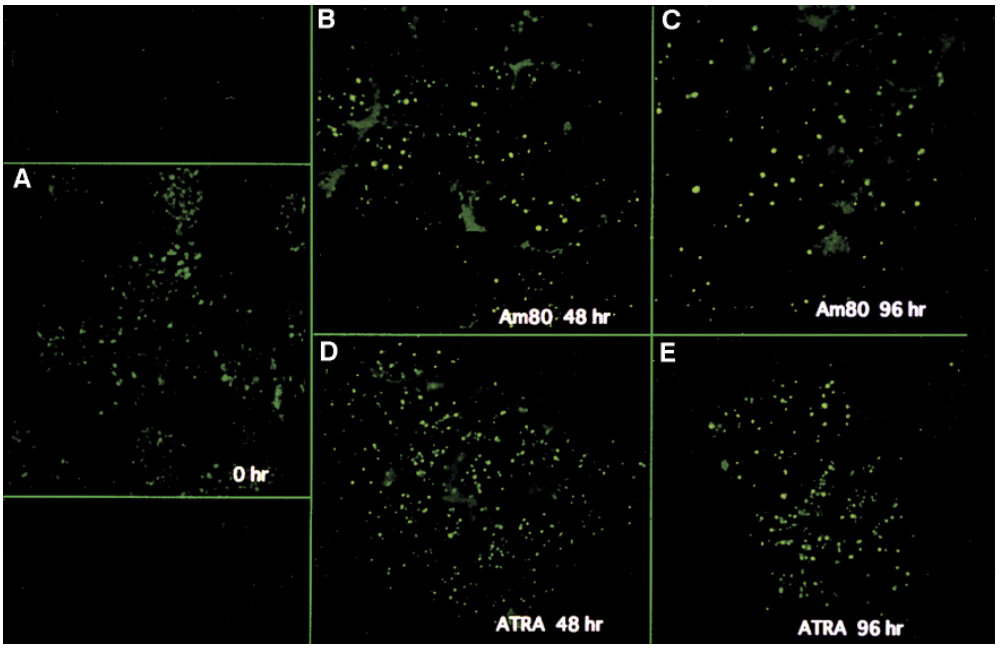

Fig. 2A–E PML immunostaining of APL cells from a patient with an Am80-induced CR (patient 2). Immunostaining features of APL cells from a patient who relapsed after an ATRA-induced CR showed the microgranular pattern (A). Exposure to 0.01 mM Am80 for 48 (B) and 96 h (C) in vitro restored the normal speckled immunostaining pattern, whereas exposure to 0.01 mM ATRA for 48 (D) and 96 h (E) did not change the microgranular pattern. On the basis of these in vitro results, we predicted that Am80 would be effective clinically in this patient, who achieved a CR at day 43

treatment with 0.01 mM Am80 (Fig. 2). Patient 2 achieved a CR after 43 days with Am80 therapy, whereas patient 1 died due to disease progression.

In five individuals (patients 8, 9, 12, 13, and 14) the samples responded similarly to both ATRA and Am80 in vitro; these patients achieved CRs with Am80 therapy. The samples from patients 1, 4, 5, 6, 7, 16, and 17 did not show relocalization of the PML-immunostaining pattern with 0.01 mM ATRA or Am80, and these patients did not achieve a CR. It is noteworthy that the three patients (2, 3, and 15) whose samples were sensitive to Am80 despite a poor response to ATRA in vitro achieved clinical CRs.

