Retinoic acid (RA) and As_2O_3 treatment in transgenic models of acute promyelocytic leukemia (APL) unravel the distinct nature of the leukemogenic process induced by the PML-RARα and PLZF-RARα oncoproteins

Eduardo M. Rego*, Li-Zhen He*, Raymond P. Warrell, Jr.†, Zhu-Gang Wang*, and Pier Paolo Pandolfi*‡

*Department of Human Genetics, Molecular Biology Program, and †Department of Medicine, Molecular Therapeutics Program, Memorial Sloan–Kettering Cancer Center, New York, NY 10021

Communicated by Paul A. Marks, Memorial Sloan–Kettering Cancer Center, New York, NY, June 26, 2000 (received for review November 19, 1999)

Acute promyelocytic leukemia (APL) is associated with chromosomal translocations always involving the *RARα* gene, which variably fuses to one of several distinct loci, including *PML* or *PLZF* (X genes) in t(15;17) or t(11;17), respectively. APL in patients harboring t(15;17) responds well to retinoic acid (RA) treatment and chemotherapy, whereas t(11;17) APL responds poorly to both treatments, thus defining a distinct syndrome. Here, we show that RA, As_2O_3, and RA + As_2O_3 prolonged survival in either leukemic PML-RARα transgenic mice or nude mice transplanted with PML-RARα leukemic cells. RA + As_2O_3 prolonged survival compared with treatment with either drug alone. In contrast, neither in PLZF-RARα transgenic mice nor in nude mice transplanted with PLZF-RARα cells did any of the three regimens induce complete disease remission. Unexpectedly, therapeutic doses of RA and RA + As_2O_3 can induce, both *in vivo* and *in vitro*, the degradation of either PML-RARα or PLZF-RARα proteins, suggesting that the maintenance of the leukemic phenotype depends on the continuous presence of the former, but not the latter. Our findings lead to three major conclusions with relevant therapeutic implications: (*i*) the X-RARα oncoprotein directly determines response to treatment and plays a distinct role in the maintenance of the malignant phenotype; (*ii*) As_2O_3 and/or As_2O_3 + RA combination may be beneficial for the treatment of t(15;17) APL but not for t(11;17) APL; and (*iii*) therapeutic strategies aimed solely at degrading the X-RARα oncoprotein may not be effective in t(11;17) APL.

Acute promyelocytic leukemia (APL) is associated with reciprocal translocations that always involve the retinoic acid (RA) receptor α (*RARα*) gene on chromosome 17 (1, 2). RARα variably translocates and fuses to several distinct loci, including the promyelocytic leukemia (*PML*) gene located on chromosome 15 [t(15;17)], or to the promyelocytic leukemia zinc-finger (*PLZF*) gene on chromosome 11 [t(11;17)] (hereafter referred as X genes) (1, 2). PML-RARα and PLZF-RARα have the capacity of heterodimerizing with PML or PLZF, respectively (3–5). Similarly, the RARα portion of X-RARα is able to mediate heterodimerization with retinoid X receptor (RXR; an obligate heterodimeric partner for various nuclear receptors), as well as DNA and ligand binding through the RARα RA- and DNA-binding domains (1, 2, 4). X-RARα has, therefore, the potential ability to interfere with both X and RAR/RXR pathways.

APL in patients harboring t(15;17) is uniquely sensitive to the differentiating action of RA, becoming the paradigm for therapeutic approaches using differentiating agents (1, 2, 6). Treatment with RA alone induces disease remission transiently, and relapse is inevitable if remission is not consolidated with chemotherapy. Furthermore, relapse is often accompanied by RA resistance (1, 2, 6, 7). APL patients harboring t(11;17) show a distinctly worse prognosis with little or no response to treatment with RA, thus defining a distinct APL syndrome (8).

The X-RARα fusion proteins form corepressor complexes with NCoR/SMRT-Sin3A-histone deacetylases, which are less sensitive to RA (9–12). PLZF-RARα, by means of its PLZF moiety, can form corepressor complexes that are insensitive to pharmacological doses of RA (9–12).

RA can also induce the caspase-mediated degradation of the PML-RARα fusion protein (13, 14). Thus, the differential response to RA can be attributed to the differential repressive ability of the two X-RARα fusion proteins.

As in human APL, leukemia in PML-RARα transgenic mice (TM) responds well to RA, which can induce complete disease remission, whereas leukemia in PLZF-RARα TM responds poorly to RA and complete disease remission is never attained (9).

Arsenic trioxide (As_2O_3) is extremely effective in the treatment of APL (7, 15). As_2O_3 may induce the degradation of the PML-RARα protein through ubiquitination of the PML moiety (16–20). This event could be critical in mediating the biological effects of As_2O_3 in APL. However, NB4–306 cells, a RA-resistant cell line derived from NB4 that no longer expresses the PML-RARα fusion protein, responded to As_2O_3 as the parental NB4 cells did (20, 21). In addition, *in vitro*, As_2O_3 shows antitumoral and proapoptotic activity in cancer cells that do not harbor t(15;17) (21–24).

RA and As_2O_3 are both very effective antileukemic drugs in t(15;17) APL. RA triggers differentiation whereas, As_2O_3 induces both apoptosis and partial differentiation of the leukemic blasts (7, 15–21, 25). However, it is still unclear whether combination of these drugs may be more effective in the treatment of t(15;17) APL, and whether therapy-resistant leukemia such as t(11;17) APL responds to As_2O_3 and/or As_2O_3 + RA. Furthermore, the mechanisms by which the X-RARα oncoproteins mediate response or resistance to treatment still need to be established. To address these questions, we have used PML-RARα and PLZF-RARα mouse models of leukemia.

MEDICAL SCIENCES

Abbreviations: RA, retinoic acid; RAR, RA receptor; APL, acute promyelocytic leukemia; PLZF, promyelocytic leukemia zinc finger; TM, transgenic mice; NM, nude mice; PB, peripheral blood; BM, bone marrow; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP end labeling; UN, untreated; C.I., confidence interval.

‡To whom reprint requests should be addressed. E-mail: p-pandolfi@ski.mskcc.org.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.180290497. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.180290497

Materials and Methods

TM and Diagnosis of Leukemia. We used leukemic hCG-PML-RARα and hCG-PLZF-RARα TM. The generation of these TM and the characterization of their hemopoiesis have been described (9, 26). Diagnosis of leukemia was made on the basis of the following concomitant criteria: (*i*) presence of blasts/promyelocytes (>1%) in the peripheral blood (PB); (*ii*) leukocytosis (WBC $> 30 \times 10^3/\mu$l); and (*iii*) anemia (hemoglobin $<$ 10 g/dl) and/or thrombocytopenia (platelets $< 500 \times 10^3/\mu$l).

