

RXR α ligand Z-10 induces PML-RAR α cleavage and APL cell apoptosis through disrupting PML-RAR α /RXR α complex in a cAMP-independent manner

Lin Xu^{1,*}, Zhiping Zeng^{1,*}, Weidong Zhang¹, Gaoang Ren¹, Xiaobin Ling¹, Fengyu Huang¹, Peizhen Xie¹, Ying Su^{1,2}, Xiao-kun Zhang^{1,2}, Hu Zhou¹

¹School of Pharmaceutical Sciences, Fujian Provincial Key Laboratory of Innovative Drug Target Research, Xiamen University, Xiamen, Fujian, China

²Cancer Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California, USA

*These authors have contributed equally to this work

Correspondence to: Hu Zhou, email: huzhou@xmu.edu.cn
Xiao-kun Zhang, email: xkzhang@xmu.edu.cn

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ABSTRACT

The major oncogenic driver of acute promyelocytic leukemia (APL) is the fusion protein PML-RAR α originated from the chromosomal translocation t(15;17). All-trans retinoic acid (ATRA) and arsenic trioxide cure most patients by directly targeting PML-RAR α . However, major issues including the resistance of ATRA and arsenic therapy still remain in APL clinical management. Here we showed that compound Z-10, a nitro-ligand of retinoid X receptor α (RXR α), strongly promoted the cAMP-independent apoptosis of both ATRA-sensitive and resistant NB4 cells via the induction of caspase-mediated PML-RAR α degradation. RXR α was vital for the stability of both PML-RAR α and RAR α likely through the interactions. The binding of Z-10 to RXR α dramatically inhibited the interaction of RXR α with PML-RAR α but not with RAR α , leading to Z-10's selective induction of PML-RAR α but not RAR α degradation. Z-36 and Z-38, two derivatives of Z-10, had improved potency of inducing PML-RAR α reduction and NB4 cell apoptosis. Hence, RXR α ligand Z-10 and its derivatives could target both ATRA-sensitive and resistant APL cells through their distinct acting mechanism, and are potential drug leads for APL treatment.

INTRODUCTION

Acute promyelocytic leukemia (APL) originates from the specific chromosomal translocations mostly between chromosomes 15 and 17, leading to the occurrence of the fused oncogene promyelocytic leukemia - retinoic acid receptor- α (PML-RAR α) [1]. The corresponding chimeric protein PML-RAR α is most often the only driving factor of APL initiation [2, 3]. PML-RAR α binds to RAR α cognate DNA elements and inhibits RAR α -mediated transcription of differentiation genes, leading to the differentiation block of promyelocytes [2, 4]. PML is the organizer of a nuclear structure known as the PML nuclear bodies (NBs), which is important for maintaining cellular homeostasis [5, 6]. Another pathogenic activity of PML-RAR α is to disrupt the PML nuclear bodies through its interaction with PML [7].

All-trans-retinoic acid (ATRA) induces the transcriptional activity of PML-RAR α , leading to the induction of RAR α -targeted genes and the differentiation of promyelocyte [2]. ATRA also triggers proteasome- and caspase-mediated degradation of PML-RAR α to contribute its efficacy [8–10]. However, ATRA at pharmacological concentration strongly stimulates the transactivation and degradation of RAR, resulting in some RAR-related adverse effects [11, 12]. Also, long term exposure of ATRA often results in the relapse likely due to the mutated RAR α moiety in PML-RAR α [13, 14]. Arsenic trioxide binds to the PML moiety, leading to SUMO-dependent and ubiquitin-mediated degradation of PML-RAR α [15, 16]. The reduction of PML-RAR α sensitizes APL cells to the apoptotic signals triggered by arsenic [17]. Because arsenic targets to the PML moiety, it can overcome ATRA-resistance in some cases [13].

However, APL driven by the PLZF-RAR α and PML-RAR α with point mutations in the PML moiety such as A216V are resistant to arsenic [18, 19]. Thus, novel drugs with different therapeutic mechanisms are desperately needed to overcome the resistance and adverse effects of ATRA and/or arsenic.

Retinoid X receptor- α (RXR α) is a unique nuclear receptor due to its ability to form heterodimers with many other nuclear receptors [20]. It has been elucidated that PML-RAR α forms homodimer mediated by the PML moiety, and RXR α binds to PML-RAR α through the RAR α moiety to form heterotetramer [21]. RXR α is required for the sumoylation of PML-RAR α and PML RAR α efficient binding to DNA, both of which are necessary for PML-RAR α -mediated transformation [22–25]. Several RXR α ligands such as SR11237 and BMS749 trigger the differentiation and apoptosis of APL cells, but they have to work together with cAMP for efficient action [26, 27], leading to the additional side effects from cAMP. Thus, whether some RXR α ligands alone could show striking anti-APL effect is worthy of investigation.

We recently reported that Z-10, a nitro-ligand of RXR α , binds to RXR α with a distinct binding mode and induces a unique conformational change of RXR α [28]. In the current study, we report that Z-10 induced PML-RAR α degradation and the apoptosis of both ATRA-sensitive and resistant APL cells in a cAMP-independent manner.

RESULTS

Z-10 induces NB4 cell apoptosis

RXR α ligands together with cAMP have shown striking anti-APL activities [26, 27]. Recently, we unraveled that Z-10 is a RXR α nitro-ligand with strong growth inhibition and apoptotic induction of MCF-7 breast cancer cells [28]. We therefore explored the activities of Z-10 on the differentiation and apoptosis induction of NB4 cells, a human APL cell line with PML-RAR α oncogene [29] (Figure 1). Differentiation induction of Z-10 on NB4 cells was examined by flow cytometry following CD11b the surface differentiation marker staining. As previously reported, ATRA at 1 μ M concentration significantly triggered NB4 cell differentiation, as shown by the increased CD11b staining (0.65% CD11b positive cells at DMSO treatment to 30.35% at ATRA treatment) (Figure 1B and Supplementary Figure 1). In comparison, Z-10 at 1 and 2 μ M concentrations only had, if there was, minor effect (Figure 5A and Supplementary Figure 3). Z-10 at 3 μ M and arsenic at 1 μ M had much weaker effect than ATRA at 1 μ M on differentiation induction of NB4 cells (Figure 1B and 5A and Supplementary Figure 1 and 3). The apoptotic effect of Z-10 on NB4 cells was examined through Annexin V-FITC/PI double staining assay. Both Z-10 and arsenic at 1 μ M significantly promoted NB4

cell apoptosis comparing with ATRA (8.07%, 7.38% and 4.76% NB4 cells underwent apoptosis in the presence of Z-10, arsenic, and ATRA, respectively) (Figure 1C). In addition, the apoptotic induction of Z-10 was in a dose-dependent manner (Figure 1C). These results suggested that Z-10 was of potent capability on inducing NB4 cell apoptosis.

