

Accelerated Degradation of PML-Retinoic Acid Receptor α (PML-RARA) Oncoprotein by All-*trans*-Retinoic Acid in Acute Promyelocytic Leukemia: Possible Role of the Proteasome Pathway¹

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Abstract

Acute promyelocytic leukemia (APL) is associated with a chromosomal translocation t(15;17) and successfully differentiated by all-*trans*-retinoic acid (ATRA) *in vivo* as well as *in vitro*. The PML-retinoic acid receptor α (RARA) oncoprotein, which is generated by the translocation, blocks the differentiation, and ATRA is thought to modulate the dominant negative function of PML-RARA. However, the molecular effect of ATRA on PML-RARA is unknown. In this study, we showed by means of immunoblotting that the expression of PML-RARA decreased within 12 h in APL cells treated with ATRA at concentrations greater than 0.1 μ M. The decrease of PML-RARA was associated with restoration of the normal subcellular PML localization. PML-RARA transcripts were not down-regulated by ATRA. However, lactacystin, a specific inhibitor of the proteasome, almost completely inhibited the decrease of PML-RARA. These data indicate that the PML-RARA degradation is accelerated by pharmacological concentrations of ATRA, suggesting that ATRA allows APL cells to differentiate by relieving the differentiation block.

Introduction

ATRA³ is a potent differentiating agent in APL (1, 2). Clinical studies have demonstrated high rates of complete remission even in chemotherapy-resistant APL. Morphological and molecular findings have indicated that terminal maturation and the apoptotic death of the leukemia clone were followed by the recovery of normal hematopoiesis during treatment with ATRA. However, although ATRA therapy has been applied to other cancers, it has been only slightly effective. The presence of t(15;17) seems to play a key role in ATRA sensitivity (1-3).

The chromosomal translocation t(15;17), which is a hallmark of APL, is a reciprocal rearrangement between the PML gene on chromosome 15 and the RARA gene on chromosome 17 (4, 5). Both genes are disrupted mainly within their restricted introns, and fusion genes are formed (4-6). Fused PML-RARA and RARA-PML transcripts are generated, of which the former product is probably associated with the leukemogenesis of APL (reviewed in Ref. 7). Structurally, PML-RARA retains the dimerization property with PML or retinoid X receptor. Thus, the functions of PML and/or retinoid X receptor are sequestered by PML-RARA in a dominant negative manner, which would be associated with the differentiation block. On the other hand, PML-RARA has a ligand receptor domain for ATRA and is thought

to be functionally modulated by binding ATRA. Under pharmacological concentrations of ATRA, the *in vitro*-transfected PML-RARA gene reportedly overstimulated the expression of RARA target genes (7).

Studies on the subcellular localization of PML and PML-RARA have brought about novel insight into the molecular mechanism of the leukemogenesis in APL as well as of the therapeutic effectiveness of ATRA (8, 9). PML-RARA and PML are aberrantly localized as microgranules in the nuclei and cytoplasm of APL cells, whereas in normal cells, PML is found as a discrete speckled pattern in nuclei. Treatment with ATRA restores the proteins to their normal localization, which is associated with subsequent differentiation (10). These data suggest that the effectiveness of ATRA is explained by the loss of the dominant negative function of PML-RARA.

However, a further question remains about the molecular mechanism of ATRA, with regard to its effect on PML-RARA. In this study, we analyzed the expression of PML, PML-RARA, and RARA in the APL cell line NB4 during exposure to ATRA. The expression of PML-RARA was decreased by ATRA. The decrease of PML-RARA was highly correlated with the restoration of the subcellular localization of PML. Furthermore, an inhibition assay suggested that the decrease of PML-RARA was due to accelerated degradation by the proteasome pathway.

Materials and Methods

Cell Culture and Agents. The APL cell line NB4 (11) was obtained from Dr. M. Lanotte (Hôpital Saint-Louis, Paris, France) and cultured in RPMI 1640 with 10% FCS at 37°C in a humidified CO₂ incubator. Kasumi-1, the M2 cell line with t(8;21), and MEG01, the megakaryocytic leukemia cell line with t(9;22), were provided by Drs. N. Kamada (Hiroshima University, Hiroshima, Japan) and M. Ogura (Aichi Cancer Center, Nagoya, Japan), respectively. ATRA was purchased from Sigma Chemical Co. (St. Louis, MO). Treatment with ATRA was performed at concentrations of 1, 0.1, and 0.01 μ M, and the cells were harvested at 6-h intervals. Lactacystin (produced by S. O.) was added to the culture 2 h before the ATRA treatment.