Nude Mice (NM) Transplantation. NU/J Hfh 11^{nu} 4-to 8-week-old NM (The Jackson Laboratory) were injected with 5×10^7 leukemic cells i.p. The leukemic cells were obtained from the bone marrow (BM) and spleen of moribund leukemic PLZF-RARα or PML-RARα TM. For these experiments, three independent leukemias from each TM genotype were used. No differences in treatment response were observed between recipients of different donors. The morphology, immunophenotype, and expression of the oncoproteins by the transplanted leukemic cells were identical to those observed in leukemic TM. By sacrificing NM at various times after transplantation, we detected leukemic infiltration in the BM and spleen in 100% of the NM by day 25. Thus, the various treatments were started 25 days after transplant.

Treatment with Arsenic and RA. To determine the dose of As_2O_3 to be administered, wild-type (C57BL/6) and NM ($n = 6$, per group) were treated with 1, 2.5, 5, 7.5, and 10 μg/g of body weight per day As_2O_3 (Sigma) with or without 1.5 μg/g per day of RA (Sigma) daily i.p. for 4 weeks, after which three animals per group were killed and a thorough pathological analysis of all organs was performed. As_2O_3 (1 or 2.5 μg/g per day) with or without RA did not cause signs of toxicity, and the remaining animals remained healthy after more than 1 year of follow-up. As_2O_3 (7.5 and 10 μg/g per day) with or without RA resulted in lethality in both NM and wild-type mice. Lethality was probably caused by liver failure, because hepatic necrosis was constantly found. NM perfectly tolerated 5 μg/g per day of AS_2O_3. In wild-type mice, however, this dose caused liver damage, but did not result in death. On the basis of these data, leukemic PLZF-RARα or PML-RARα TM were treated, at presentation, for 21 consecutive days with an i.p. daily injection of (*i*) RA at 1.5 μg/g per day; (*ii*) As_2O_3 at 2.5 μg/g per day; or (*iii*) As_2O_3 + RA at the same doses. NM were treated as above with (*i*) RA at 1.5 μg/g per day; (*ii*) As_2O_3 at 5 μg/g per day; or (*iii*) As_2O_3 + RA at the same doses. TM and NM were followed on a daily basis, and automatic and differential counts of PB were performed weekly. The animals were considered in hematological remission according to the following criteria: hemoglobin levels ≥ 12 g/dl; WBC counts ≤ 30,000/μl with ≤1% blasts, and platelets ≥ 500,000/μl. All these criteria were fulfilled in two consecutive weekly tests.

Apoptosis and Differentiation. NM were transplanted as described with either PML-RARα or PLZF-RARα leukemic cells, and 25 days later they were treated for 3 days with As_2O_3 at 5 μg/g per day, RA at 1.5 μg/g per day, or As_2O_3 + RA at the same doses. Approximately 6 h after the last dose, NM were killed and the leukemic cells were then sorted from the liver by using a MoFlo flow cytometer (Cytomation, Fort Collins, CO). The cells were sorted on the basis of these immunophenotypic features: $CD45^+$, $CD3e^-$, and $B220^-$ (all antibodies were purchased from PharMingen). On sorting, more than 90% myeloid cells were obtained as judged by morphological analysis (Fig. 4*B*). Apoptotic cells were scored by the terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling method (TUNEL) by using the *In Situ* Cell Death Detection Kit (Boehringer Mannheim). The TUNEL assay was performed according to the instructions of the supplier. 4′,6-Diamidino-2-phenylindole (DAPI; Sigma) was used to reveal nuclei. Myeloid differentiation on various treatments was evaluated: (*i*) by morphological analysis scoring the number of mature myeloid cells; and (*ii*) by measuring with flow cytometry the expression levels of the CD11b surface marker (clone M1/70, PharMingen) in the sorted leukemic cells.

Establishment of the Lee Cell Line from a Leukemic PLZF-RARα TM. BM cells were collected from PLZF-RARα TM with leukemia and cultured in DMEM supplemented with 30% FCS, 2% pokeweed mitogen-stimulated murine spleen cell-conditioned medium (StemCell Technologies, Vancouver), 2 mM L-glutamine, and 1% penicillin–streptomycin at 37°C in a 5% CO_2 atmosphere. Without pokeweed mitogen-stimulated murine spleen cell-conditioned medium, cells died within 1 month. After six passages, the concentration of FCS was reduced to 20%, and recombinant stem cell factor and IL-3 (PeproTech, Rocky Hill, NJ) were added to the cultures at a final concentration of 10 and 5 ng/ml, respectively. Morphological and flow cytometric surface marker analyses as well as *in vitro* RA responses revealed features that paralleled those observed *in vivo* in leukemic blasts from PLZF-RARα TM.

Cell Cultures. NB4 cells were grown in RPMI medium 1640 supplemented with 10% FCS and 1% penicillin–streptomycin and maintained at 37°C in a 5% CO_2 atmosphere. Lee cells were cultured as described. The cells were treated with As_2O_3 and/or RA at the indicated doses.

Immunocytofluorescence and Western Blot Analyses. Transplanted NM and leukemic TM were treated for 2 or 7 days with As_2O_3, RA, or As_2O_3 + RA. Leukemic cells were sorted from the liver of NM or harvested from the BM of leukemic TM, cytospun onto slides, and subjected to confocal indirect immunofluorescence analysis with a rabbit polyclonal anti-RARα antibody (a kind gift from P. Chambon, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France) as described (9). Staining with 4′,6-diamidino-2-phenylindole was performed to reveal nuclei. For Western blot analysis, whole-cell lysates were prepared by direct lysis in enzyme immunoassay buffer. Proteins were electrophoresed on an SDS/8% polyacrylamide gel, transferred to a nitrocellulose membrane, and immunostained with anti-RARα and anti-β-actin antibodies.

RNA Analysis. Total RNA was prepared by using Trizol reagents (GIBCO/BRL). For the Northern blot analysis, denatured total RNA (25 μg) was hybridized with the human *PLZF Ava*I probe 3′, following standard procedures.