Z-10 destabilizes PML-RAR α

Through the induction of PML-RAR α degradation, arsenic promotes the apoptosis of APL cells [17]. Thus, we explored whether Z-10 affected the stability of PML-RAR α . Figure 2A and 2B showed that Z-10 time- and dose- dependently induced PML-RAR α reduction in the absence of cAMP. In the presence of cycloheximide (CHX), Z-10 still induced PML-RAR α reduction in a time-dependent manner, indicating that Z-10 destabilized PML-RAR α protein (Figure 2C). The small fragments of PML-RAR α induced by Z-10 suggested that Z-10 destabilized PML-RAR α through cleavage (Figure 2A-2C). Z-10-induced PML-RAR α reduction and cleavage were strongly inhibited by z-VAD-FMK but only slightly prevented by MG132, implying that caspases but not proteasome was mainly responsible for the degradation and cleavage of PML-RAR α (Figure 2D). Z-11 (Figure 1A), an isomer of Z-10, also binds to RXR α but induces a distinct RXR α conformation [28]. Different from Z-10, Z-11 failed to trigger PML-RAR α reduction (Figure 2E), indicating the selectivity of RXR α nitro-ligands. Additionally, the strong ability of Z-10 on inducing PML-RAR α reduction was unique among RXR α ligands examined (Figure 2F). Therefore, results from above study showed that Z-10 down-regulated PML-RAR α mainly through caspase-mediated cleavage in a cAMP independent manner, which might trigger the apoptosis of NB4 cells. This may also explain why Z-10 induced dramatic apoptosis in NB4 cells but not in THP1 cells that do not possess PML-RAR α oncoprotein (Supplementary Figure 2).

Z-10 inhibits the interaction of PML-RAR α and RXR α

We evaluated whether Z-10 activated caspases to cleave PML-RAR α . Caspase activator PAC-1 could induce PML-RAR α degradation only when caspase3 was highly activated, showing from the simultaneous events of PML-RAR α decreased and cleaved caspase3 increased expressions. However, PML-RAR α degradation induced by Z-10 was an earlier event before the weak activation of caspase3 (Figure 3A). This suggested that Z-10-induced PML-RAR α degradation may not, at least not completely, depend on caspase activation. The binding of RXR α to PML-RAR α has been implicated in the development of APL [2]. We then examined the possibility of RXR α regulating PML-RAR α stability.

When NB4 cells were transfected with siRNA to reduce RXR α expression, the expression levels of both PML-RAR α and RAR α were accordingly reduced (Figure 3B), suggesting that RXR α binding stabilizes PML-RAR α and RAR α . We hypothesized that Z-10 could bind to RXR α and inhibit the interaction of RXR α and PML-RAR α ,

thereby leading to the instability of PML-RAR α . Our co immunoprecipitation assay showed that PML-RAR α and RXR α overexpressed in Cos-7 cells formed strong complex, which was largely disrupted by Z-10 (Figure 3C-3D and 4C). It has been reported that Z-10 fails to bind to RXR α /C432S mutant with cysteine 432 substituted