Northern Blotting. Total cellular RNA was extracted using guanidine thiocyanate. The mRNA fraction containing polyadenylic acid was isolated using an oligodeoxythymidylic acid-cellulose column, separated on a formaldehyde-agarose gel, then transferred to a nylon membrane. The membrane was incubated with ³²P-labeled probes and washed four times in 2× SSC [1× SSC = 0.15 M NaCl and 0.015 M Na citrate (pH 7.0)] with 0.1% SDS at room temperature, then once 0.2× SSC with 0.2% SDS at 52°C. Autoradiography proceeded at -80°C for 1-6 days. The probes for the RARA and PML genes were provided by Drs. P. Chambon (Institut National de la Santé et de la Recherche Médicale, Strasbourg, France) and A. Kakizuka (Kyoto University, Kyoto, Japan), respectively.

Production of Rabbit Anti-PML and Anti-RARA Antibodies. An antiserum against PML was produced by immunizing rabbits with the GST-PML fused protein expressed in *Escherichia coli*. The inclusion bodies containing

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The abbreviations used are: ATRA, all-*trans* retinoic acid; APL, acute promyelocytic leukemia; RARA, retinoic acid receptor α ; GST, glutathione S-transferase.

the fused protein were isolated and dissolved in an 8 M urea buffer. The fused protein was resolved by electrophoresis for purification using a Prepcell 491 (Bio-Rad Laboratories, Hercules, CA). The fused protein (100 μ g) was injected into rabbits with Freund's adjuvant five times at 2-week intervals. The antiserum was affinity purified on a GST-PML column then passed through a GST column.

Rabbit antiserum against RARA was produced by immunization with the thyroglobulin-conjugated synthetic polypeptides corresponding to the C-terminal 20 amino acids of RARA. The fused protein (100 μ g) was injected into rabbits with Freund's adjuvant three times at 2-week intervals. The antiserum was then affinity purified.

Immunoblotting. Crude nuclear extracts were prepared from NB4 cells. The cells were washed with cold PBS and resuspended in buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM DTT] for 10 min at 4°C. After centrifugation for 1 min at 600 \times g, the cells were lysed in buffer A containing 0.2% NP40, 1.5 mg/ml protease inhibitors, and 0.2 mM phenylmethylsulfonyl fluoride. The nuclei were pelleted by centrifugation for 1 min at 600 \times g, and nuclear proteins were extracted in buffer B [20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.5 mM DTT, 0.42 M NaCl, 25% (v/v) glycerol, 1.5 mg/ml protease inhibitors, and 0.2 mM phenylmethylsulfonyl fluoride] for 20 min on ice. After centrifugation for 3 min at 15,000 \times g for 4°C, the supernatant nuclear extract was portioned and frozen at -80°C.

The nuclear extracts were separated by 8% SDS-PAGE and electroblotted onto Immobilon polyvinylidene difluoride (Millipore, Bedford, MA). The membranes were incubated in PBS with 3% nonfat dry milk then incubated with the rabbit anti-PML antibody (2 μ g/ml) for 2 h at room temperature. The membranes were washed with PBS containing 0.5% Tween 20 and incubated with peroxidase-conjugated goat antirabbit IgG (Bio-Rad, Richmond, CA) diluted at 1:500 for 2 h at room temperature. After washing, they were stained with a Konica immunostaining kit (Konica Co., Tokyo, Japan). For the blocking experiment, the diluted antibody (2 μ g/ml) was preincubated with 20 μ g/ml GST-PML protein for 1 h at room temperature.

Immunostaining. Cells suspended in RPMI 1640 containing 10% FCS were prepared using a Cytospin (Tomy-Seiko Co., Tokyo, Japan). Specimens were completely dried and fixed with periodate-lysine-paraformaldehyde [0.01 M NaIO₄, 0.075 M phosphate buffer, and 2% paraformaldehyde (pH 6.2)] for 20 min at 4°C. After washing with PBS, the anti-PML antibody (5 μ g/ml) was dropped on the specimen and incubated for 60 min at room temperature. After washing with PBS, 1:100 diluted fluorescence-labeled goat antirabbit IgG (Cappel, West Chester, PA) was added to the slide and incubated for 60 min at room temperature. After washing with PBS, the immunofluorescence was detected using a fluoromicroscope (BH-2; Olympus Co., Tokyo, Japan).