Statistics. Statistical analysis was performed by using the SPSS 9.0 software (SPSS, Chicago). Survival analysis was based on Kaplan–Meyer estimation and groups were compared by the log-rank test. The comparison between the percentage of CD11b-positive and of TUNEL-positive cells in treated and untreated NM was performed by the Mann–Whitney *u* test. All quoted *P* values are two sided, and confidence interval (C.I.) refers to 95% boundaries.

Results

The Combination of As_2O_3 and RA Is More Effective in the Treatment of Leukemia in PML-RARα TM Than Either Drug Alone. PML-RARα leukemic TM were treated with a daily i.p. dose of As_2O_3, RA, or As_2O_3 + RA. In any of the three therapeutic arms, survival was significantly longer than in untreated (UN) animals ($P <$ 0.0001) (mean survival time of UN: 9.3 days, 95% C.I. = 6.6–12 days; RA: 44.3 days, 95% C.I. = 36.7–51.9 days; As_2O_3: 36.7 days, 95% C.I. = 29.5–43.9 days; As_2O_3 + RA: 72 days, 95% C.I. = 47.2–96.8 days) (Fig. 1*A*). Although in mice treated with RA alone the mean survival was longer than in those treated with As_2O_3 alone, this difference was not statistically significant ($P =$ 0.26), whereas the mean survival of leukemic PML-RARα TM

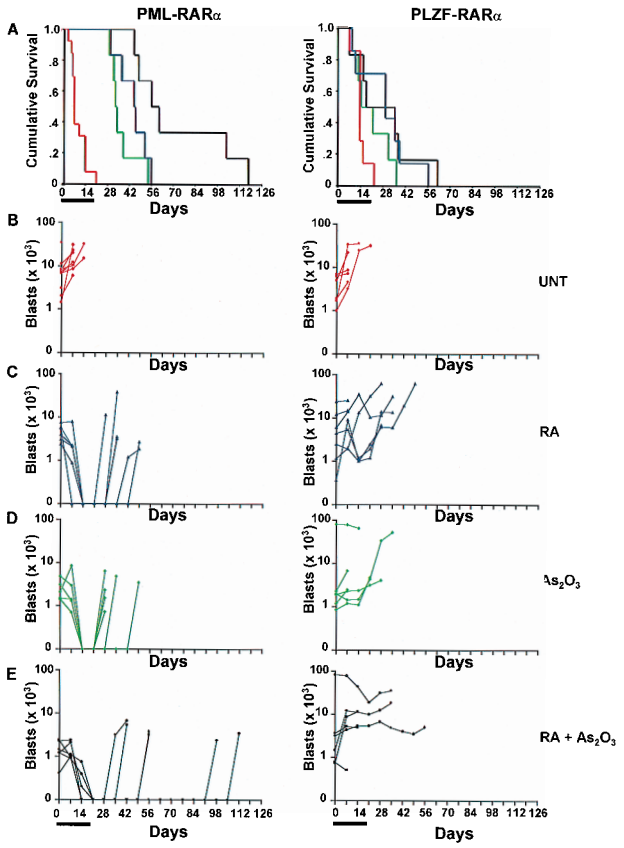

Fig. 1. Effects of treatment with As_2O_3, RA, or As_2O_3 + RA on the survival of leukemic TM PML-RARα and PLZF-RARα. (*A*) Cumulative survival of leukemic TM PML-RARα (*Left*) and PLZF-RARα (*Right*) injected daily i.p. with RA (blue line), As_2O_3 (green line), or RA + As_2O_3 (black line). Control animals received no treatment (red line). The black bar on the abscissa represents the 21-day period of treatment. (*B–E*) Absolute number of blasts (log scale) in the PB of leukemic PML-RARα (*Left*) and PLZF-RARα (*Right*) TM. (*B*) Untreated PML-RARα ($n = 13$) and PLZF-RARα ($n = 7$) TM. (*C*) RA-treated PML-RARα ($n = 6$) and PLZF-RARα ($n = 7$) TM. (*D*) As_2O_3-treated TM ($n = 6$). (*E*) RA + As_2O_3-treated TM ($n = 6$).

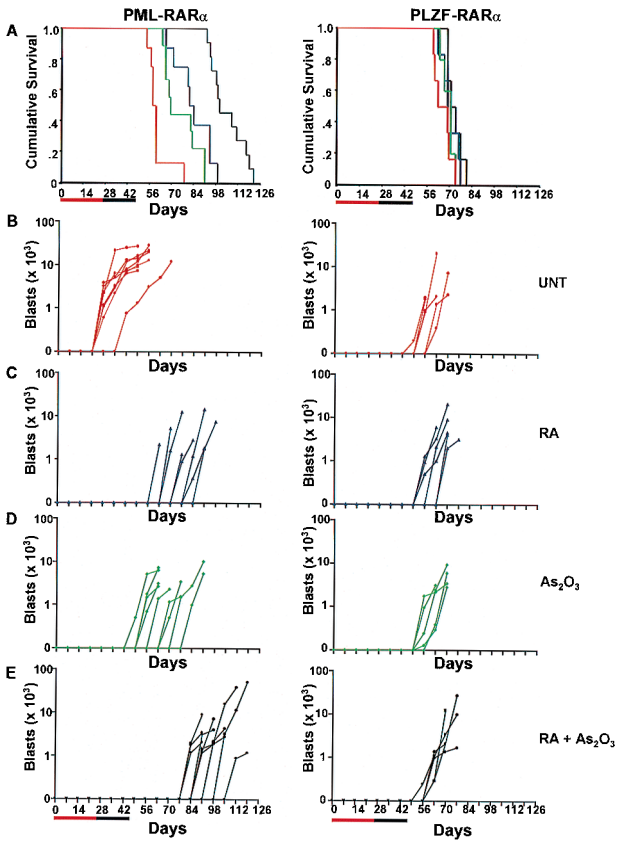

Fig. 2. Effects of treatment with As_2O_3, RA, or As_2O_3 + RA on the survival of NM transplanted with leukemic cells PML-RARα and PLZF-RARα. (*A*) Cumulative survival of NM transplanted with leukemic cells obtained from PML-RARα (*Left*) or PLZF-RARα (*Right*) leukemic TM and treated with daily i.p. injections of As_2O_3 (green line), RA (blue line), or RA + As_2O_3 (black line). Control animals received no treatment (red line). The red bar on the abscissa represents the 25-day period between the transplantation and the beginning of the treatment and the black bar represents the 21-day treatment period. (*B–E*) Absolute number of blasts (log scale) in the PB of leukemic PML-RARα (*Left*) and PLZF-RARα (*Right*) NM. (*B*) Untreated PML-RARα ($n = 8$) and PLZF-RARα ($n = 6$) NM. (*C*) RA-treated PML-RARα ($n = 8$) and PLZF-RARα ($n = 6$) NM. (*D*) As_2O_3-treated PML-RARα ($n = 9$) and PLZF-RARα ($n = 6$) NM. (*E*) RA + As_2O_3-treated NM PML-RARα ($n = 11$) and PLZF-RARα ($n = 6$).

treated with As_2O_3 + RA was significantly longer than the one observed with RA alone ($P = 0.04$) or As_2O_3 alone ($P = 0.03$).

