8-CPT-cAMP/all-trans retinoic acid targets t(11;17) acute promyelocytic leukemia through enhanced cell differentiation and PLZF/RARα degradation

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The refractoriness of acute promyelocytic leukemia (APL) with t(11;17)(q23;q21) to all-trans retinoic acid (ATRA)-based therapy concerns clinicians and intrigues basic researchers. By using a murine leukemic model carrying both promyelocytic leukemia zinc finger/retinoic acid receptor-α (PLZF/RARα) and RARα/PLZF fusion genes, we discovered that 8-chlorophenylthio adenosine-3′,5′-cyclic monophosphate (8-CPT-cAMP) enhances cellular differentiation and improves gene trans-activation by ATRA in leukemic blasts. Mechanistically, in combination with ATRA, 8-CPT-cAMP activates cAMP/protein kinase A, causing phosphorylation of PLZF/RARα at Ser765 and resulting in increased dissociation of the silencing mediator for retinoic acid and thyroid hormone receptors/nuclear receptor corepressor from PLZF/RARα. This process results in changes of local chromatin and transcriptional reactivation of the retinoic acid pathway in leukemic cells. Meanwhile, 8-CPT-cAMP also potentiated ATRA-induced degradation of PLZF/RARα through its Ser765 phosphorylation. In vivo treatment of the t(11;17) APL mouse model demonstrated that 8-CPT-cAMP could significantly improve the therapeutic effect of ATRA by targeting a leukemia-initiating cell activity. This combined therapy, which induces enhanced differentiation and oncoprotein degradation, may benefit t(11;17) APL patients.

leukemogenesis | PKA pathway | PML/RARα | SMRT | Ncor1

Acutely promyelocytic leukemia (APL) comprises up to 10–15% of acute myeloid leukemia (AML), and its unique morphological, cytogenetic, and clinical features make it a distinct AML subtype (1). The majority of these patients carry the hallmark t(15;17)(q22;q12) chromosomal translocation, which gives rise to the promyelocytic leukemia/retinoic acid receptor-α (PLZF/RARα) fusion gene. These leukemic cells undergo induced terminal differentiation both in vitro and in vivo upon treatment of all-trans retinoic acid (ATRA), a natural pan-retinoic acid-receptor agonist. Modern therapeutic modalities integrating ATRA in both remission-induction and postremission stages have greatly improved the 5-yr survival of these patients up to 90% or even higher (2). In contrast, the 1–2% of APL patients, with t(11;17)(q23;q21) translocation that fuses the promyelocytic leukemia zinc finger (PLZF) to the RARα gene, respond poorly to ATRA treatment. In addition, these APL patients are refractory to concurrent cytotoxic chemotherapy and generally have poor outcomes (3). These features make t(11;17) APL a distinct variant from typical APL. Effective therapeutic regimens for this subtype of APL are imperatively needed.

There have been sporadic case-reports documenting successful differentiation of t(11;17) APL using ATRA in combination with other differentiation inducers, highlighting the possibility and significance of reversing retinoic acid (RA) resistance in the treatment of this peculiar leukemia (4, 5). However, combinatorial differentiation inducers are usually chosen on an empirical basis, and their therapeutic mechanisms are still mostly undefined. This choice can be largely attributed to the scarcity of clinical samples and lack of a suitable disease model.

To facilitate pathophysiological mechanistic studies and the therapeutic exploration of APL with the PLZF/RARα fusion gene, we established a murine leukemic model with both PLZF/RARα and RARα/PLZF fusions expressed in myeloid cells by transgene technology (6). This t(11;17) APL model largely recapitulated the phenotype of human disease. We and others have previously shown that cAMP and the PKA pathway plays important roles in drug-induced APL cell differentiation (7–9). We found that 8-chlorophenylthio adenosine-3′,5′-cyclic monophosphate (8-CPT-cAMP) could enhance the therapeutic efficacy of ATRA through both induction of differentiation and enhanced PLZF/RARα protein degradation. These activities significantly extended the survival of leukemic mice, raising hopes that they could be used in patients.

Results

Combined Treatment with 8-CPT-cAMP and ATRA Induces PLZF/RARα APL Cell Differentiation. We and others have shown that transgenic mice expressing PLZF/RARα under the control of a minigene cassette derived from the human Cathepsin G gene promoter develop chronic myeloid leukemia-like phenotypes (10, 11). We subsequently generated transgenic mice expressing both PLZF/RARα and RARα/PLZF fusion genes. The double-transgenic (DT) mice developed myeloid leukemia resembling the human t(11;17) APL as regards accumulation of immature myeloblastic cells in hematopoietic tissues, impairment of normal hematopoiesis, and infiltration of nonhematopoietic organs, including liver, lung, gastrointestinal tract, and kidney (6). These APL can be transplanted to syngeneic recipient mice by transferring leukemic blasts from DT mice bone marrow (BM) or spleen (Fig. S1 A–D). As few as 105 unselected nucleated BM cells could invariably induce overt disease in lethally irradiated recipient mice (Fig. S1E). Leukemic blasts could be morphologically traced in BM and spleen of recipient mice 10 d posttransplantation (Fig. S1F). A cDNA microarray analysis of CD34+ BM cells from both PLZF/RARα (three PLZF-RARα and two PLZF/RARα–RARα/PLZF) animals as well as PML/RARα (four animals) transgenic mice with leukemia


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revealed distinct gene-expression patterns (Fig. S2), consistent with the distinct nature of the driving fusions.

To identify compounds that might synergize with ATRA to induce APL cell differentiation, we screened several potential differentiation inducers with ATRA, using a nitroblue tetrazolium (NBT) reduction assay of primary BM blasts as an endpoint. We observed a remarkable synergy between 8-CPT-cAMP and ATRA for cellular differentiation (Fig. S3A) \( (P = 0.0003) \). Morphological analysis of the leukemic cells confirmed enhanced cellular differentiation by these two drugs (Fig. S3B).