Results

Reactivity of the Anti-PML and Anti-RARA Antibodies. The specificity of the anti-PML and anti-RARA antibodies was confirmed by immunoblotting against leukemia cell lines. The anti-PML antibody detected a band with a molecular mass of 110 kDa only in NB4 cells (Fig. 1A), and this band was diminished when the antibody was incubated with the GST-PML fused protein beforehand (Fig. 1B). The molecular mass was the same as that predicted for PML-RARA (5, 12). Endogenous PML isoforms, which have different molecular masses, were indistinguishable from nonspecific bands. The anti-RARA antibody recognized the 110-kDa band and endogenous RARA isoforms at 50–55 kDa against NB4 cells (Fig. 1D). The anti-RARA antibody did not give the significant immunofluorescence, whereas the anti-PML antibody detected the discrete speckled pattern in nuclei of HL60 cells, as reported previously (8, 9).

Immunoblotting and Immunostaining of PML, PML-RARA, and RARA in NB4 Cells during Exposure to ATRA. NB4 cells were incubated with ATRA, and then the expression of PML, PML-RARA, and RARA was analyzed by immunoblotting and immunostaining. ATRA at a concentration of 1 μ M remarkably decreased the intensity of the PML-RARA band after 6 h. A 0.1 μ M concentration reduced the intensity of the PML-RARA band after 12 h (Fig. 1C). The decrease of PML-RARA was also confirmed using the anti-

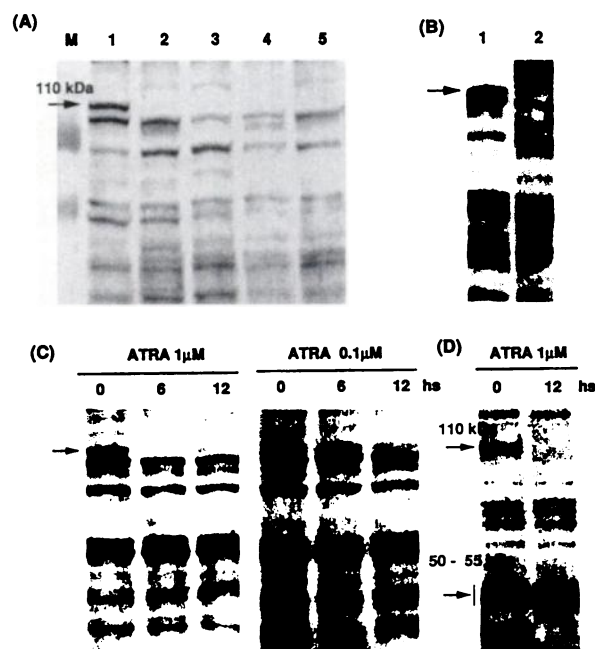


Fig. 1. Immunoblots of PML, PML-RARA, and RARA. A, analysis of the nuclear extracts from various cell lines using anti-PML antibody. Lanes 1–5, NB4, KG1, Kasumi-1, MEG01, and K562, respectively. A 110-kDa band corresponding to PML-RARA (arrow) was detected in NB4. B, analysis of the nuclear extracts of NB4 cells using anti-PML antibody without (Lane 1) or with (Lane 2) preincubation with GST-PML fusion protein. PML-RARA (arrow) band disappeared using anti-PML antibody preincubated with the GST-PML fusion protein. C, analysis of PML and PML-RARA in NB4 cells treated with ATRA. The PML-RARA band (arrow) almost completely disappeared after treatment with 1 μ M ATRA for 6 h. Treatment with 0.1 μ M ATRA also reduced the intensity of the PML-RARA band. D, analysis of PML-RARA and RARA in NB4 cells before and after ATRA treatment using the anti-RARA antibody. The disappearance of the PML-RARA band (top arrow) was confirmed using anti-RARA antibody. Several isoforms of RARA were also detected (bottom arrow).

RARA antibody (Fig. 1D), whereas endogenous RARA bands were stable during exposure to ATRA.

The immunostaining features identified by the anti-PML antibody were changed by 1 μ M ATRA after 6 h; the number of small granules was decreased, the size of granules was increased, and the cytoplasmic staining was reduced. After 24 h, the immunostaining pattern was restored to the normal pattern (Fig. 2A). Exposure to 0.1 μ M ATRA for 24 h brought about a similar immunostaining change to 1 μ M ATRA for 6 h (Fig. 2B). ATRA at 0.01 μ M did not significantly affect the immunoblotting and immunostaining features until 36 h (data not shown). At any concentration examined in this study, the intensity of the PML-RARA band was decreased after exposure to ATRA, and then the immunostaining pattern was changed.