RA and As_2O_3, alone or in combination, induced disease remission in 100% of leukemic PML-RARα TM as demonstrated by the disappearance of the leukemic cells from the PB (Fig. 1 *B–E*) and the return of the WBC, hemoglobin, and platelet counts to normal (data not shown). Disease remission was also confirmed by killing mice and performing differential bone marrow counts in each therapeutic arm at the time when the blasts disappeared from the PB (not shown). Spontaneous disease remission was never observed among untreated mice. After relapse, leukemic infiltration of spleen, liver, and BM was consistently detected in the necropsy.

RA, As_2O_3, or As_2O_3 + RA Are Ineffective in Leukemic PLZF-RARα TM. PLZF-RARα leukemic TM were treated with a daily i.p dose of As_2O_3, RA, or As_2O_3 + RA. Leukemic mice survived longer when treated. However, the mean survival time did not differ statistically between untreated and treated mice ($P = 0.1$) (mean survival time of UN: 14.6 days, 95% C.I. = 11.1–18 days; RA: 30.3 days, 95% C.I. = 17.9–42.6 days; As_2O_3: 21 days, 95% C.I. = 11.7–30.3 days; As_2O_3 + RA: 29.67 days, 95% C.I. = 13.4–45.9 days) (Fig. 1*A*). Moreover, RA, As_2O_3, or their combination never induced disease remission, as shown by the constant presence of leukemic cells in the peripheral blood (Fig. 1 *B–E*).

As_2O_3 + RA Prolongs the Survival of NM Transplanted with PML-RARα Leukemic Cells. In NM transplanted with PML-RARα leukemic cells, either RA or As_2O_3 treatments resulted in a significantly ($P < 0.001$) longer mean survival than that observed in UN mice (Fig. 2*A*). The treatment with RA or As_2O_3 alone resulted in comparable mean survival time (UN: 60.6 days, 95% C.I. = 55.5–65.7 days; As_2O_3: 75.6 days, 95% C.I. = 68.3–82.8 days; RA: 83.6 days, 95% C.I. = 75.6–91.6 days). When treated with the As_2O_3 + RA combination, transplanted NM survived longer than those treated with either drug alone (105 days, 95% C.I. = 98.6–111.4 days; $P = 0.004$ for the comparison between As_2O_3 + RA with RA; $P < 0.0001$ for As_2O_3 + RA versus As_2O_3). Prolonged survival induced by the various drugs was accompanied by a longer interval between the transplantation and the appearance of blasts in the PB (Fig. 2 *B–E*).

RA, As_2O_3, or As_2O_3 + RA Are Ineffective in the Treatment of NM Transplanted with PLZF-RARα Leukemic Cells. As observed in PLZF-RARα TM, neither of the treatments prolonged survival in

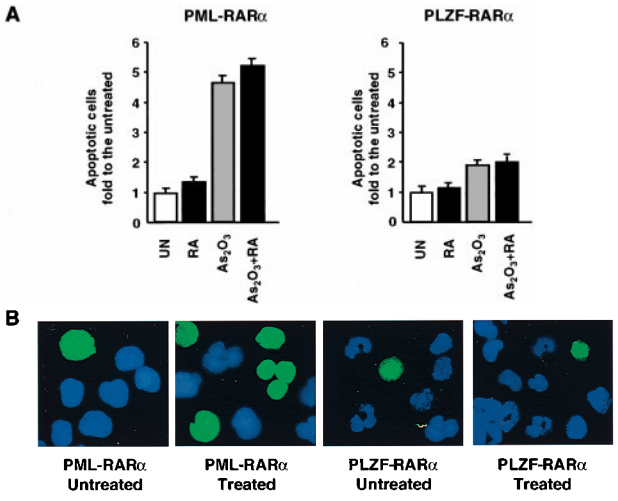

Fig. 3. Effects of treatment with As_2O_3, RA, or As_2O_3 + RA on the induction of apoptosis in leukemic cells sorted from the liver of transplanted NM. (*A*) The percentage of apoptotic cells in the sorted leukemic cells from the NM transplanted with PML-RARα (*Left*) or PLZF-RARα (*Right*) leukemic cells and treated as indicated was evaluated by scoring the number of TUNEL-positive cells. The results are expressed as -fold relative to the untreated animals. Mean percentage ± standard deviation (SD) of PML-RARα leukemic apoptotic cells in the liver: UN: 3.3% ± 0.5%; RA: 4.6% ± 0.8%; As_2O_3: 16% ± 4%; and As_2O_3 + RA: 18% ± 4.5%. Mean percentage ± SD of PLZF-RARα leukemic apoptotic cells in the liver: UN: 4% ± 1.1%; RA: 4.4% ± 1.2%; As_2O_3: 7.7% ± 1.5%; and As_2O_3 + RA: 8% ± 1.8%. (*B*) TUNEL-positive cells (green fluorescence) in sorted leukemic cells from the liver of NM transplanted with PML-RARα or PLZF-RARα cells and treated with As_2O_3 + RA for 3 days. Staining with 4′,6-diamidino-2-phenylindole was performed to reveal nuclei. (×1,000.)