To validate this cross-talk in a human leukemic system, we used U937 cell sublines harboring stably transfected, conditionally inducible fusion genes (U937-mt-PLZF/RAR\(\alpha\) and U937-mt-P/R9) and their parental control (U937-mt-Bulk) (12) (Fig. S3C). Using the NBT reduction assay, we confirmed that U937-mt-PLZF/RAR\(\alpha\) cells are quite resistant to ATRA-triggered differentiation, but efficiently mature when 8-CPT-cAMP is added. The quantitative extent of leukemic cell maturation represented by the percentage of NBT reduction-positive cells (Fig. 1A) (39.3 ± 1.8\% and 60.0 ± 1.4\% at 5 and 7 d, \( P = 0.0003 \) and \( P = 0.0002 \), respectively) was equivalent to that of PML/RAR\(\alpha\)-expressing U937-mt-P/R9 cells upon ATRA treatment. Moreover, morphological analysis of U937-mt-PLZF/RAR\(\alpha\) cells further validated enhanced differentiation induction effect of combined treatment (Fig. 1B), as did FACS analysis of CD11b and CD11c expression (Fig. S3E and F).

Next, by using a retinoid-responsive luciferase reporter (pRARE-tk-luc), we found that 1 \( \mu \)M ATRA modestly activated RA-response in U937-mt-PLZF/RAR\(\alpha\) cells, but the combination of 8-CPT-cAMP and ATRA dramatically increased reporter activation (Fig. 1C) \( (P = 0.0001) \). U937-mt-P/R9 cells were significantly more responsive to ATRA treatment \( (P = 0.009) \) and also superactivated by coexposure to 8-CPT-cAMP. Collectively, transcriptional response to 8-CPT-cAMP and ATRA in PLZF/RAR\(\alpha\)- or PML/RAR\(\alpha\)-expressing leukemic cells mirrors differentiation (Fig. 1C). We then investigated in U937-mt-PLZF/RAR\(\alpha\) cells the effect of combined treatment in well-known RA target genes, RAR\(\alpha\)2, RAR\(\beta\)2, CEBP\(\alpha\), and TGM2. Real-time PCR and semiquantitative RT-PCR again demonstrated that 8-CPT-cAMP significantly enhanced the transactivating effect.
of ATRA (Fig. 1D and Fig. S3D), in line with the results with artificial reporters.

Silencing Mediator for RA and Thyroid Hormone Receptor Dissociation from PLZF/RARα Through Ser765 Phosphorylation by PKA Is Facilitated by 8-CPT-cAMP. 8-CPT-cAMP activates the PKA pathway and the latter targets RARs (Fig. 2A), prompting us to examine the role of PKA pathway activation in ATRA and 8-CPT-cAMP synergy. Serine/threonine-specific phosphorylation was enhanced in U937-mt-PLZF/RARα cells treated with 8-CPT-cAMP (Fig. 2B) and cAMP-response element binding protein (13) became phosphorylated on Ser133 upon 8-CPT-cAMP treatment; both were further enhanced by ATRA (Fig. S4A).

The cAMP/PKA pathway phosphorylates RARα at Ser369 (14). To test the potential impact of this phosphorylation on PLZF/RARα-mediated transcriptional regulation, we constructed a mutant S765A in its RARα moiety (equivalent to Ser369 of RARα). Then, we examined their affinity to silencing mediator for RA and thyroid hormone receptor/nuclear receptor corepressor (SMRT/NcoR1) in mammalian two-hybrid experiments in COS-7 cells. As shown in Fig. 2, Upper and Fig. S4B, coexpression of PLZF/RARα-WT and SMRT or NCoR1 could remarkably increase the transcriptional activities of pGal4-(RE)5-tk-Luc reporter in COS-7 cells, indicating a direct association between PLZF/RARα and SMRT or NCoR1 proteins. ATRA treatment (10 nM) had minor effects on the association of PLZF/RARα and SMRT, whereas the addition of 8-CPT-cAMP with ATRA significantly loosened SMRT binding to PLZF/RARα (P = 0.019) (Fig. 2C, Upper). Notably, this was reverted by 10 μM H89, a potent selective PKA inhibitor. Of note, the PLZF/RARα-S765A mutant itself failed to bind SMRT (Fig. 2C, Lower), suggesting that Ser765 is essential for both corepressor interaction and 8-CPT-cAMP response. Similar results were found when analyzing the binding of PLZF/RARα to NcoR1 regarding the 8-CPT-cAMP response, although the interaction pattern between PLZF/RARα and NcoR1 was different from that between PLZF/RARα and SMRT (Fig. S4B).

Using ChIP assays, we tested if this combined treatment could affect the amount of corepressors recruited to regulatory regions of RA responsive genes, presumably by PLZF/RARα oncoprotein. As shown in Fig. 2D, ATRA treatment mildly affected the association of SMRT protein with retinoic acid response element (RARE)-containing promoter regions of RARα2, RARβ2, and CEBPs in U937-mt-PLZF/RARα cells. Nevertheless, the combined treatment greatly enhanced the dissociation of SMRT from the promoter regions of these genes. For NCoR1, treatment with ATRA decreased its promoter binding, but addition of 8-CPT-cAMP did not increase corepressor release. The acetylation levels of histone 3/4 were significantly increased under drug combination compared with single-drug treatment (Fig. 2D), consistent with enhanced transcriptional activations.