When the effects of 1 mM ATRA and Am80 were examined, relocalization was observed in two samples (patients 7 and 11) that showed a poor response, if any, to 0.01 mM ATRA and Am80. The sample from patient 7 was sensitive to 1 mM ATRA and Am80 in vitro and exhibited an incomplete speckled staining pattern despite the lack of response to 0.01 mM retinoid; part of the sample from patient 11 responded to 1 mM retinoid despite its poor

Table 3 Clinical characteristics and in vitro response [++ Rapid and complete restoration to the normal speckled pattern, + incomplete speckled staining with fine granules, ± poor response, – no response, NE not evaluable (degraded cells), PD progressive disease, CR complete remission, NR no response, PR partial response]

Patient	Time since prior ATRA therapy (months)	BM blasts at relapse (%)	In vitro response to[a]		Clinical outcome (day)
			Am80	ATRA	
1	30	96.2	–	–	PD
2	24	91.6	++	±	CR (43)
3	22	83.5	+	±	CR (28)
4	2	25.0	–	–	NR
5	27	82.1	–	–	PD
6	20	88.0	–	–	NR
7	14	61.5	–	–	NR
8	30	23.0	++	++	CR (20)
9	18	97.0	++	++	CR (46)
10	16	42.0	NE	NE	CR (26)
11	3	66.5	±	±	PR
12	25	14.0	+	+	CR (40)
13	25	92.8	++	++	CR (37)
14	22	26.2	+	+	CR (41)
15	18	88.0	+	±	CR (46)
16	25	44.0	–	–	NR
17	14	96.0	–	–	NR
18	45	89.0	±	±	CR (58)
19	12	17.2	NE	NE	CR (29)

[a] An in vitro response indicates that the PML-immunostaining pattern was restored to normal after treatment with 0.01 mM Am80 or ATRA for 48 h

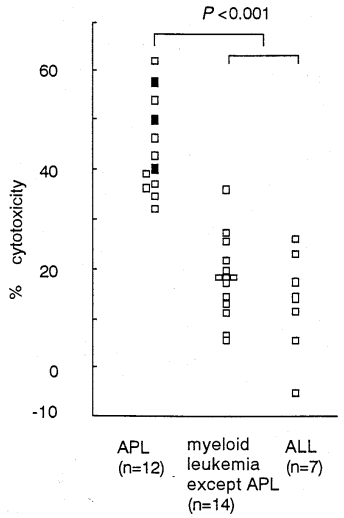

Fig. 3 Cytotoxic effects of 1 mM As_2O_3 on leukemia cells from clinical samples. Leukemia cells were cultured in the presence or absence of 1 mM As_2O_3. The number of viable cells was counted after 72 h, and cytotoxicity was expressed as a percentage of the decrease in the number of viable cells as compared to respective controls. Closed marks indicate APL samples refractory to ATRA treatment in vitro. Myeloid leukemia except APL includes M1, 1; M2, 3; M4, 3; M6, 1; blast crisis of chronic myelogenous leukemia, 3; blast crisis of chronic myelomonocytic leukemia, 3. Acute lymphoblastic leukemia (ALL) includes 5 B-lineage and 2 T-lineage leukemias

response to 0.01 nM retinoid. However, these patients were clinically resistant to Am80 therapy. These findings suggest that clinical failure is due in part to an inability to maintain effective differentiating concentrations in vivo during RA treatment and to heterogeneity of the APL cell population in terms of RA sensitivity. The results of these studies also indicate that PML immunostaining is useful not only as a simple and rapid APL diagnostic tool but also as a method to examine in vitro sensitivity to RA and predict the clinical response to therapy.

Previous studies have shown that APL patients who relapse after ATRA-induced CRs are frequently refractory to a second course of ATRA therapy and that APL cells from patients resistant to ATRA are also resistant to other RA isomers such as 9-cis RA [18, 23]. In comparison, the 58% rate of CR reinduction obtained with Am80 is an improvement, although the issue of the features of APL cells at relapse that explain Am80 sensitivity and ATRA resistance remain to be identified. Thus, Am80 may be an effective compound for the treatment of APL and is a promising alternative retinoid for patients who have relapsed after ATRA-induced CRs, particularly in conjunction with a method of predicting sensitivity to Am80 on the basis of PML immunostaining.

As_2O_3

Recently, reports from China on the effectiveness of arsenic compounds in APL treatment have been published [2, 11, 25]. In a recent clinical trial using As_2O_3, CRs were achieved by 15 of 16 APL patients who had relapsed after ATRA-induced and chemotherapy-maintained CRs [2].