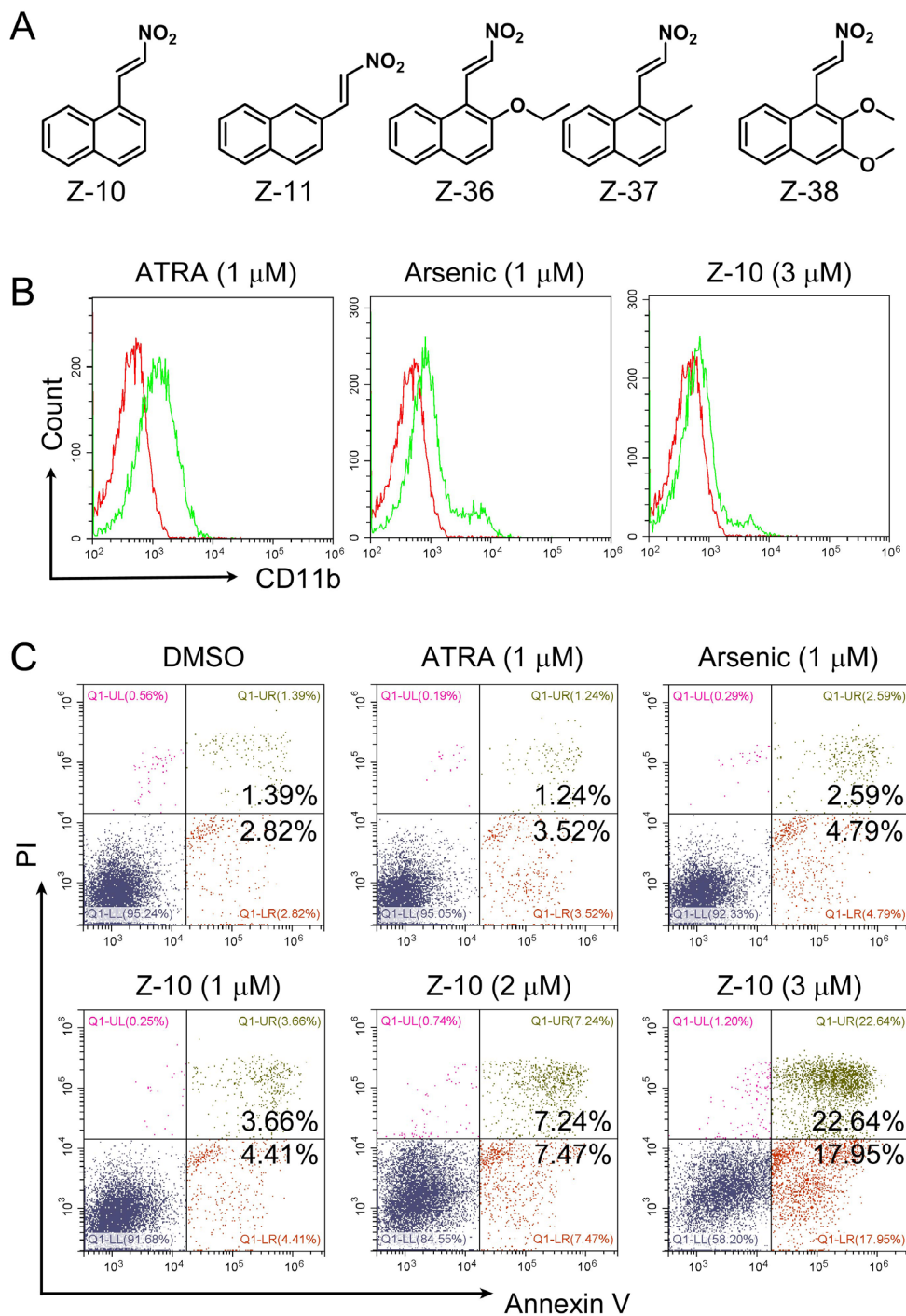


Figure 1: NB4 cell apoptosis induction by Z-10. **A.** The structures of Z serial compounds. **B.** NB4 cells were incubated with ATRA (1 μ M), arsenic (1 μ M) or Z-10 (3 μ M) for two days, and CD11b-positive cells were counted by flow cytometry. Cells treated with DMSO were plotted in red, and cells treated with compounds were plotted in green. **C.** Detection of apoptotic cells by Annexin V-FITC and Propidium iodide (PI) double staining in NB4 cells after incubated with ATRA, arsenic or Z-10 at the indicated concentrations for 36 hours.

with serine [28]. We found that Z-10 also failed to inhibit the interaction of PML-RAR α and RXR α /C432S (Figure 3D), which indicated that Z-10 inhibiting RXR α and PML/RAR α interaction relied on its binding to RXR α . In Cos-7 cells, overexpressed RXR α prevented Z-10 induced PML-RAR α degradation to a certain extent (Figure 3E). Therefore, RXR α binding could stabilize PML-RAR α , and the disruption of the RXR α /PML RAR α complex by Z-10 resulted in the instability of PML-RAR α . Our data also suggested that the basal activity of caspases was able to induce PML-RAR α cleavage once the PML-RAR α /RXR α complex was disrupted by Z-10.

Z-10 has no effect on RAR α stability

Because ATRA could bind to both PML-RAR α and RAR α , one unavoidable side effect of ATRA

originates from its strong induction of RAR α activation and degradation when patients are treated with ATRA at pharmacologic concentrations [30, 31]. Consistently, we found that ATRA at 1 μ M potently induced RAR α degradation in NB4 cells (Figure 4A). However, Z-10 only induced PML-RAR α degradation and had no apparent effect on the expression level of RAR α in NB4 cells (Figure 4A). Similarly, Z-10 dramatically promoted the degradation of overexpressed PML-RAR α but not RAR α in Cos-7 cells (Figure 4B). As expected, Z-10 failed to inhibit the interaction of RAR α and RXR α as did to the interaction of PML RAR α and RXR α (Figure 4C). Thus, Z-10 induction of PML-RAR α but not RAR α degradation was due to its selective inhibition of RXR α interaction with PML-RAR α but not with RAR α .