To investigate the mechanism of enhanced cell differentiation induced by 8-CPT-cAMP and ATRA in PLZF/RARα cells, we compared the dynamic interaction status of the same gene locus, a RARE region of RARα2, between U937-mt-PLZF/RARα cells and U937-mt-P/R9 cells (Fig. S4C). Consistent with previous observations, ATRA alone was enough to activate RARβ2 gene by elevating acetylated histone 3/4, increasing trimethylated H3K4Me3, and decreasing trimethylated H3K27Me3 in U937-mt-P/R9 cells; 8-CPT-cAMP could not further enhance these effects. In contrast, in U937-mt-PLZF/RARα cells, 8-CPT-cAMP potentiated the ATRA-induced increment in acetylated histone 3 and trimethylated H3K4Me3, and contrary to its effect in PML/RARα-expressing cells, increased trimethylated H3K27Me3.

ATRA-Induced Degradation of PLZF/RARα Protein Through Ser765 Phosphorylation Is Enhanced by 8-CPT-cAMP. Phosphodiesterase inhibitors elevate endogenous cAMP and facilitate ATRA-induced PML/RARα degradation through Ser873 phosphorylation
PLZF/RARα levels were decreased by treatment with 8-CPT-cAMP, ATRA, as well as both at most of the time points analyzed (Fig. 3A). PLZF/RARα mRNA expression was unchanged in these conditions. We then used inhibitors of degradation pathways, including caspases (z-VAD-fmk), lysosome (chloroquine), proteasome (MG132), and PKA (H89). A pan-caspase inhibitor, z-VAD-fmk, but not the MG132 proteasome inhibitor, reverted PLZF/RARα degradation by both 8-CPT-cAMP and ATRA, although H89 selectively inhibited 8-CPT-cAMP-induced PLZF/RARα degradation (Fig. 3B), suggesting that PKA activation favors the turnover of PLZF/RARα. To confirm this hypothesis, we overexpressed either wild-type or mutant PLZF/RARα by transient transfection in 293T cells before 24-h treatment with 8-CPT-cAMP and/or ATRA (Fig. 3C). As expected, absence of S765 phosphorylation impeded ATRA-induced PLZF/RARα protein degradation. Taken together, these data show that 8-CPT-cAMP enhances ATRA-induced degradation of PLZF/RARα proteins through S765 phosphorylation.

In Vivo Combination Treatment with 8-CPT-cAMP and ATRA Improves the Survival of t(11;17) APL Mice. To assess the in vivo relevance of this 8-CPT-cAMP/ATRA synergy, we treated t(11;17) APL transplantable mouse model with 8-CPT-cAMP and ATRA. First, we conducted a preliminary short-term experiment (Fig. S5A). The mice treated with the combination showed significantly decreased spleen index (Fig. S5B). Moreover, PLZF/RARα expression was sharply decreased in BM cells (Fig. S5C), likely reflecting both clearance of APL blasts and the fact that these two drugs induced degradation of PLZF/RARα in ex vivo treatment (Fig. S5D). FACS analysis of BM cells further confirmed that normal hematopoiesis was partially restored upon combined treatment (Fig. S5E). Pathological analysis, moreover, demonstrated greatly reduced leukemic cell infiltration in both the liver and spleen in mice that received combined treatment (Fig. S5F), suggestive of a differentiation-enhancing and antiproliferative potency of this combined therapy.

Next, we attempted to evaluate the long-term effect on t (11;17) APL leukemia-initiating cells (LIC) in the transplantable model. We first defined the LICs by isolating the leukemic BM cell populations with an appropriate combination of cell surface markers. Using a stepwise sorting strategy and limited-dilution assay, we characterized cell populations with the strongest leukemia-initiating ability in vivo (for details, see Fig. S6 and Table S1). Myeloid-lineage committed cells (Mac-1+/Gr-1−/c-Kit+) had more primitive features, such as round-oval nucleus with a high nuclear-cytoplasmic ratio, expressed higher levels of PLZF/RARα, and were highly clonogenic (Fig. S6F). In contrast, cells from the Mac-1+/Gr-1+/c-Kit− fraction had a mature myeloid appearance with lower PLZF/RARα expression (Fig. S6 B–E) and were poorly clonogenic (Fig. S6F).

Next, sublethally irradiated animals inoculated with 5 x 10^5 transplantable t(11;17) APL cells were treated with 8-CPT-cAMP (20 mg·kg−d) and ATRA (10 mg·kg·d) for 22 d beginning on day 5 postinoculation (Fig. 4A). As shown in Fig. 4B, within a 120-d observation, those treated with the 8-CPT-cAMP/ATRA combination showed a significantly prolonged survival time compared with those treated with ATRA-alone (median 115 d vs. 52 d, P = 0.0207), demonstrating that the 8-CPT-cAMP/ATRA synergy for differentiation and PLZF/RARα degradation could be translated into a sharp survival benefit.

As shown in the treatment scheme (Fig. 4A), sorted BM cells (Mac-1+/Gr-1−/c-Kit+) were transplanted to secondary recipients either immediately after the termination of treatment (day 27) or 2 wk after the termination of treatment (day 41). In the day 27 transplant recipients, no significant differences were found in survival between the ATRA-alone and combined therapy groups (Fig. 4 C, ii). On day 41, only two groups of primary mice survived (ATRA alone and in combination). Although there was no statistical difference in spleen index between these two groups (Fig. 4 D, i), both the frequency and absolute numbers of the LIC-containing subpopulation were significantly reduced by combined treatment (Table S2). During nearly 100-d follow-up, we found that mice receiving combined therapy showed a significantly prolonged survival time in contrast to ATRA alone, especially at 1,000-cell dose (Fig. 4 D, ii) (P = 0.006). Of note, two mice were additionally treated with a standard dose of Ara-C (250 mg·kg−d) for 5 d, which produced a good response (Fig. 4 C, i) but was not as efficient as combined 8-CPT-cAMP/ATRA therapy in eliminating LICs (Table S2).