Effects of Lactacystin on the Instability of PML-RARA. To study whether the decreasing effect on PML-RARA by ATRA was due to decreased production or accelerated degradation, we examined the PML and PML-RARA transcripts by Northern blot. The amount of the PML-RARA transcripts was stable during ATRA exposure, although the RARA transcripts were slightly decreased at 6 and 12 h (Fig. 3). The amount of the PML transcripts was stable during the ATRA exposure (data not shown).

Accordingly, we supposed that PML-RARA bound with ATRA might become unstable and undergo accelerated degradation. The degradation of most cellular proteins is catalyzed by the nonlysosomal ubiquitin-proteasome pathway, which is dependent on ATP and closely involved in the proteolysis of aberrantly generated products (13). To investigate whether proteasomes are involved in the decrease of PML-RARA by ATRA, we examined the effect of the *Streptomyces* metabolite lactacystin (14), a highly specific inhibitor of the

proteasome (15). The decrease of PML-RARA induced by ATRA was dose-dependently inhibited by lactacystin (Fig. 4). Lactacystin at 10 μM almost completely inhibited the decrease of PML-RARA induced by 1 μM ATRA. These data suggest that ATRA accelerates the degradation of PML-RARA in the proteasome pathway.

Discussion

This study showed that PML-RARA is promptly degraded in response to the pharmacological concentration of ATRA and confirmed the notion that PML-RARA is the direct target for ATRA therapy. Accordingly, ATRA might allow APL cells to differentiate by relieving the differentiation block caused by PML-RARA.

The dominant negative characteristics of PML-RARA have been supposed to cause the differentiation block in APL. The dominant negative mechanism depends on abnormal molecules, which themselves are negative or nonfunctional variants, sequestering or blocking the function of remaining normal molecules. Therefore, the phenotype is supposed to stand on a precarious balance between normal and abnormal molecules. A decrease in the abnormal molecules might cancel or attenuate the dominant negative phenotype. However, thus far, the function of PML-RARA has been studied using *in vitro*-transfected cells in which the *PML-RARA* gene is exogenously and

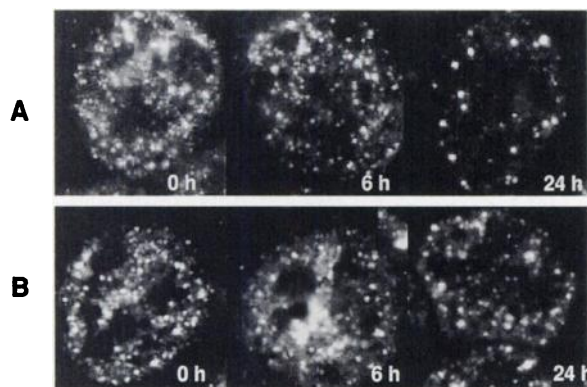


Fig. 2. Immunostaining in NB4 cells treated with ATRA using anti-PML antibody. A, 1 μM ATRA. B, 0.1 μM ATRA. Immunostaining features in NB4 cells showed the microgranular pattern. Exposure to 1 μM ATRA for 24 h restored the normal immunostaining pattern. Exposure to 0.1 μM ATRA for 24 h increased the granule size and decreased their numbers.

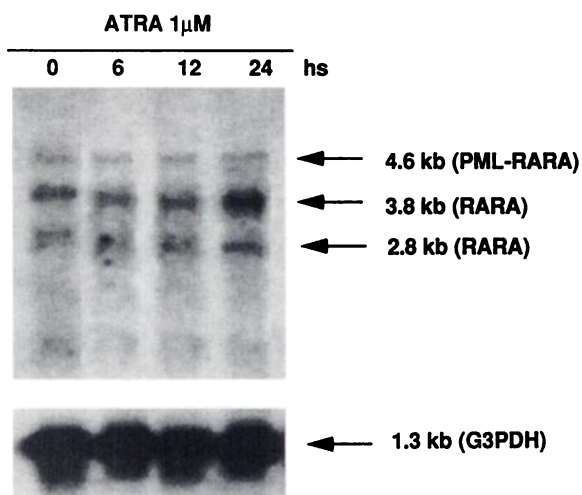


Fig. 3. Northern blots of the PML-RARA and RARA transcripts in NB4 cells incubated with ATRA. The amount of the PML-RARA transcripts was stable, whereas the RARA transcripts were slightly down-regulated at 6 and 12 h. The applied mRNA was calibrated using a cDNA for glycerol-3-phosphate dehydrogenase as a probe.