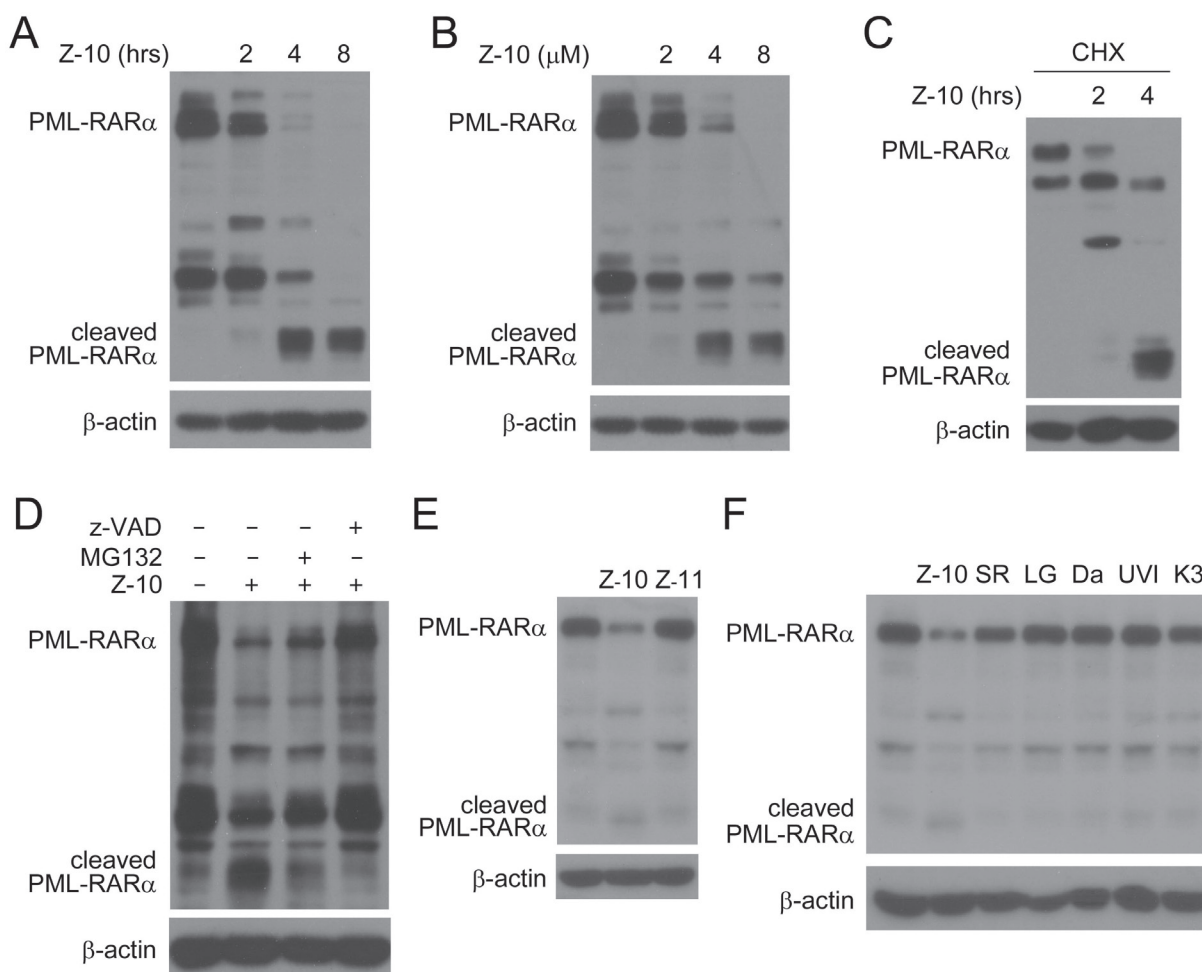


Figure 2: PML-RAR α reduction induced by Z-10 through cleavage. A-B. NB4 cells were treated with 5 μ M of Z-10 for the indicated time (A) or treated for 4 hours with the indicated concentrations (B), and PML-RAR α expression was analyzed by western blot using anti-RAR α antibody. The expression of β -actin was used as a loading control. C. NB4 cells were treated with 50 μ g/mL cycloheximide (CHX) for 4 hours and 5 μ M Z-10 for 2 or 4 hours. Protein expression was analyzed by western blot. D. NB4 cells were treated with 5 μ M Z-10 together with 20 μ M MG132 or 20 μ M z-VAD-FMK (z-VAD) for 4 hours, and protein expression was examined by western blot. E-F. NB4 cells were treated with 5 μ M compounds as indicated for 4 hours, and PML-RAR α protein was detected by western blot. SR, LG, Da, UVI and K3 represent SR11237, LG100745, Danthron, UVI3003 and K-8003, respectively.

Z-10 induces PML-RAR α degradation and apoptosis in ATRA-resistant NB4 cells

ATRA is a widely used and effective clinical drug for APL [32, 33]. However, a fraction of APL is refractory to ATRA due to long-term ATRA treatment and PML-RAR α mutations [13, 14]. As previously reported, ATRA could strongly induce the differentiation of NB4 cells but not NB4-LR1 and NB4-LR2 cells, two ATRA-resistant cell lines [14, 34] (Figure 5A and Supplementary Figure 3). Consistently, ATRA promoted PML-RAR α degradation

only in NB4 cells but not in NB4-LR1 or NB4-LR2 cells (Figure 5B and 5D). However, Z-10 was able to induce PML-RAR α degradation in all the three cell lines (Figure 5B-5E). Also, Z-10 dose- and time-dependently induced PML-RAR α reduction and cleavage in both NB4-LR1 and NB4-LR2 cell lines mainly through caspase-mediated pathway (Figure 5C-5F). Similarly, Z-10 did not affect the expression of RAR α in NB4-LR1 cells (Figure 5D). Although Z-10 did not show apparent differentiation induction of NB4-LR1 and NB4-LR2 cells (Figure 5A and Supplementary Figure 3), it significantly promoted