In an independently derived DT model previously described (15), a shorter (10 d) treatment initiated 7 d after APL inoculation, sharply prolonged survival and exhibited a clear-cut synergy between 8-CPT-cAMP and ATRA, which normalized spleen weight but was not sufficient to eradicate the disease (Fig. S7 A, i and ii). In fact, BM transplantation experiments in secondary recipients to assess the effect of treatment on LIC activity demonstrated a clear loss of clonogenic activity after 8 d of...
combined treatment, particularly with the highest dose of ATRA (Fig. S7 A, iii). In contrast, 5-Aza-2-Deoxycytidine (DAC), an inhibitor of DNA methylation that had little effect on its own, was dramatically synergistic with ATRA to clear the disease, as assessed by spleen weight and the DAC/ATRA combination could actually eradicate leukemia-initiating activity in secondary recipients (Fig. S7B).

Collectively, these data suggest that the ATRA/8-CPT-cAMP combination favors survival by targeting t(11;17) APL LICs in vivo.

Discussion

Here we used a unique model of APL with PLZF/RARα and RARα/PLZF fusion genes (6). We demonstrate that ATRA triggers both differentiation and PLZF/RARα degradation, both of which are sharply enhanced by PKA activation. It was previously reported that the c-Myc gene is a direct target up-regulated by PLZF/RARα oncoprotein by dominant-negative inhibition of wild-type PLZF (16). We indeed found clues of c-Myc deregulation in our microarray of CD34+ leukemia cells from t(11;17) APL mice (Fig. S2). Full-length PLZF can transcriptionally repress c-Kit expression in CD34+ cells, similar to Myc (17). Reversal of c-Kit repression by PLZF/RARα or RARα/PLZF could account for elevated KIT expression in PLZF/RARα leukemia cells, possibly contributing to their immature features. Enrichment of LICs within the Mac-1+/Gr-1int/c-Kit+ population could illustrate a hierarchical structure within the leukemia. Such disordered immunophenotype of the leukemia blasts is distinct from that of hematopoietic stem cell-like LIC in BCR/ABL+ chronic myeloid leukemia (18). Nevertheless, our results are consistent with PML/RARα-positive APL, where a population of committed myeloid cells (Gr-1int/c-Kit+/FcγRIII/II+/CD34+) was identified as the LIC compartment in different t(15;17) APL models (19, 20). These LICs morphologically resemble our LIC-enriched cell, reflecting possible common origin and oncopogenic mechanism.

Using this model of t(11;17) APL, we found that 8-CPT-cAMP could significantly enhance both the transcriptional activity and cellular differentiation effects of ATRA both ex vivo and in vivo. In APL, ATRA target genes are repressed by PML/RARα through recruitment of a transcriptional corepressor complex containing histone deacetylases (HDACs) (21). The PLZF moiety of PLZF/RARα can also independently recruit SMRT/NcoR1 through its N-terminal POZ/BTB domain (22, 23). Although the effects of various HDAC inhibitors have been tested in APL with t(11;17) and other AMLs, such as those with t(8;21), these agents induce apoptosis rather than differentiation of leukemic blasts (24). In our experiments, addition of 8-CPT-cAMP to ATRA specifically increased acetylation levels of histone 3/4 in ATRA-regulated genes and also enhanced dissociation of SMRT from PLZF/RARα fusion protein. Thus, 8-CPT-cAMP–triggered PKA signaling (25) may cross-talk with the RA pathway and modulate the differentiation of APL cells. PKA-enhanced leukemia differentiation may reflect the “desubordination” of RXRα over RARα by inducing corepressor release from the RARα component of RXR–RARα heterodimers (26). It was also reported that RARα Ser369 phosphorylated by PKA facilitates Ser77 phosphorylation by CDK7/CyclinH complex, which then further potentiates RARα’s transcription ability (14). Indeed, we provide evidence that in the presence of ATRA, PKA-induced Ser765 phosphorylation in PLZF/RARα decreases SMRT/NcoR1 affinity to the fusion protein, thereby facilitating ATRA-induced transactivation.

Besides the effect on transcriptional regulation, substrate phosphorylation by PKA can also affect RARα metabolism. In
(15;17) APL, phosphorylation of PML/RARα Ser873 by PKA led to PML/RARα degradation and, eventually, to eradication of LICs in vivo (15, 27). Similarly, our results demonstrate that 8-CPT-cAMP and ATRA sharply reduce PLZF-RARα levels, contributing to clearance of r(11;17) APL. The primary mechanisms proposed to be implicated in APL response to ATRA are cell differentiation and oncprotein degradation (28, 29). Enforced PML-RARα by means of ATRA through ATRA binding facilitates granulocyte maturation of myeloid cell lines (30, 31). Accordingly, although standard-dose ATRA can induce complete differentiation of APL cells with partial degradation of PML/RARα protein, it fails to eradicate LICs (2, 15). By elevating the intracellular concentration of ATRA, the application of high-dose ATRA or liposomal ATRA results in full PML/RARα degradation and LIC clearance (9, 32, 33). Similarly, ATO, cAMP, or phosphodiesterase inhibitors can also clear r(15;17) APL LICs via complete degradation of the PML/RARα protein (15). Here, we provide evidence that combined therapy of 8-CPT-cAMP and ATRA can induce enhanced differentiation and oncprotein degradation in this model, and raise hopes for better clinical management of r(11;17) APL.

Materials and Methods

Mouse Model and in Vivo Drug Treatment. Six- to eight-week-old female C57BL/ 6J mice were preconditioned with high-energy γ-ray irradiation at a sublethal dose of 4.0 Gy at the day before leukemic cells inoculation. Nucleated cells collected from spleens of mice with full-blown leukemia were injected into each recipient mouse via tail vein to establish a transplantable leukemia mouse model. For in vivo drug treatment, ATRA was administrated through oral gavage at the dosage of 10 or 20 mg·kg−1·d−1. 8-CPT-cAMP was injected intravenously at the dosage of 20 or 40 mg·kg−1. Mice of the untreated group received mock treatment of placebo. For the Ara-C treatment cohort, 250 mg·kg−1 Ara-C was injected intravenously in 5 d. All the animal experiments were approved by The Animal Care & Welfare Committee of Shanghai Jiao Tong University School of Medicine.