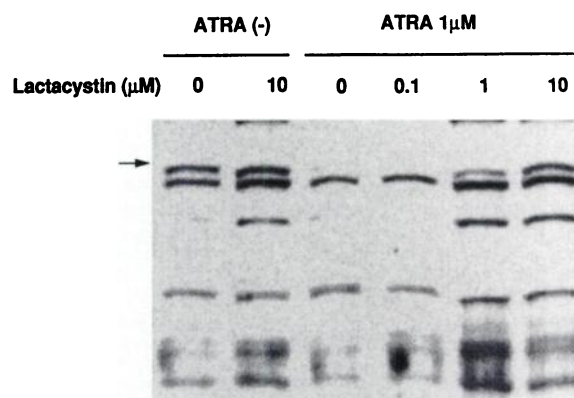


Fig. 4. Effect of lactacystin on PML-RARA in NB4 cells incubated with ATRA. Lactacystin (10 μM) almost completely inhibited the decrease of PML-RARA (arrow) by ATRA.

highly expressed. The transfected cells could represent the differentiation block but might be an unsuitable model for differential therapy because of the high abnormal:normal molecule ratio.

The decrease of PML-RARA by ATRA was inhibited by the proteasome-inhibitor lactacystin. The proteasome degrades many critical regulatory proteins that must be rapidly destroyed for normal growth and metabolism (13). As substrates of the proteasome, transcriptional factors, cyclins, and cell surface proteins have been listed thus far (reviewed in Ref. 16). Highly abnormal proteins that arise due to mutation or postsynthetic damage are also rapidly eliminated by the proteasome. For the decay in the proteasome, posttranslational modification of the target protein, namely ubiquitination, is necessary. Stepwise catalysis by enzymes E1-E3 play roles in binding the ubiquitin polymer to the target protein, although the mechanism of substrate recognition remains unclear. In APL cells, ATRA may change the three-dimensional structure of PML-RARA and may indirectly promote the ubiquitination. However, it is unknown whether the rapid degradation of PML-RARA by ATRA is actually mediated by the ubiquitin pathway. PML-RARA bound with ATRA might be the direct target of the proteolytic attack catalyzed by the proteasome. Indeed, the proteasome is known to degrade ornithine decarboxylase, which has a very rapid turnover, by association with the inhibitory protein antizyme without ubiquitination (17). Further biochemical studies of the decay of PML-RARA in the ubiquitin and/or proteasome pathway are necessary. A rare variant of APL, which has t(11;17) or PLZF-RARA, is unresponsive to ATRA (18), although PML-RARA has a ligand receptor domain (19). The reason for this is unknown, but the ligand-dependent ubiquitination or the proteasome itself might not work in PLZF-RARA. Understanding the mechanism involved in the ubiquitin-proteasome pathway might broaden the biological applications to molecule-targeted therapy.

The view that ATRA allows APL cells to differentiate by relieving the differentiation block might be an important clue for considering subsequent differentiation therapy. Thus far, many agents, including retinoids, hormones, cytokines, and anticancer drugs, patently induce the differentiation of myeloid leukemia cells *in vitro*. However, *in vivo*, most differentiating agents have little effect, except for the effect of ATRA against APL. A distinctive oncoprotein that blocks differentiation might be a suitable target molecule.