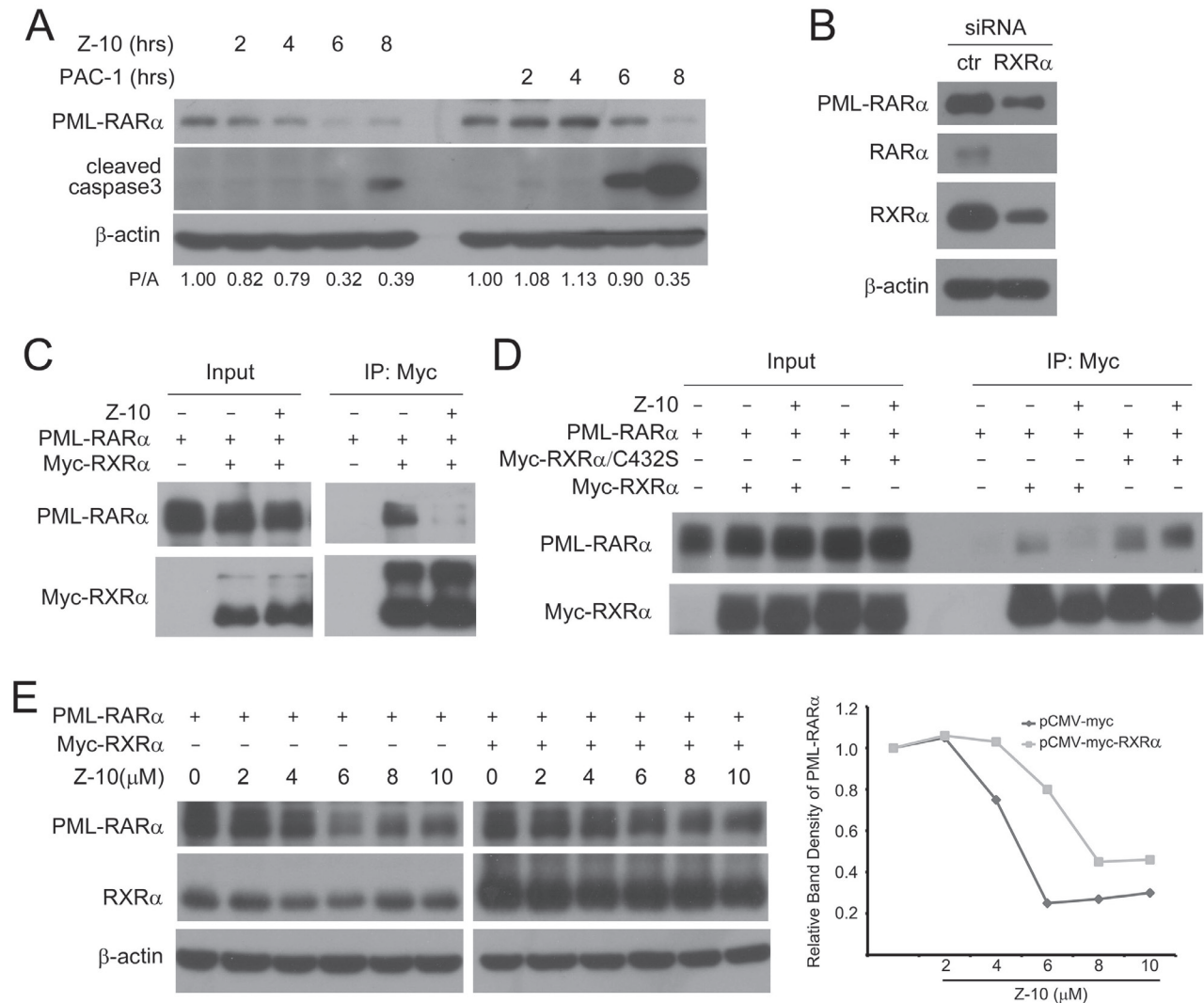


Figure 3: The inhibition of PML-RAR α /RXR α complex formation by Z-10. **A.** NB4 cells were treated with 5 μ M Z-10 or 50 μ M PAC-1 for the indicated time, and the expression of PML-RAR α and cleaved caspase3 was analyzed by western blot. Relative protein band densities after normalization to β -actin signal were shown at the bottom of the figure (P/A represents PML-RAR α / β -actin). **B.** NB4 cells were infected with sh RXR α or sh control retrovirus for three days. Protein expression levels were analyzed by western blot. **C-D.** Cos-7 cells were transfected with the indicated plasmids for 24 hours and then treated with 5 μ M of Z-10 for 4 hours. The complex formations were examined by co-immunoprecipitation and western blot using the indicated antibodies. **E.** Cos-7 cells were transfected with PML-RAR α expression plasmid alone or together with Myc-RXR α plasmid for 24 hours, and treated with Z-10 at the indicated concentrations for 4 hours. Protein expression levels were analyzed by western blot. Relative PML-RAR α band densities after normalization to β -actin were plotted.

the apoptosis of these two cell lines in a dose-dependent manner (Figure 5G). Thus, Z-10 could induce PML-RAR α degradation and apoptosis in ATRA-resistant NB4 cells.

Z-36 and Z-38 are optimized Z-10 derivatives

To improve the potency of Z-10, we synthesized a series of Z-10 derivatives and examined their effects in NB4 cells. Among the synthesized Z-10 derivatives examined, we found that Z-37 had similar ability as Z-10 while Z-36 and Z-38 possessed stronger ability than Z-10 on inducing PML-RAR α degradation (Figure 1A and Figure 6B). In consistent with their ability on degrading PML-RAR α , Z-36 and Z-38 showed stronger potency on inhibiting NB4 cell viability (The IC₅₀ of Z-10, Z-36 and Z-38 were 4.284, 0.7476 and 0.713 μ M, respectively). Z-36 and Z-38 also displayed stronger apoptosis induction of NB4 and NB4-LR1 cells than Z-10 (Figure 6D). Z-10 and its derivatives exhibited similar stimulating effect of RXR α transactivation (Figure 6A), suggesting that their anti-APL ability

was not directly correlated to their activation of RXR α transactivation.

DISCUSSION

Although ATRA and arsenic have improved the overall survival rate of APL patients [35, 36], there exist major issues in the APL treatment such as ATRA and arsenic resistance as well as their off target side effects [18, 19, 37]. In our current study, we provided a potential drug lead that may overcome ATRA-resistance and ATRA-related side effects through its distinct acting mode.

Z-10, as the first identified nitro-ligand of RXR α , has distinct properties from classic RXR α ligands in both molecular structures and RXR α -dependent activities [28]. cAMP-dependent and PKA-catalyzed phosphorylation of PML-RAR α is crucial for some RXR α ligands to efficiently induce PML-RAR α degradation and APL cell apoptosis [38, 39]. Here, we showed that Z-10 is unique among RXR α ligands in that it has strong anti-APL

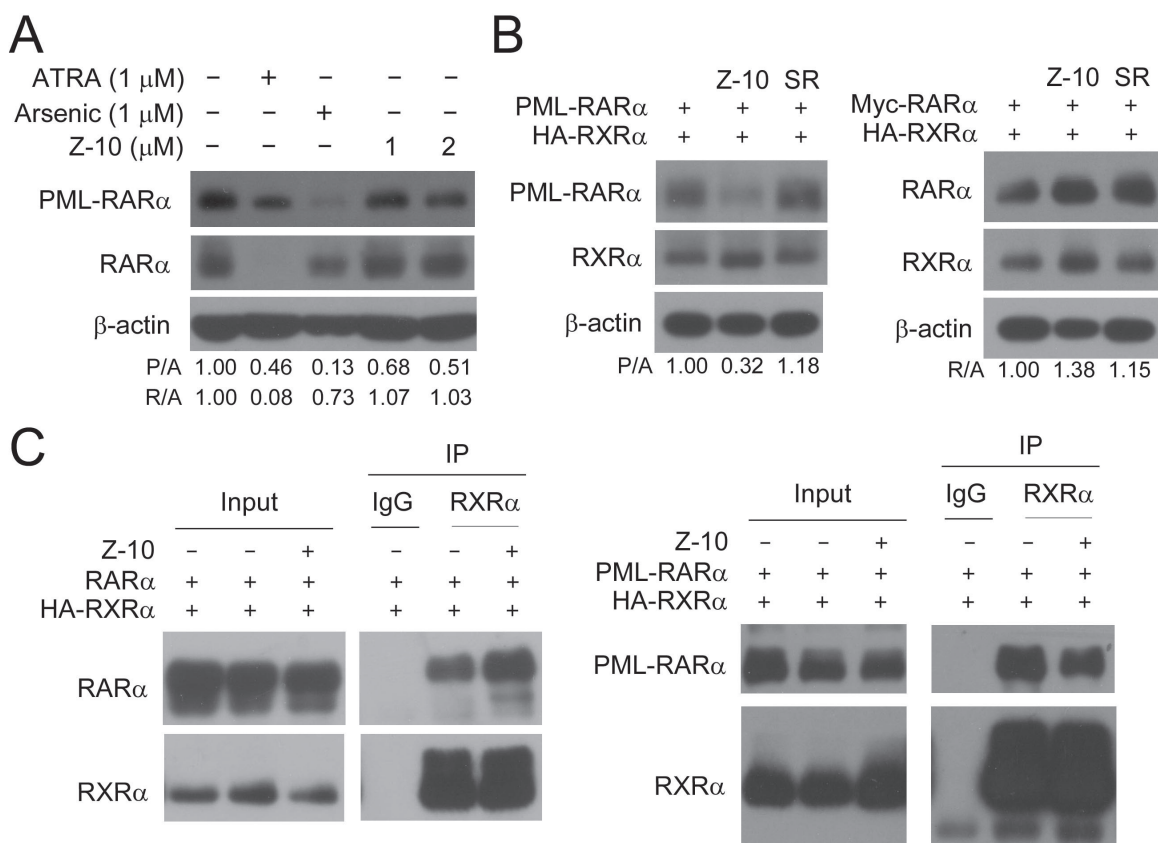


Figure 4: Z-10 had no effect on the stability of RAR α . **A.** NB4 cells were incubated with Z-10, arsenic or ATRA for 48 hours. The PML-RAR α and RAR α protein was detected by anti-RAR α antibody with β -actin as a loading control. Relative protein band densities after normalization to β -actin signal were shown at the bottom of the figure (P/A represents PML-RAR α / β -actin, and R/A represents RAR α / β -actin). **B.** Cos-7 cells were transfected with the indicated plasmids for 24 hours, and then treated with 5 μ M of Z-10 or SR11237 (SR) for 6 hours. Protein expression levels were analyzed by western blot. **C.** Cos-7 cells were transfected with the indicated plasmids for 24 hours and then treated with 5 μ M of Z-10 for 4 hours. The complex formations were examined by co-immunoprecipitation using the indicated antibodies.