Supporting Information

Jiao et al. 10.1073/pnas.1222863110

SI Materials and Methods

Cell Culture, Reagents, and Antibodies. U937-mt-PLZF/RARα and U937-mt-P/R9 cells, which conditionally express promyelocytic leukemia zinc finger/retinoic acid receptor-α (PLZF/RARα) and promyelocytic leukemia/retinoic acid receptor-α (PML/RARα) fusion proteins, respectively, were kindly provided by P. G. Pelicci (University of Milano, Milan, Italy). In parallel, U937-mt-Bulk cells were used as parental control. All cell lines were maintained in RPMI 1640 supplemented with 10% (vol/vol) FBS (Gibco BRL) at 37 °C in a fully humidified atmosphere with 5% CO2. COS-7 cells were maintained in DMEM supplemented with 10% (vol/vol) FBS. Primary murine leukemia cells were flushed from the tibia and femur of mice afflicted by full-blown myeloid leukemia, and cultured in DMEM containing 20% (vol/vol) FBS. All-trans retinoic acid (ATRA), TSA, PMA, DMSO, theophylline, H89 (Sigma-Aldrich), and 8-CPT-cAMP (BI-OLOG Life Science) were prepared as described previously (1). The following antibodies were used: RARα (sc-551), Lamin B (sc-6216) (all from Santa Cruz), PLZF (ab39354), silencing mediator for retinoic acid and thyroid hormone receptors (SMRT; ab24551), nuclear receptor corepressor (NCoR1; ab24552), acetylated H3 (ab47915), trimethylated H3K4 (ab8580), and H3K27 (ab6002) (all from Abcam), acetylated H4 (ab-866, Millipore), p-(Ser/Thr) PKA substrates (9621), p-Ser133-CREB (9104), CREB (9104) (all from Cell Signaling), and β-actin (a1978, Sigma).

Microarray Analysis. We performed a cDNA microarray analysis to profile the gene expression patterns in CD34+ bone marrow (BM) cells from both PLZF/RARα (three PLZF-RARα and two PLZF/RARα-RARα/PLZF animals) and PML/RARα (four PML-RARα animals) transgenic mice with leukemia. Primary BM cells were harvested from the indicated transgenic mice with leukemia and subjected to immunomagnetic-positive selection using the CD34 monoclonal antibody (Miltenyi Bio-tech). Total RNA from the sorted cells was hybridized to the CodeLink Uniset Mouse I Bioarray chips (Amersham). Labeled chips were read by GenePix 4000B microarray scanner (Molecular Devices). Normalized data were filtered for minimal expression and then tested for gene-set enrichment using Gene Set Enrichment Analysis (GSEA) v2.0 (www.broad.mit.edu/gsea). GSEA enrichment results were filtered for statistical significance using a nominal P-value threshold of 0.05.

Immunophenotyping, Cytochemistry, and Histology. For immunophenotypic analysis, fresh 1 × 10^6 BM cells were stained with anti-mouse Mac-1-FITC, Gr-1-PerCP-cy5.5, c-Kit-APC, CD34-PE, Sca-1-PE antibodies, and antilineage kit (BD Pharmingen) separately or in combination, and then analyzed on an LSR II system (BD Biosciences). For morphological analysis, cytopsin slides containing aliquots of 3–4 × 10^4 cells of each sample were stained with Wright–Giemsa staining solution before microscopic inspection. For the nitroblue tetrazolium (NBT) reduction test, cells were incubated in PBS containing 0.1% NBT and 0.5 μg/mL PMA for 30 min at 37 °C. Cells were rinsed with PBS three times before being spun to slides for microscopic examination. For histopathological analysis, sections of selected organs were prepared and stained with H&E by using standard protocol, as previously described (2).

Luciferase Reporter Analysis. The pRARE-tk-Luc reporter plasmid, which was designed to drive the expression of firefly luciferase reporter under the control of a synthetic core consensus sequence derived from the retinoic acid response element (RARE) in the human RARβ2 gene, has been described previously (3). pRL-SV40 was used as internal control for transfection efficiency evaluation (Promega). Transient transfection of U937 cells was carried out using GenePulsor II electroporation system (Bio-Rad). Before electroporation, U937-mt-P/R9 and U937-mt-PLZF/RARα cells were cultured in medium containing 100 μM ZnSO4 for 12 h to induce the expression of the PML/RARα and PLZF/RARα fusion protein, respectively. For electroporation, the complete medium was removed and 2 × 10^6 cells were resuspended in RPMI 1640 containing pRARE-tk-Luc (10 μg) and pRL-SV40 (2.5 μg). The electroporation parameters were 950 μF and 220 V at room temperature. Twelve hours after transfection, cells were treated with indicated materials for an additional 24 h. Then luciferase activities of each sample were detected with Dual-Luciferase Reporter Assay System (Promega) using Lumat LB9507 luminometer (Berthold).

Mammalian Two-Hybrid Analysis. Plasmids for mammalian two-hybrid analysis including pGAL(Re)-tk-Luc, pNLVP16, pVP16-PLZF/RARα, pGal4 empty vector, pGal4-SMRT and pGal4-NCoR1 were kindly provided by A. Zelent (Institute of Cancer Research, Sutton, United Kingdom) and R. N. Cohen (University of Chicago, Chicago, IL) (4, 5). The pVP16-PLZF/RARα-S765A mutant was constructed by site-directed mutagenesis kit (Stratagene). COS-7 cells maintained in 24-well plates were transiently transfected using SuperFect (Qiagen) with 50 ng pVP16-PLZF/RARα and 50 ng pGal4-SMRT or pGal4-NCoR1, in company with 600 ng pGAL(Re)-tk-Luc and 1.5 ng pRL-SV40. After 18 h, cells were treated with ATRA and/or 8-CPT-cAMP for additional 6 h. The activities of luciferase were detected as above.