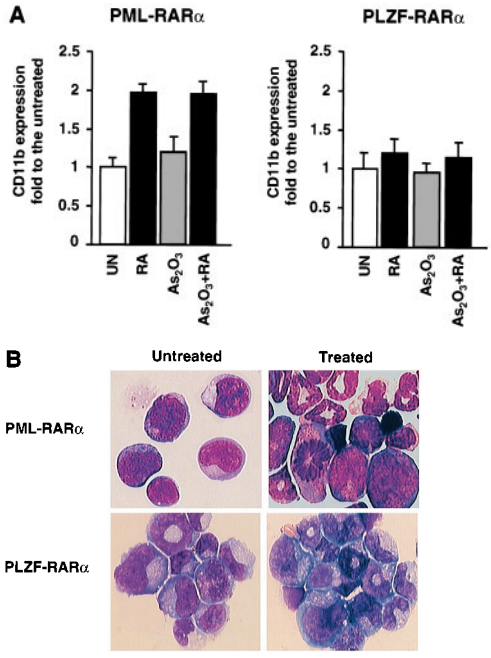

Fig. 4. Effects of treatment with As_2O_3, RA, or As_2O_3 + RA on the induction of differentiation in leukemic cells sorted from the liver of transplanted NM. (*A*) The percentage of $CD11b^+$ cells in the sorted leukemic cells from the NM transplanted with PML-RARα (*Left*) or PLZF-RARα (*Right*) leukemic cells and treated as indicated was determined by flow cytometry. Results are expressed as -fold relative to the untreated animals. Mean percentage ± SD of PML-RARα leukemic cells expressing CD11b in the liver: UN: 25.6% ± 4.5%; RA: 53.5% ± 10.2%; As_2O_3: 33.9% ± 8%; and As_2O_3 + RA: 54.5% ± 12%. Mean percentage ± SD of PLZF-RARα leukemic cells expressing CD11b in the liver: UN: 36.4% ± 4.4%; RA: 43.2% ± 4.1%; As_2O_3: 35.1% ± 5.2%; and As_2O_3 + RA: 44.3% ± 6.5%. (*B*) Morphological features of PML-RARα or PLZF-RARα leukemic cells sorted from the liver of transplanted NM and treated with As_2O_3 + RA for 3 days. The Cytospin preparations were stained with Wright–Giemsa stain. (×1,000.)

transplanted NM in a significant manner (UN: 66.2 days, 95% C.I. = 61.7–70.7 days; As_2O_3: 69.4, 95% C.I. = 65.9–72.8 days; RA: 70.2 days, 95% C.I. = 65.9–74.5 days; As_2O_3 + RA: 73.5 days, 95% C.I. = 69.7–77.3 days). The differences between the three arms were not significant (P = 0.15). In addition, no significant difference was observed among the various treatments in the interval time between transplantation and the detection of blasts in peripheral blood (Fig. 2 *B–E*).

Distinct Biological Response to As_2O_3, RA, or As_2O_3 + RA Treatments in PML-RARα or PLZF-RARα Leukemia Models. In NM transplanted with either PML-RARα or PLZF-RARα leukemic cells, both As_2O_3 and As_2O_3 + RA treatments resulted in increased apoptosis of the leukemic blasts (Fig. 3). However, whereas in the PML-RARα model As_2O_3 and As_2O_3 + RA caused a marked increase in apoptosis (>5-fold), in the PLZF-RARα model, the increase was modest (up to 2-fold) (Fig. 3).

The differential response of the PML-RARα and PLZF-RARα leukemic cells to the various treatments was even more pronounced when we evaluated the differentiating ability of the various drugs. In the PML-RARα NM model, we observed a significant increase in the number of mature myeloid cells (UN: 28.1% ± 8%; RA: 54.5% ± 12.3%; As_2O_3: 35.7% ± 8.3%; As_2O_3 + RA: 57.7% ± 13.2%) as well as in the levels of CD11b expression on treatment with RA or with As_2O_3 + RA, compared with that detected in mice untreated or treated with As_2O_3 alone (Fig. 4). In contrast, in the PLZF-RARα NM model, none of the treatments induced a significant increase in the number of mature myeloid cells (UN: 37.1% ± 12.1%; RA: 41.1% ± 8.1%; As_2O_3: 39% ± 11.7%; As_2O_3 + RA: 43.9% ± 8%) or in the CD11b expression in the leukemic cells, as compared with cells from untreated NM (Fig. 4).

RA or RA + As_2O_3 Can Induce, Both *in Vivo* and *in Vitro*, the Complete Degradation of the PML-RARα or PLZF-RARα Oncoproteins. We next studied the effects of RA, As_2O_3, or their combination on the stability of the PML-RARα or PLZF-RARα oncoproteins in our leukemia models. Immunofluorescence analysis was performed on the cells from the BM of leukemic TM and on the leukemic cells sorted from transplanted NM on various treatments. By using an anti-RARα antibody that recognizes the X-RARα fusion proteins, a characteristic nuclear microspeckled staining pattern was detected in leukemic cells from both PML-RARα and PLZF-RARα TM (ref. 9 and Fig. 5*A*). In the PML-RARα TM and NM models, RA, As_2O_3, or RA + As_2O_3 treatments resulted in the disappearance of the nuclear microspeckled signal after 2 or 7 days of treatment, in agreement with the notion that these drugs can induce the degradation of the PML-RARα protein (Fig. 5*A* and not shown). Surprisingly, however, in both PLZF-RARα TM and NM models of leukemia, RA + As_2O_3 or RA treatments also induced the disappearance of the nuclear microspeckled signal, whereas As_2O_3 alone did not (Fig. 5*A* and not shown), suggesting that RA can induce the degradation of the PLZF-RARα fusion protein as well. Western blot analysis demonstrated that the PLZF-RARα oncoprotein is persistently absent in actively growing leukemic cells treated for 7 days with RA + As_2O_3 (Fig. 5*A*). This result also rules out the possibility that a novel subpopulation of leukemic blasts resistant to the RA + As_2O_3-induced degradation of the PLZF-RARα protein is selected throughout treatment.