activity in the absence of cAMP (Figure 1 and Figure 2). This should, at least in part, be attributed to its induction of a distinct RXR α conformation, resulting in the dissociation of PML-RAR α from RXR α (Figure 3C-3D and Figure 4C). The cAMP-independent PML-RAR α degradation by Z-10 also suggested that Z-10 may represent a promising

drug lead in a distinct class of RXR α ligands for APL treatment.

RXR α forms strong heterotetramers with PML-RAR α , and disruption of their interaction inhibits APL initiation and development [24, 25]. RXR α has been shown to be required for PML-RAR α sumoylation and efficiently binding to DNA, both of which are essential

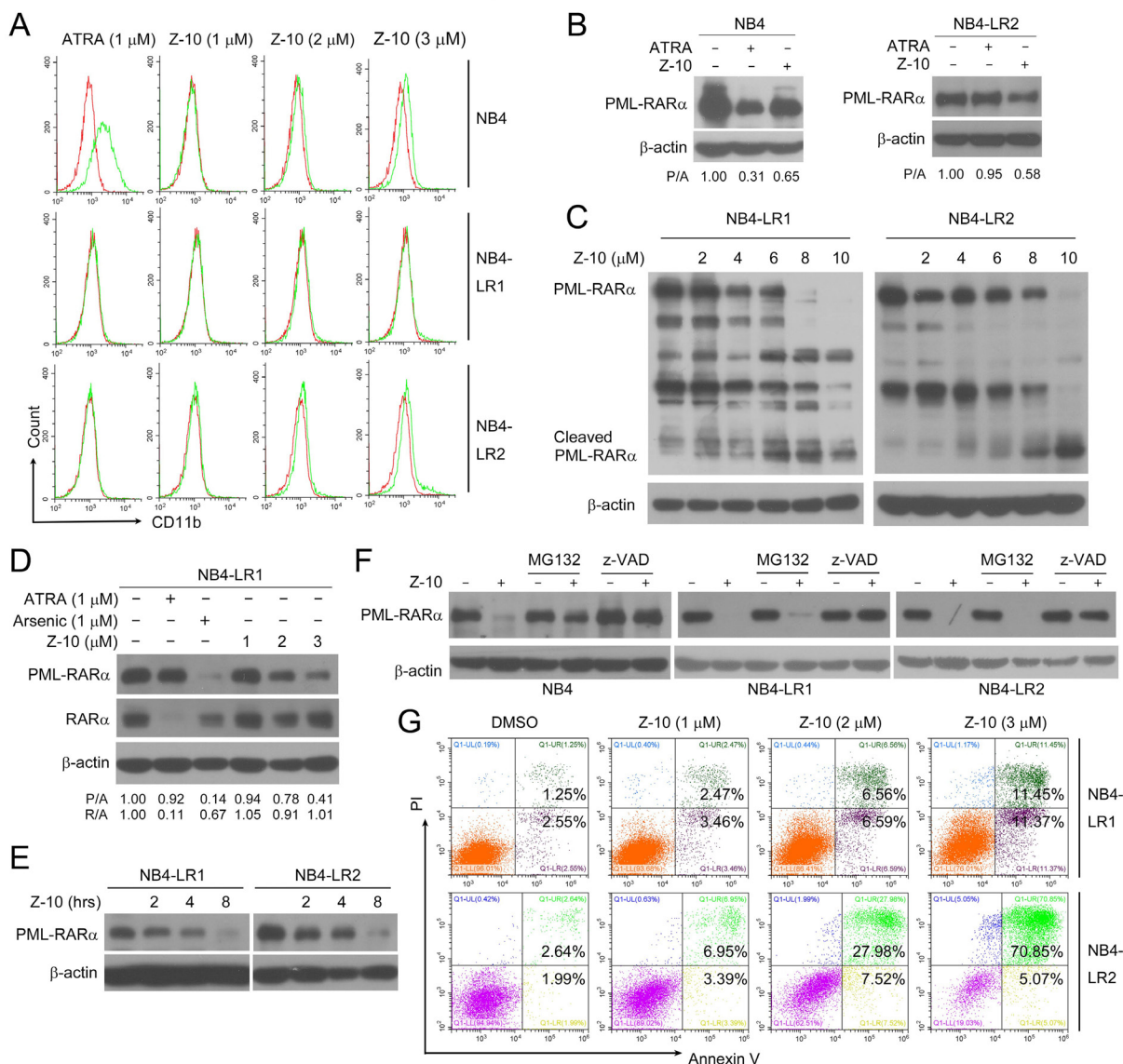


Figure 5: The induction of PML-RAR α degradation and apoptosis by Z-10 in ATRA-resistant NB4 cells. **A.** NB4, NB4-LR1 or NB4-LR2 cells were incubated with Z-10 or ATRA for two days and CD11b-positive cells were counted by flow cytometry. Cells treated with DMSO were plotted in red, and cells treated with compounds were plotted in green. **B.** NB4 and NB4-LR2 cells were incubated with 1 μ M of Z-10 or ATRA for 48 hours. The PML-RAR α protein was detected by anti-RAR α antibody with β -actin as loading control. **C.** NB4-LR1 and NB4-LR2 cells were treated with Z-10 at the indicated concentration for 4 hours followed by western blot to examine protein expression. **D.** NB4-LR1 cells were incubated with Z-10, ATRA or arsenic at the indicated concentration for 48 hours. The PML RAR α and RAR α protein was detected by western blot using anti-RAR α antibody. Relative protein band densities after normalization to β -actin signal were shown at the bottom of the figure (P/A represents PML-RAR α / β -actin, and R/A represents RAR α / β -actin). **E.** Western blot analysis of PML-RAR α expression in NB4-LR1 and NB4-LR2 cells treated with 5 μ M of Z-10 for the indicated hours. **F.** NB4, NB4-LR1 and NB4-LR2 cells were treated with 5 μ M Z-10 together with 20 μ M MG132 or 20 μ M z-VAD-FMK (z-VAD) for 4 hours, and protein expression was examined by western blot. **G.** NB4-LR1 and NB4-LR2 cells were incubated with Z-10 at the indicated concentrations for 36 hours, and the detection of apoptotic cells was carried out by Annexin V-FITC/PI double staining.