ChIP. After ZnSO4 induction, U937-mt-PLZF/RARα cells were treated with ATRA and/or 8-CPT-cAMP, as described above. Next, 2 × 10^5 cells of each sample were collected for ChIP assay, as previously described (6). Specific primer pairs spanning RARE sites of individual genes are listed below.

RT-PCR and Western Blot. Real-time RT-PCR assays were carried out by using SYBR Green PCR Master Mix reagents on ABI PRISM 7000 SDS (Applied Biosystems). For Western blot, 20 μg proteins of each sample were fractioned by denatured SDS/PAGE and then transferred onto PVDF membrane (GE Healthcare). Specific antibodies were detected by using an ECL detection kit (Millipore). PCR Primers and ChIP-PCR primers of human or murine genes are listed below.


Fig. S1. Establishment of a unique t(11;17) acute promyelocytic leukemia (APL) transgenic and transportable murine model. (A) Morphological features of cells collected from bone marrow (Upper) and spleen (Lower) of wild-type C57BL/6J mouse (Left), transgenic leukemic mouse (Center), and recipient of leukemia transplantation (Right) (magnification, 1,000×). (B) RT-PCR and Western blot results confirmed expression of the PLZF/RARα and RARα/PLZF fusion genes at the mRNA and protein level in tissues from a transplantable mouse model. (WT, wild-type; DT, dual transgene; BM, bone marrow; SP, spleen). (C) FISH analysis displays the integration of exogenous transgenes in the genome of the transplantable model. Detection of both PLZF/RARα and RARα/PLZF fusion genes in leukemic cells from mouse BM (magnification, 1,000×). (D) Cytogenetic analysis of the leukemic blasts cannot detect any gross chromosomal abnormalities. Cytogenetic analysis of leukemic cells from transplantable model with both PLZF/RARα and RARα/PLZF fusion genes is shown. (E) Survival analysis of recipient mice inoculated with different doses of transplantable leukemic cells. Different doses (a dilution from 20 to 1 x 10⁶ cells) of nucleated cells collected from spleens of mice with full-blown leukemia were injected into each recipient mouse via tail vein. (F) Wright–Giemsa staining of BM and spleen cells from the mice inoculated with 1 x 10⁶ leukemic cells at days 5, 10, and 17 postinoculation, respectively (magnification, 1,000×). A normal C57BL/6J mouse was used as a wild-type control.
Fig. S2. GSEA of the microarray data of two distinct APL transgenic mouse models. (A) Heat map representation of the top 50 up- and down-regulated genes in t(11;17) APL compared with t(15;17) APL. KIT and CDKN2D (arrow) is up- and down-regulated in t(11;17) APL, respectively. (B) Representative gene sets were listed by normalized enrichment score (NES) in each APL model. GSEA plots showed particular gene sets that enriched in PML/RARα APL (Upper) or PLZF/RARα APL (Lower), respectively. (C) Significant target genes were listed with normalized expression score.
Fig. S3. 8-CPT-cAMP potentiates ATRA-mediated gene transactivation. (A) Results of NBT reduction assay of murine primary leukemic cells after 48-h treatment of differentiating agents [including 1 μM SAHA, 25 nM TSA, 0.5 μM VPA, 1 nM PMA, 1% (vol/vol) DMSO, 2 mM theophylline, 2 μM piclamilast, 50 μM 8-CPT-cAMP, 0.5 μM eriocalyxin B, 1 μM oridonin] with or without 1 μM ATRA. (B) Morphological analysis of primary mouse leukemia blasts from t(11;17) APL transplantable mouse model after 8-CPT-cAMP (50 μM) and/or ATRA (1 μM) treatment for 2 d (magnification, 1,000×). (C) Result of Western blot with anti-RARα antibody showed the expression of PML/RARα or PLZF/RARα fusion proteins in U937-mt-Bulk (lanes 1 and 2), U937-mt-P/R9 (lanes 3 and 4), and U937-mt-PLZF/RARα (lanes 5 and 6) cells, respectively, after ZnSO₄ exposure for 12 h (Upper, * denotes unspecific band), and the expression pattern of PLZF/RARα in U937-mt-PLZF/RARα cells (with lanes 1–9 representing samples after 0, 12, 24, 48, 72, 96, 120, 144, and 168 h of ZnSO₄ exposure, respectively). The amounts of Lamin B were used as internal control indicating equivalent loadings (Lower). (D) Semiquantitative RT-PCR results of RARα2, RARβ, CEBPα, and TGM2 genes in U937-mt-PLZF/RARα cells after 8-CPT-cAMP (100 μM) and/or ATRA (1 μM) for 1 and 2 d. 18s rRNA was used as an internal control. (E and F) The expression of CD11b, CD11c, and CD64 on U937-mlt-PLZF/RARα cells after 8-CPT-cAMP (50 or 100 μM) and/or ATRA (1 μM) for 72 h. Each typical result out of three independent assays was shown.
Fig. S4. 8-CPT-cAMP enhances ATRA-induced gene transactivation through its Ser765 phosphorylation. (A) Phosphorylated CREB level is elevated upon 8-CPT-cAMP addition. Both Ser133-phosphorylated and total CREB proteins were detected by Western blot analysis. Loading was calibrated by β-actin (Upper). Quantitative analysis of the ratio of Ser133-phosphorylated and total CREB level was presented by calculating the band’s integrated optical density. (B) 8-CPT-cAMP couldn’t potentiate the ATRA-induced dissociation between NcoR1 and PLZF/RARαS765A mutant. A similar experiment was performed as above. Results are expressed as mean ± SD of two independent experiments. ChIP assays in U937-mt-PLZF/RARα (C, i) U937-mt-P/R9 (C, ii) cells after being treated with indicated agents. N.S. refers to “not significant” in comparison with Student t test.
Combined treatment with 8-CPT-cAMP and ATRA reduces the tumor burden in t(11;17) APL transplantable mouse model in vivo. (A) Scheme of the short-term high-dose drug treatment in vivo. (B) Spleen index of mice receiving drug treatment in vivo. (C) Western blot of PLZF/RARα protein in bone marrow cells from each treatment group was done by anti-RARα antibody (Upper). Equal loading was assessed by β-actin (Lower). (D) PLZF/RARα protein was degraded by combined therapy ex vivo. BM cells from t(11;17) APL mice were treated with 8-CPT-cAMP (100 μM) and/or ATRA (1 μM) ex vivo for 12 h. (E) Flow cytometry analysis of bone marrow cells from each treatment group by Mac-1 and Gr-1. Representative data of two independent experiments were shown. (F) Leukemic cell infiltrations of organs (BM, spleen, and liver) among mice receiving different treatments. Both cytospin (Upper two panels; magnification, 1,000×) and biopsy results (Lower two panels; magnification, 100×) of infiltration organs were presented.
Fig. S6. Identification and characterization of leukemia-initiating cell (LIC) population in t(11;17) APL transplantable mouse model. (A) Illustration of the sorting strategy of LIC population in BM from t(11;17) APL transplantable mouse model. (B) Sorted cells in step 1 were stained by Wright–Giemsa staining, and four representative pictures of each population were taken by an Olympus BX51 microscope (original magnification, 1,000×). (C) Cells isolated from step 2 (Mac-1+/Gr-1+/c-Kit+ and Mac-1+/Gr-1−/c-Kit−) were stained by Wright–Giemsa method. Four representative pictures of each population were taken by Olympus BX51 microscope (magnification, 1,000×). (D) Expression level of PLZF/RARα protein in both c-Kit+ and c-Kit− subsets. (E) Qualitative real-time RT-PCR determined the expression of RA-response genes (RARα2, RARβ2, CEBP{alpha}, and TGM2) (Upper), and granulocyte-specific genes [Cathepsin G, Elastase2 (Ela2), Myeloperoxidase (MPO), Lactoferrin, and MMP9] (Lower) in sorted cells from Mac-1+/Gr-1+ and Mac-1+/Gr-1− subpopulations. Representative data of two independent experiments are shown. (F) Survival curves of secondary recipients inoculated by different sorted populations from indicated steps. (i) 200-cell and 50-cell doses of sorted populations were transplanted in step 1 isolation. (ii) 200-cell and 50-cell doses of sorted populations were transplanted in step 2 isolation. (iii) Four c-Kit+ populations were isolated and transplanted at 50-cell dose.
**In vivo combined treatment with 8-CPT-cAMP, 5-Aza-2-Deoxycytidine (DAC) and different doses of ATRA improves the survival of transplantable t(11;17) APL mice by promoting clearance of LICs.**