To confirm that the disappearance of the microspeckled signal observed in the PLZF-RARα leukemic cells on RA or RA +

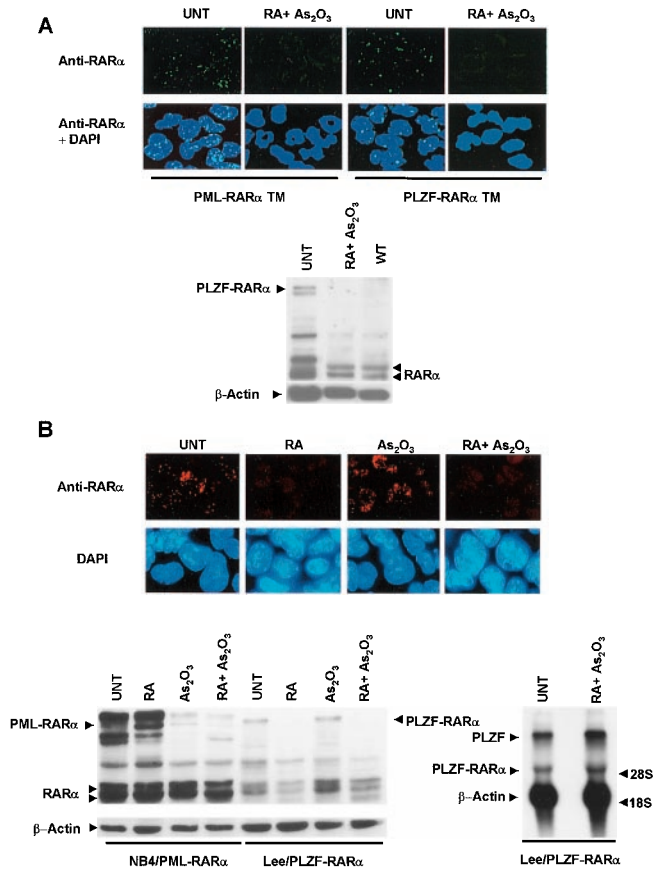

Fig. 5. *In vivo* and *in vitro* effects of RA or RA + As_2O_3 treatments on the PML-RARα and PLZF-RARα oncoproteins. (*A Upper*) Indirect immunofluorescence with an anti-RARα polyclonal antibody on BM cells from leukemic PML-RARα and PLZF-RARα TM-treated for 7 days with As_2O_3 + RA at the same doses used for the trials. In the PLZF-RARα TM, the WBC counts at leukemia presentation were 80,000 cells per μl (4,000 blasts per μl) and a week later 320,000 cells per μl (9,600 blasts per μl) demonstrating the resistance to treatment of this leukemia. The PML-RARα TM, on the contrary, responded well to As_2O_3 + RA treatment. WBC counts at leukemia presentation 38,500 cells per μl (1,925 blasts per μl) and a week later 16,200 cells per μl with no blasts detected in the PB. The green dots reveal the microspeckled distribution of the fusion proteins. Staining with 4′,6-diamidino-2-phenylindole was performed to reveal nuclei. (×1,000.) (*A Lower*) Cellular extracts from BM cells from the leukemic PLZF-RARα TM shown in *A* were subjected to Western blot analysis with an anti-RARα antibody (see *Materials and Methods*). Arrowheads indicate the PLZF-RARα, RARα, and β-actin proteins. (*B Upper*) Immunofluorescence analysis with an anti-RARα antibody on Lee cells treated for 24 h with RA (10^{-7} M), As_2O_3 (10^{-6} M), or As_2O_3 + RA at the same doses, or untreated (vehicle). (×1000.) (*B Lower Left*) Western blot analysis of protein extracts from NB4 and Lee cells treated as in *B Upper*. Arrowheads indicate the PML-RARα, PLZF-RARα, and RARα and β-actin proteins. (*B Lower Right*) Northern blot analysis of total RNA extracted from Lee cells treated with RA (10^{-7} M) plus As_2O_3 (10^{-6} M) for 3 h or untreated (vehicle). Arrowheads indicate the PLZF, PLZF-RARα, and β-actin RNA.

As_2O_3 is not caused by a subcellular redistribution of the oncoprotein, but is the result of its degradation, and to compare the effects of the various drugs on PML-RARα and PLZF-RARα, we took advantage of a cell line established from a leukemic PLZF-RARα TM (Lee) and the APL NB4 cell line, which express the PML-RARα oncoprotein (27). Lee cells express PLZF-RARα, which is detected in its classical nuclear microspeckled distribution (Fig. 5*B*). RA was used at subpharmacological doses (10^{-7} M), which could induce only a partial degradation of PML-RARα in NB4 (Fig. 5*B* and refs. 13 and 14). As observed *in vivo*, however, at this dose of RA, in Lee cells either this drug or RA + As_2O_3, but not As_2O_3, induced the disappearance of the nuclear microspeckled staining and the PLZF-RARα fusion protein as demonstrated by immunofluorescence and Western blot analysis, respectively (Fig. 5*B*). On treatment with RA + As_2O_3, the complete disappearance of the microspeckled staining pattern could already be demonstrated after 3 h (not shown). At this time point, Northern blot analysis showed identical amounts of PLZF-RARα RNA in treated and untreated Lee cells (Fig. 5*B*), suggesting that the disappearance is likely caused by protein degradation. In NB4 cells, in concordance with what was observed in cells from PML-RARα NM and TM, As_2O_3 and RA + As_2O_3 induced the complete degradation of the PML-RARα fusion protein, whereas a subpharmacological dose of RA only partially degraded PML-RARα (Fig. 5*B*). Immunofluorescence analysis in NB4 cells revealed the disappearance of the microspeckled nuclear signal on As_2O_3 and RA + As_2O_3, and its weakening on RA, corroborating what was observed by Western blot analysis (not shown). Thus, despite the unresponsiveness to RA and RA + As_2O_3 observed in the PLZF-RARα TM and NM models of leukemia, both regimens could induce the degradation of the PLZF-RARα oncoprotein.

Discussion

The first conclusion that can be drawn from the data presented here is that RA and As_2O_3 in combination might be effective in the treatment of t(15;17) APL, which represents the most frequent genetic subtype of APL. This is clearly demonstrated by the fact that *in vivo*, in both PML-RARα leukemic TM and NM transplantation models, the two drugs are effective when administered together. These data are in agreement with the notion that RA and As_2O_3 can both induce the degradation of the PML-RARα fusion protein (13, 14, 17–20, 28–30), as we have confirmed *in vivo* in our mouse models. As_2O_3 targets PML-RARα for degradation through the PML moiety (17, 18), whereas RA targets through both the PML and the RARα moieties (13, 14, 28–30). Synergy between RA and As_2O_3 was also reported in a murine transplantation model of PML-RARα leukemia (31). Furthermore, in our PML-RARα animal models, RA acts as an inducer of differentiation, whereas As_2O_3 acts as a proapoptotic agent. In combination, the two drugs do not antagonize their respective activities, thus providing a rationale for combining these drugs in APL treatment. Thus, although caution should be taken in extrapolating from murine models, an important clinical implication of our findings is that RA + As_2O_3 may be effective for the treatment of t(15;17) APL, even at presentation. NM seemed to respond to treatment less efficiently than the leukemic TM, which may be caused by differences in immune response and drug metabolism between NM and TM. However, despite these differences, similar conclusions can be drawn from trials in both TM and NM models.