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References

1. Warrell, R. P., Frankel, S. R., Miller, W. H., Scheinberg, D. A., Itri, L. M., Hittelman, W. N., Vyas, R., Andreeff, M., Tafuri, A., Jakubowski, A., Gabrilove, J., Gordon, M. S., and Dmitrovsky, E. Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-*trans*-retinoic acid). *N. Engl. J. Med.*, *324*: 1385–1393, 1991.
2. Degos, L., Dombret, H., Chomienne, C., Daniel, M. T., Miclea, J. M., Chastang, C., Castaigne, S., and Fenaux, P. All-*trans*-retinoic acid as a differentiating agent in the treatment of acute promyelocytic leukemia. *Blood*, *85*: 2643–2653, 1995.
3. Miller, W. J., Kakizuka, A., Frankel, S. R., Warrell, R. J., DeBlasio, A., Levine, K., Evans, R. M., and Dmitrovsky, E. Reverse transcription polymerase chain reaction for the rearranged retinoic acid receptor α clarifies diagnosis and detects minimal residual disease in acute promyelocytic leukemia. *Proc. Natl. Acad. Sci. USA*, *89*: 2694–2698, 1992.
4. de The, H., Chomienne, C., Lanotte, M., Degos, L., and Dejean, A. The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor α gene to a novel transcribed locus. *Nature (Lond.)*, *347*: 558–561, 1990.
5. Kakizuka, A., Miller, W. J., Umesono, K., Warrell, R. J., Frankel, S. R., Murty, V. V., Dmitrovsky, E., and Evans, R. M. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR α with a novel putative transcription factor, PML. *Cell*, *66*: 663–674, 1991.
6. Yoshida, H., Naoe, T., Fukutani, H., Kiyoi, H., Kubo, K., and Ohno, R. Analysis of the joining sequences of the t(15;17) translocation in human acute promyelocytic leukemia: sequence non-specific recombination between the *PML* and *RARA* genes within identical short stretches. *Genes Chromosomes & Cancer*, *12*: 37–44, 1995.
7. Grignani, F., Fagioli, M., Alcalay, M., Longo, L., Pandolfi, P. P., Donti, E., Biondi, A., Lo Coco, F., Grignani, F., and Pelicci, P. G. Acute promyelocytic leukemia: from genetics to treatment. *Blood*, *83*: 10–25, 1994.
8. Dyck, J. A., Maul, G. G., Miller W. H., Jr., Chen, J. D., Kakizuka, A., and Evans, R. M. A novel macromolecular structure is a target of the promyelocytic-retinoic acid receptor oncoprotein. *Cell*, *76*: 333–343, 1994.
9. Weis, K., Rambaud, S., Lavau, C., Jansen, J., Carvalho, T., Carmo-Fonseca, M., Lamond, A., and Dejean, A. Retinoic acid regulates aberrant nuclear localization of PML-RAR α in acute promyelocytic leukemia cells. *Cell*, *76*: 345–356, 1994.
10. Naoe, T., Yoshida, H., Kitamura, K., Chino, M., Osumi, K., and Ohno, R. Delayed and insufficient restoration of PML and PML-RAR α subcellular localization after exposure *in vitro* to retinoic acids in all-*trans* retinoic acid-resistant APL cells. *Leukemia (Baltimore)*, *9*: 1610–1611, 1995.
11. Lanotte, M., Martin, T. V., Najman, S., Balerini, P., Valensi, F., and Berger, R. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). *Blood*, *77*: 1080–1086, 1991.
12. de The, H., Lavau, C., Marchio, A., Chomienne, C., Degos, L., and Dejean, A. The PML-RAR α fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. *Cell*, *66*: 675–684, 1991.
13. Goldberg, A. L. Functions of the proteasome: the lysis at the end of the tunnel. *Science (Washington DC)*, *268*: 522–523, 1995.
14. Omura, S., Fujimoto, T., Otaguro, K., Matsuzaki, K., Moriguchi, R., Tanaka, H., and Sasaki, Y. Lactacystin, a novel microbial metabolite, induces neurogenesis of neuroblastoma cells. *J. Antibiot. (Tokyo)*, *44*: 113–116, 1991.
15. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science (Washington DC)*, *268*: 726–731, 1995.
16. Ciechanover, A. The ubiquitin-proteasome proteolytic pathway. *Cell*, *79*: 13–21, 1994.
17. Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., and Ichihara, A. Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature (Lond.)*, *360*: 593–599, 1992.
18. Licht, J. D., Chomienne, C., Goy, A., Chen, A., Scott, A. A., Head, D. R., Michaux, J. L., Wu, Y., DeBlasio, A., Miller, W., Jr., Zelenetz, A. D., Willman, C. L., Chen, Z., Chen, S.-J., Zelent, A., Macintyre, E., Veil, A., Cortes, J., Kantarjian, H., and Waxman, S. Clinical and molecular characterization of a rare syndrome of acute promyelocytic leukemia associated with translocation (11;17). *Blood*, *85*: 1083–1094, 1995.
19. Chen, Z., Brand, N. J., Chen, A., Chen, S. J., Tong, J. H., Wang, Z. Y., Waxman, S., and Zelent, A. Fusion between a novel Krüppel-like zinc finger gene and the retinoic acid receptor- α locus due to a variant t(11;17) translocation associated with acute promyelocytic leukaemia. *EMBO J.*, *12*: 1161–1167, 1993.