(A) A transplantable t(11;17) APL model was independently established and nude mice were treated as previously described (1), using APLs from PLZF/RARα-RARα/PLZF mice (2). (i) The survival curve of primary mice treated with indicated therapies for 10 d, 7 d after inoculation. (ii) Spleen weight of moribund mice killed at day 8. (iii) The survival curve of the secondary recipients inoculated with BM APL cells derived from treated mice at day 8. (B) Similar experiments were carried out using DAC (5 mg kg⁻¹) given intraperitoneally, and ATRA. (i) Spleen weight of mice killed at day 4. (ii) The survival curve of the secondary recipients of day 4 for LIC assessment. RA10, ATRA 10 mg kg⁻¹; RA100, ATRA 100 mg kg⁻¹.

Table S1. Identification of LIC population by limited dilution assay in BM transplantation experiment

<table>
<thead>
<tr>
<th>Cell-sorting strategy</th>
<th>Sorted population (donor n)</th>
<th>Cell doses</th>
<th>APL incidence of recipients*</th>
<th>LIC frequency (95% CI)</th>
<th>P value†</th>
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</thead>
<tbody>
<tr>
<td><strong>First sorting step‡</strong></td>
<td>Mac-1⁺/Gr-1⁺ (n = 2)</td>
<td>1,000</td>
<td>3/3 (100%)</td>
<td>1 in 124 (54–283)</td>
<td>0.0174</td>
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<td></td>
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<td>200</td>
<td>3/5 (60%)</td>
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<td>50</td>
<td>4/7 (57%)</td>
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<tr>
<td></td>
<td>Mac-1⁺/Gr-1⁻ (n = 2)</td>
<td>1,000</td>
<td>2/2 (100%)</td>
<td>1 in 668 (219–2041)</td>
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<td>1/5 (20%)</td>
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<td>50</td>
<td>0/7 (0%)</td>
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<tr>
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<td>Mac-1⁺/Gr-1⁻ (n = 2)</td>
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<td>2/3 (67%)</td>
<td>1 in 730 (259–2,059)</td>
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<td>0/7 (0%)</td>
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<tr>
<td></td>
<td>Mac-1⁻/Gr-1⁻ (n = 2)</td>
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<td>4/4 (100%)</td>
<td>1 in 2,745 (1,626–4,567)</td>
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<td>50</td>
<td>0/7 (0%)</td>
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<tr>
<td></td>
<td><strong>Second sorting step§</strong></td>
<td>Mac-1⁺/Gr-1⁻/c-Kit⁺ (n = 2)</td>
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<td>3/3 (100%)</td>
<td>1 in 59 (39–89)</td>
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<td>6/7 (86%)</td>
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<td>Mac-1⁺/Gr-1⁻/c-Kit⁻ (n = 2)</td>
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<td>2/2 (100%)</td>
<td>1 in 432 (155–1207)</td>
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<td>2/5 (40%)</td>
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<td>0/6 (0%)</td>
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<tr>
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<td>c-Kit⁺ populations‡</td>
<td>c-Kit⁺ /Mac-1⁺/Gr-1⁺ (n = 2)</td>
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<td>c-Kit⁺ /Mac-1⁻/Gr-1⁺ (n = 2)</td>
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<td>c-Kit⁺ /Mac-1⁺/Gr-1⁻ (n = 2)</td>
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<td>c-Kit⁺ /Sca-1⁺/Lin⁻ (n = 2)</td>
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<td>0/6 (0%)</td>
<td>N.A.</td>
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</table>

N.A., not available.