RA and As_2O_3 activities converge toward the functional and physical inactivation of PML-RARα, which correlates with the efficacy of this treatment. An etiopathogenetic implication of these findings is that the PML-RARα oncogenic function is critical for the maintenance of the malignant phenotype. It is, of course, possible that other biological effects of RA and As_2O_3 may also contribute to the efficacy of the combined treatment. Furthermore, because the animal models studied here do not harbor the reciprocal product of the t(15;17), RARα-PML, we cannot exclude that this fusion protein may also play a critical role in the maintenance of the leukemic phenotype in human APL, as suggested by the fact that one APL case has been reported in which only the reciprocal RARα-PML product was expressed (32).

In contrast, RA, As_2O_3, or their combination does not induce disease remission in leukemic PLZF-RARα TM. These regimens are also ineffective in the PLZF-RARα NM model. Not only do PLZF-RARα leukemias respond poorly to RA, As_2O_3, or RA + As_2O_3, but the leukemic blasts harboring the PLZF-

RARα display a marked protection from the differentiating and proapoptotic effects of these treatments. Thus, the different NH_2-terminal moiety of the two X-RARα oncogenes directly mediates differential response to both RA and As_2O_3. The unresponsiveness to As_2O_3 might be explained by the inability of the drug to induce the degradation of the PLZF-RARα oncoprotein. Surprisingly, however, RA with or without As_2O_3 is effective, both *in vivo* and *in vitro*, in inducing the degradation of PLZF-RARα. Effective degradation of PLZF-RARα by RA has also been reported *in vitro* in cells from one t(11;17) human APL patient (33). These findings unravel a striking difference between leukemias associated with PML-RARα or PLZF-RARα. Whereas PML-RARα leukemia appears to depend on the presence of the oncoprotein, in PLZF-RARα leukemia, the malignant phenotype does not appear to depend entirely on the continuous presence of the oncoprotein, although it cannot be excluded that undetectable amounts of the PLZF-RARα oncoprotein may suffice to exert its leukemogenic effects.

The absence of the PLZF-RARα oncoprotein in treated leukemic cells originates a paradox: how can PLZF-RARα dictate unresponsiveness to treatment, and in its absence, these effects still be maintained? Three explanations can be entertained. (*i*) In PML-RARα leukemia, RA may be effective by inducing transactivation and cellular differentiation by means of the oncoprotein before its degradation. This mechanism is not effective in the case of the PLZF-RARα because this molecule acts as a potent transcriptional repressor even in the presence of RA (9–12). (*ii*) In a multistep model of leukemogenesis, it is formally possible that the additional genetic events which cooperate with the two X-RARα fusion proteins are distinct. These events might render PLZF-RARα, but not PML-RARα, redundant for maintenance of the malignant phenotype. (*iii*) PLZF-RARα might affect transcription through epigenetic mechanisms. Aberrant transcriptional regulation would be, therefore, maintained throughout cell replication even in the absence of the PLZF-RARα oncoprotein. This is supported by the fact that PLZF-RARα through the PLZF moiety can directly interact with nuclear corepressors and NCoR/SMRT-Sin3A-histone deacetylase, thus leading to chromatin remodeling (9–12). In this regard, it is also important to notice that methylation has been associated with epigenetic transcriptional inactivation and cancer pathogenesis (34). A link between gene methylation and histone deacetylation has now been established by a protein known as MeCP2, which binds the mSin-3/NCoR/SMRT-Sin3A-histone deacetylase complex to methylated cytosines (35). Thus, aberrant reorganization of chromatin and/or gene methylation might be propagated through cell division in the promyelocytic blasts. Irrespective of the mechanisms, these findings predict that therapeutic strategies aiming solely at targeting the expression or the stability the PLZF-RARα oncoprotein, such as approaches with hammerhead ribozyme (36), might be ineffective in t(11;17) APL, and that the inactivation/degradation of the fusion protein should be accompanied by a proper costimulus. The complete remission obtained in a t(11;17) APL patient with RA administered in combination with granulocyte colony-stimulating factor (37) might represent a promising precedent.

In summary, our data provide strong support to the tenet that cancer treatment has to be tailored on the basis of the specific molecular mechanisms underlying the pathogenesis of each cancer subtype. To this end, mouse models are invaluable tools to unravel these mechanisms as well as to test *in vivo* the efficacy of novel therapeutic strategies.

We thank G. Cattoretti, P. Chambon, C. Cordon-Cardo, T. Delohery, and M. Lanotte for help, materials, and advice. P.P.P. is a Scholar of the Leukemia Society of America. E.M.R. is the recipient of a Fundação de Amparo à Pesquisa do Estado de São Paulo Fellowship. This work is supported by National Cancer Institute Grant CA-08748 and National Institutes of Health Grants CA71692 and CA74031 to P.P.P.