We have studied the in vitro effect of arsenic on the APL cell line NB4 [14]. NB4 cells were cultured in the presence or absence of 1 nM to 10 mM As_2O_3, and the number of viable cells and cell morphology were examined daily. Little effect was observed at As_2O_3 concentrations of <0.1 mM, and at 0.1 mM As_2O_3 treated NB4 cells showed partial differentiation. However, at As_2O_3 concentrations of ≥ 0.1 mM the growth of NB4 cells was suppressed, and 1 mM As_2O_3 caused a gradual decrease in cell number and induced acute cell death. NB4 cells cultured with 1 mM As_2O_3 for 48 h showed apoptotic features, whereas 0.1 mM As_2O_3 caused other morphologic changes: nuclei became indented with coarse chromatin, showing morphologic features similar to those of metamyelocytes and band neutrophils. However, nitroblue tetrazolium (NBT) reduction activity and cell-surface marker expression revealed that the morphologic changes differed from those typically seen during differentiation into granulocytes caused by ATRA: 0.1 mM As_2O_3 neither increased maturation marker CD11b, CD11c, or CD18 expression on myeloid cells nor induced NBT reduction activity in NB4 cells. Furthermore, DNA fragmentation assay indicated that As_2O_3 concentrations of ≥ 1 mM induced time- and dose-dependent DNA fragmentation and that cell death was preceded by the formation of apoptotic bodies and DNA fragmentation. These results suggest that the As_2O_3 concentration is critical for its growth-inhibitory and cytotoxic effects on NB4 cells.

To investigate whether arsenic-induced growth suppression and apoptosis were specific to APL cells, we studied the growth-inhibitory effect of metal compounds, including As_2O_3, As_2O_5, $CdCl_2$, GeO_2, $Hg(CH_3COO)_2$, SeO_2, and $ZnCl_2$, on myeloid cell lines. As observed in NB4 cells, 0.1 mM As_2O_3 suppressed the growth of Kasumi-1 and KG-1 cells, but neither morphologic change nor apoptotic cell death was observed in these cells. Although 1 mM As_2O_3 had antiproliferative activity of varying degrees in the myeloid cell lines tested, significant growth suppression ($\geq$70% growth inhibition as compared to controls after treatment for 48 h) and apoptotic cell death were selectively observed in NB4 cells. NB4 cells tended to be more sensitive than other cell lines to metal ions: 0.1 mM Cd^{2+} and Zn^{2+} suppressed NB4 cell proliferation in a fashion similar to that seen for 0.1 mM As^{3+} but did not induce similar morphologic changes. Interestingly, As_2O_5 had a >10-fold weaker growth-inhibitory effect than did As_2O_3 on NB4 cells. It was noteworthy that NB4/RA cells, which do not show cellular differentiation in the presence of 1 mM ATRA, had the same sensitivity to metal compounds as did parental NB4 cells, exhibiting induced apoptotic cell death at 1 mM As_2O_3.

When clinical leukemia samples were examined, all APL samples, including six obtained from newly diagnosed patients and six acquired from patients who had relapsed after ATRA therapy, were sensitive to arsenic, regardless of

their ATRA sensitivity (Fig. 3). Some cells underwent differentiation-like morphologic or apoptotic changes after 72-h culture. In contrast, no significant cytotoxic response was observed in the other leukemia samples tested, with the exception of one sample that was phenotypically M4 with a normal 46XY karyotype.

The effectiveness of arsenic was limited mainly to APL cells among the myeloid leukemia samples and cell lines tested. However, some other types of leukemia or solid tumors may also be sensitive to arsenic, although the PML-RARa protein may be the target of As_2O_3, similar to the situation with ATRA. Further study is necessary to determine whether only APL is sensitive to arsenic and to elucidate the pathways by which As_2O_3 induces APL cell apoptosis and/or differentiation.

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