*APL incidence represented as “Recipients died from APL/Total recipients.”

†Comparison between groups of Mac-1⁺/Gr-1⁺ and Mac-1⁻/Gr-1⁻ treatment in first BM transplantation (BMT) experiment. Comparison between groups of Mac-1⁺/Gr-1⁺/c-Kit⁺ and Mac-1⁻/Gr-1⁻/c-Kit⁻ treatment in second BMT experiment. Two-tailed P value was estimated by limited-dilution assay analysis (L-software; StemCell). Comparison of the differences of the survival curves of four groups. Two-tailed P value was calculated by Long-rank algorithm for survival analysis.

‡Follow-up time was up to 90 d.

§Follow-up time was up to 135 d.

¶Follow-up time was up to 120 d.
Table S2. 8-CPT-cAMP and ATRA combinatorial therapy can target LIC (Mac-1+/Gr-1+/c-Kit+) and reduce LIC frequency and absolute number in t(11;17) APL model

<table>
<thead>
<tr>
<th>BMT</th>
<th>Treatment (donor n)</th>
<th>Cell doses</th>
<th>APL incidence of recipients*</th>
<th>LIC frequency (95% CI)</th>
<th>P value†</th>
<th>Whole BM3 (×10⁶ cells)</th>
<th>Mac-1+/Gr-1+/c-Kit+ (×10⁶ cells)</th>
<th>Mac-1+/Gr-1+/c-Kit+ absolute number of LIC5 (×10⁶ cells)</th>
<th>P value*</th>
</tr>
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<tbody>
<tr>
<td>First BMT†</td>
<td>Untreated (n = 2)</td>
<td>1,000</td>
<td>3/3 (100%)</td>
<td>1 in 26 (10–64)</td>
<td>P = 0.2625</td>
<td>18.25 ± 3.54</td>
<td>45.00 ± 1.84</td>
<td>8.18 ± 1.26</td>
<td>0.3146 ± 0.048</td>
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<tr>
<td></td>
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<td>200</td>
<td>5/5 (100%)</td>
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<td></td>
<td></td>
<td>50</td>
<td>6/7 (86%)</td>
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<td></td>
<td>8-CPT-cAMP (n = 2)</td>
<td>1,000</td>
<td>3/3 (100%)</td>
<td>1 in 72 (32–160)</td>
<td>22.50 ± 1.77</td>
<td>36.00 ± 4.95</td>
<td>8.06 ± 0.48</td>
<td>0.1119 ± 0.0066</td>
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<td></td>
<td>ATRA (n = 2)</td>
<td>1,000</td>
<td>3/3 (100%)</td>
<td>1 in 417 (164–1063)</td>
<td>28.38 ± 0.88</td>
<td>19.70 ± 13.86</td>
<td>5.53 ± 3.76</td>
<td>0.013 ± 0.0090</td>
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<tr>
<td></td>
<td>cAMP+ATRA (n = 2)</td>
<td>1,000</td>
<td>2/2 (100%)</td>
<td>1 in 1047 (283–3875)</td>
<td>41.75 ± 5.30</td>
<td>11.00 ± 0.47</td>
<td>4.61 ± 0.78</td>
<td>0.004398 ± 0.00074</td>
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<td>Ara-C (n = 2)</td>
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<td>3/4 (75%)</td>
<td>1 in 331 (118–929)</td>
<td>43.25 ± 1.41</td>
<td>10.88 ± 1.41</td>
<td>4.69 ± 0.45</td>
<td>0.036 ± 0.0035</td>
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<td>2/3 (67%)</td>
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<tr>
<td>Second BMT**</td>
<td>ATRA (n = 4)</td>
<td>1,000</td>
<td>8/10 (80%)</td>
<td>1 in 428 (211–869)</td>
<td>P = 0.0054</td>
<td>39.67 ± 18.24</td>
<td>28.87 ± 17.99</td>
<td>8.99 ± 4.74</td>
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<td>200</td>
<td>4/5 (80%)</td>
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<tr>
<td></td>
<td>cAMP+ATRA (n = 3)</td>
<td>1,000</td>
<td>2/10 (20%)</td>
<td>1 in 2483 (900–6844)</td>
<td>46.11 ± 14.63</td>
<td>16.98 ± 20.01</td>
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<td>0.0024 ± 0.0021</td>
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*APL incidence represented as “Recipients died from APL/total recipients.”
†Comparison of LIC frequency between groups of ATRA and cAMP+ATRA treatment in each BMT experiment. Two-tailed P value was estimated by limited-dilution assay analysis (L-calc software; StemCell).
‡Data for LIC quantification were derived from two femurs and tibias.
§Absolute number of LICs of each group was calculated by multiplying absolute number of Mac-1+/Gr-1+/c-Kit+ by LIC frequency.
*Comparison of absolute number of LIC between groups of ATRA and cAMP+ATRA treatment in each BMT experiment. Two-tailed P value was estimated by Student t test.
{Comparison of absolute number of LIC between groups of ATRA and cAMP+ATRA treatment in each BMT experiment. Two-tailed P value was estimated by Student t test.
**Follow-up time was up to 120 d.
**Follow-up time was up to 105 d.