1. He, L.-Z., Merghoub, T. & Pandolfi, P. P. (1999) *Oncogene* **18,** 5278–5295.
2. Melnick, A. & Licht, J. D. (1999) *Blood* **93,** 3167–3215.
3. Kalantry, S., Delva, L., Gaboli, M., Gandini, D., Giorgio, M., Hawe, N., He, L.-Z., Peruzzi, D., Rivi, R., Tribioli, C., *et al.* (1997) *J. Cell. Physiol.* **173,** 288–296.
4. Perez, A., Kastner, P., Sethi, S., Lutz, Y., Reibel, C. & Chambon, P. (1993) *EMBO J.* **12,** 3171–3182.
5. Bardwell, V. J. & Treisman, R. (1994) *Genes Dev.* **8,** 1664–1677.
6. Warrell, R. P., Jr., de Thé, H., Wang, Z. Y. & Degos, L. (1993) *N. Engl. J. Med.* **329,** 177–189.
7. Soignet, S. L., Maslak, P., Wang, Z. G., Jhanwar, S., Calleja, E., Dardashti, L. J., Corso, D., DeBlasio, A., Gabrilove, J., Scheinberg, D. A., *et al.* (1998) *N. Engl. J. Med.* **339,** 1341–1348.
8. Licht, J. D., Chomienne, C., Goy, A., Chen, A., Scott, A., Head, D. R., Michaux, J. L., Wu, Y., DeBlasio, A., Miller, W. H., Jr., *et al.* (1995) *Blood* **85,** 1083–1094.
9. He, L. Z., Guidez, F., Tribioli, C., Peruzzi, D., Ruthardt, M., Zelent, A. & Pandolfi, P. P. (1998) *Nat. Genet.* **18,** 126–135.
10. Lin, R. J., Nagy, L., Inoue, S., Shao, W., Miller, W. H., Jr., & Evans, R. M. (1998) *Nature (London)* **391,** 811–814.
11. Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F. F., Zamir, I., *et al.* (1998) *Nature (London)* **391,** 815–818.
12. Guidez, F., Ivins, S., Zhu, J., Soderstrom, M., Waxman, S. & Zelent, A. (1998) *Blood* **91,** 2634–2642.
13. Nervi, C., Ferrara, F. F., Fanelli, M., Rippo, M. R., Tomassini, B., Ferrucci, P. F., Ruthardt, M., Gelmetti, V., Gambacorti-Passerini, C., Diverio, D., *et al.* (1998) *Blood* **92,** 2244–2251.
14. Raelson, J. V., Nervi, C., Rosenauer, A., Benedetti, L., Monczak, Y., Pearson, M., Pelicci, P. G. & Miller, W. H., Jr. (1996) *Blood* **88,** 2826–2832.
15. Shen, Z. X., Chen, G. Q., Ni, J. H., Li, X. S., Xiong, S. M., Qiu, Q. Y., Zhu, J., Tang, W., Sun, G. L., Yang, K. Q., *et al.* (1997) *Blood* **89,** 3354–3360.
16. Chen, G. Q., Zhu, J., Shi, X. G., Ni, J. H., Zhong, H. J., Si, G. Y., Jin, X. L., Tang, W., Li, X. S., Xong, S. M., *et al.* (1996) *Blood* **88,** 1052–1061.
17. Sternsdorf, T., Puccetti, E., Jensen, K., Hoelzer, D., Will, H., Ottmann, O. G. & Ruthardt, M. (1999) *Mol. Cell. Biol.* **19,** 5170–5178.
18. Muller, S., Miller, W. H., Jr. & Dejean, A. (1998) *Blood* **92,** 4308–4316.
19. Chen, G. Q., Shi, X. G., Tang, W., Xiong, S. M., Zhu, J., Cai, X., Han, Z. G., Ni, J. H., Shi, G. Y., Jia, P. M., *et al.* (1997) *Blood* **89,** 3345–3353.
20. Shao, W., Fanelli, M., Ferrara, F. F., Riccioni, R., Rosenauer, A., Davison, K., Lamph, W. W., Waxman, S., Pelicci, P. G., Lo Coco, F., *et al.* (1998) *J. Natl. Cancer Inst.* **90,** 124–133.
21. Wang, Z. G., Rivi, R., Delva, L., Konig, A., Scheinberg, D. A., Gambacorti-Passerini, C., Gabrilove, J. L., Warrell, R. P., Jr., & Pandolfi, P. P. (1998) *Blood* **92,** 1497–1504.
22. Zhu, X. H., Shen, Y. L., Jing, Y. K., Cai, X., Jia, P. M., Huang, Y., Tang, W., Shi, G. Y., Sun, Y. P., Dai, J., *et al.* (1999) *J. Natl. Cancer Inst.* **91,** 772–778.
23. Bazarbachi, A., El-Sabban, M. E., Nasr, R., Quignon, F., Awaraji, C., Kersual, J., Dianoux, L., Zermati, Y., Haidar, J. H., Hermine, O., *et al.* (1999) *Blood* **93,** 278–283.
24. Rousselot, P., Labaume, S., Marolleau, J. P., Larghero, J., Noguera, M. H., Brouet, J. C. & Fermand, J. P. (1999) *Cancer Res.* **59,** 1041–1048.
25. Gianni, M., Koken, M. H., Chelbi-Alix, M. K., Benoit, G., Lanotte, M., Chen, Z. & de Thé, H. (1998) *Blood* **91,** 4300–4310.
26. He, L.-Z., Tribioli, C., Rivi, R., Peruzzi, D., Pelicci, P. G., Soares, V., Cattoretti, G. & Pandolfi, P. P. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5302–5307.
27. Lanotte, M., Martin-Thouvenin, V., Najman, S., Balerini, P., Valensi, F. & Berger, R. (1991) *Blood* **77,** 1080–1086.
28. Zhu, J., Gianni, M., Kopf, E., Honoré, N., Chelbi-Alix, M., Koken, M., Quignon, F., Rochette-Egly, C. & de Thé, H. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 14807–14812.
29. Fanelle, M., Minucci, S., Gelmetti, V., Nervi, C., Gambacorti-Passerini, C. & Pelicci, P. G. (1999) *Blood* **93,** 1477–1481.
30. Yoshida, H., Kitamura, K., Tanaka, K., Omura, S., Miyazaki, T., Hachiya, T., Ohno, R. & Naoe, T. (1996) *Cancer Res.* **56,** 2945–2948.
31. Lallemand-Breitenbach, V., Guillemin, M. C., Janin, A., Daniel, M. T., Degos, L., Kogan, S. C., Bishop, J. M. & de Thé, H. (1999) *J. Exp. Med.* **189,** 1043–1052.
32. Lafage-Pochitaloff, M., Alcalay, M., Brunel, V., Longo, L., Saintly, D., Simonetti, J., Birg, F. & Pelicci, P. G. (1995) *Blood* **85,** 1169–1174.
33. Koken, M. H., Daniel, M. T., Gianni, M., Zelent, A., Licht, J., Buzyn, A., Minard, P., Degos, L., Varet, B. & de Thé, H. (1999) *Oncogene* **18,** 1113–1118.
34. Baylin, S. B., Herman, J. G., Graff, J. R., Vertino, P. M. & Issa, J. P. (1998) *Adv. Cancer Res.* **72,** 141–196.
35. Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N. & Bird, A. (1998) *Nature (London)* **393,** 386–389.
36. Nason-Burchenal, K., Takle, G., Pace, U., Flynn, S., Allopenna, J., Martin, P., George, S. T., Goldberg, A. R. & Dmitrovsky, E. (1998) *Oncogene* **17,** 1759–1768.
37. Jansen, J. H., de Ridder, M. C., Geertsma, W. M. C., Erpelinck, C. A. J., Smit, B., Slater, R., van der Reijden, B. A., de Greef, G. E., Sonneveld, P. & Löwenberg, B. (1999) *Blood* **94,** 39–45.