events for APL transformation [22, 23]. In our research, we unraveled that RXR α was also vital for the stability of PML-RAR α (Figure 3), which provided a new mechanism underlying RXR α actions in APL. It is conceivable that this contribution of RXR α also originates from its interaction with PML-RAR α . Therefore, a feasible approach to eliminate PML-RAR α is to abrogate its interaction with RXR α . Indeed, Z-10 could potentially inhibit the interaction of PML-RAR α and RXR α (Figure

3C-3D and Figure 4C), leading to the caspase-mediated cleavage of PML-RAR α (Figure 2D). We hypothesized that the binding of RXR α may preclude the cleavage of PML-RAR α through masking the cleavage sites or inhibiting the access of caspases. Figure 3A indicated that PML-RAR α reduction induced by Z-10 preceded caspase activation, implying that basal activity of caspases was enough for catalyzing PML-RAR α cleavage once the PML-RAR α /RXR α complex was disrupted.

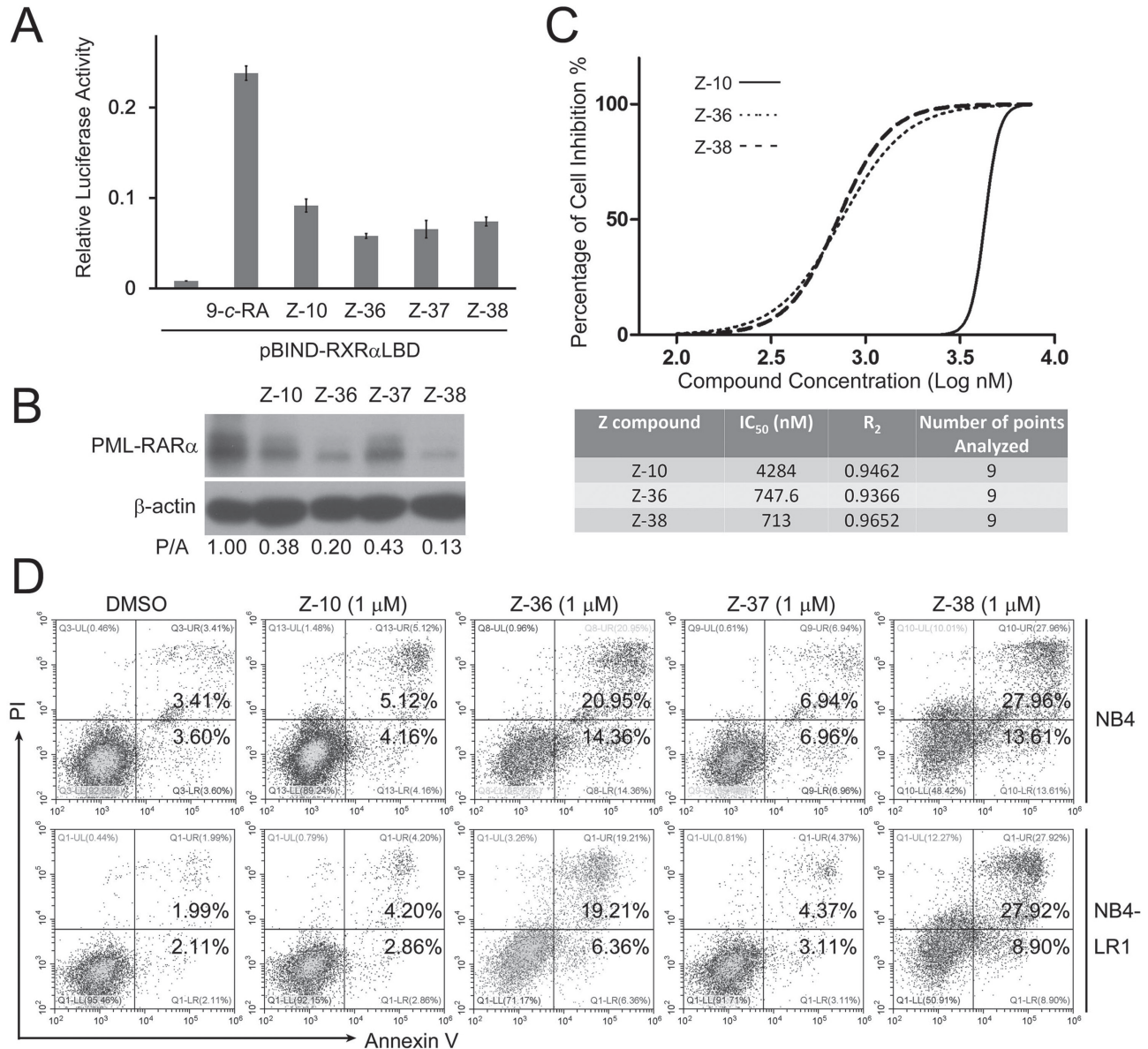


Figure 6: Z-36 and Z-38 were optimized Z-10 derivatives. **A.** HEK293T cells were transfected with pBIND-RXR α -LBD and pG5-luc for 24 hours, and treated with 0.1 μ M 9-*cis*-RA (9-*c*-RA) and the indicated compounds (5 μ M). Luciferase activities were measured 12 hours after treatment and relative luciferase activity was plotted. **B.** NB4 cells were treated with 5 μ M of the indicated compounds for 4 hours. The PML-RAR α protein was detected by anti-RAR α antibody with β -actin as loading control. **C.** NB4 cells were plated at a density of 1×10^5 cells/ml in a 96-well plate. Cells were treated with Z-10 at increased concentrations (0.1, 0.5, 1, 2, 3, 4, 5, 6, and 7.5 μ M) for 36 hours, and cell proliferation was measured by MTS assay. Data were analyzed and plotted using GraphPad Prism software. **D.** NB4 and NB4-LR1 cells were incubated with 1 μ M of compounds as indicated for 36 hours, and the detection of apoptotic cells was carried out by Annexin V-FITC/PI double staining.

Different from the heterodimer formed by RAR α and RXR α , PML-RAR α and RXR α forms heterotetramer. In the heterotetramer, PML-RAR α not only interacts with RXR α through RAR α moiety, but also interacts with each other through PML moiety [23, 25]. These may underlie the different modes of RXR α interaction with PML-RAR α and RAR α . It is conceivable that the conformational change induced by Z-10 may only affect RXR α interaction with PML-RAR α but not with RAR α , a possibility that was confirmed by our co immunoprecipitation assay (Figure 4C). Consistently, even though RXR α was vital for the stability of both PML-RAR α and RAR α , Z-10 binding to RXR α selectively reduced the stability of PML-RAR α (Figure 4A-4B and Figure 5D). This property might allow Z-10 to circumvent some of ATRA's side effects. RXR α could form heterodimers with many other nuclear receptors [40]. If Z-10 did not affect RXR α binding to RAR α , it likely has no or only minor effect on RXR α heterodimerization with other nuclear receptors.

One limitation in APL treatment is ATRA resistance [14, 41]. Due to the mutations of the RAR α moiety that directly affect the binding of ATRA, it should be difficult to solve this issue through modifying ATRA or using other RAR α ligands. Since Z-10 inhibited PML RAR α through binding to RXR α , it may overcome this problem. Indeed, our results showed that Z-10 induced PML-RAR α degradation and apoptosis in ATRA-resistant NB4 cells (Figure 5). This implied that RXR α was also important to maintain the stability of the mutated PML RAR α , and Z-10 was able to inhibit the interactions of RXR α with the mutated PML-RAR α . It remains to be investigated whether Z-10 could also overcome the arsenic-resistance.

Z-36 and Z-38 are two derivatives of Z-10 only after simple modifications (Figure 1A). However, these two derivatives had substantially improved potency (Figure 6B-6D), which suggested that Z-10 can be further optimized in term of APL treatment. Animal studies of Z-10 and its derivatives will be needed to confirm their efficacy and study their potential side effects. It will also be interesting to study the synergistic effect of Z-10 and ATRA in animal experiments.

MATERIALS AND METHODS

Reagents and antibodies

Antibodies for RAR α (sc-551), RXR α (sc-553), PARP-1/2 (sc-7150), c-Myc (sc-40) were purchased from Santa Cruz Biotechnology; Antibody for β -actin (A2228), ATRA (R2625), SR11237 (S8951), LG 100754 (SML0771), Danthron (D108103), Arsenic, and 9-*cis*-RA (R4643) were purchased from Sigma-Aldrich; Antibody for human FITC-CD11b was from eBioscience; Antibody for Cleaved caspase-3 (#9661) was from Cell Signaling Technology; PAC-1(Procaspase-activating compound-1) was from MedChem Express; UVI3003 (3303) was from

Tocris Bioscience; K-8003 and Z compounds were stored in our laboratory.

Compound synthesis

General procedure for the synthesis of Nitroolefins (Z-10, Z-11, Z-36, Z-37 and Z-38) was via the Henry reaction as previously described [28].

Cell culture

APL cell lines NB4 and NB4-derived ATRA-resistant NB4-LR1 and NB4-LR2 were cultured in RPMI 1640 medium (Thermo Fisher Scientific). African green monkey kidney fibroblast-like cell COS-7 and human embryonic kidney cell 293T were cultured in DMEM medium (Thermo Fisher Scientific). The mediums were supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 IU/ mL), and streptomycin (100 μ g/mL), and the cells were maintained at a humidified incubator at 37 °C and 5% CO₂. For experiments, cells were incubated with compounds with 0.01-0.1% DMSO treatment as control.

Plasmids and transfection

PSG5-PML-RAR α plasmid was a gift from Dr. Jun Zhu (Shanghai Jiao-Tong University School of Medicine). Myc-RXR α /C432S was constructed with standard methods. HA-RXR α , Myc-RAR α , Myc-RXR α , pBind-RXR α -LBD, pGL6-TA-RXRE, pG5 were preserved in our laboratory. COS-7 cell transfections were carried out by using TurboFect Transfection Reagent (R0531, Fermentas) according to the instructions of the manufacturer. NB4 cell transfections were carried out by using retrovirus infection, and RXR α shRNA sequence is CAAGGACTGCCTGATTGAC.

Western blot

Cells were lysed, and equal amounts of the lysates were loaded onto 8% sodium dodecylsulfate-polyacrylamide gel, electrophoresed, and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% skimmed milk in TBST (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.1% Tween 20) for 1 hour and then incubated with primary antibodies overnight at 4°C, followed by secondary antibodies for 2 hours at room temperature. The detection was performed by the ECL system (Thermo). β -actin was used as an internal control. Protein band density was quantified using software Quantity One-4.6.2.

Co-immunoprecipitation assay

Cells were harvested and lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl,

1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate and 1 mM Na_3VO_4 , with proteinase inhibitor cocktail. Immunoprecipitation was performed as previously described [28, 42, 43].

Apoptosis and differentiation assay

Three-milliliter cultures of NB4, NB4-LR1 and NB4-LR2 cells at a density of 3×10^5 cells/mL were incubated with compounds and equal amount of DMSO for 36 hours. The apoptosis of the cells was detected using FITC Annexin V Apoptosis Detection Kit I (556547, BD Biosciences) according to the instructions of the manufacturer. Cell surface differentiation antigen CD11b was measured using fluorescein isothiocyanate-labeled antibodies with isotype controls via flow cytometry.

MTS assay

NB4 cells were seeded in 96-well culture plate at a cell density of 1×10^5 cells/mL and treated with compounds for 36 hours. The cell viability was evaluated using an MTS assay kit (Promega, G3580). Briefly, MTS (10 mg/ml, 3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2 (4-sulfophenyl)-2H-tetrazolium) was added to each well followed by incubation for 2 hours at 37 °C. The absorbance was measured at a wavelength of 490 nm using an Elisa-Reader.

Mammalian one-hybrid assay

293T cells were cotransfected with pG5-luc reporter (Promega) together with the RXR α -LBD fused with the DNA-binding domain of Gal4. After 24 hours, cells were treated with DMSO, Z compounds, or 9-*cis*-RA. After 12 hours, cells were lysed by passive lysis buffer. Firefly and Renilla luciferase activities were quantitated using the Dual-Luciferase Reporter Assay System (Promega, E1960). Transfection and expression efficiency was normalized to Renilla luciferase activity.

Statistical analysis

Results were derived at least three independent experiments and shown as the mean \pm standard deviation. The student's *t*-test was applied for the statistic analysis, and *p* < 0.05 was considered significant.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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