Targeting of leukemia-initiating cells in acute promyelocytic leukemia

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Abstract: Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia (AML) with peculiar molecular, phenotypic and clinical features and unique therapeutic response to specific treatments. The disease is characterized by a single, pathognomonic molecular event, consisting of the translocation t(15;17) which gives rise to the PML-retinoic acid receptor α (RARα) hybrid protein. The development of this leukemia is mainly related to the fusion oncoprotein PML/RARα, acting as an altered RAR mediating abnormal signalling and repression of myeloid differentiation, with consequent accumulation of undifferentiated promyelocytes. The prognosis of APL has dramatically been improved with the introduction in therapy of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO). The main effect of these two drugs is linked to the targeting of either RAR moiety of the PML/RARα molecule and induction of cell differentiation (ATRA) or of the PML moiety of the fusion protein and induction of leukemic cell apoptosis, including leukemic progenitors (mostly induced by ATO). These two drugs exhibited excellent synergism and determine a very high rate of durable remissions in low/intermediate-risk APLs, when administered in the absence of any chemotherapeutic drug. The strong synergism and the marked clinical efficacy of these two agents when administered together seem to be related to their capacity to induce PML/RARα degradation and complete eradication of leukemia stem cells.

Keywords: Acute myeloid leukemia (AML); acute promyelocytic leukemia (APL); leukemia-initiating cells; differentiation; apoptosis

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Introduction

Chimeric fusion proteins generated by chromosomal translocations play an important role in the pathogenesis of many hematological malignancies by regulating cell survival, proliferation, differentiation and apoptosis. Some of these fusion events involve the RARA gene with various other genes, determining the formation of several fusion genes, such as PML/RARα, PLZF/RARα, NPM/RARα, NUP98/RARα, NUMA/RARα, STAT5B/RARα, FIP1L1/RARα, BCOR/RARα, PRKAR1A/RARα and TBLR1/RARα. This review is focused on the pathogenetic role of the most frequently observed RARA fusion protein, i.e., PML/RARα, while chimeric RARA fusions involving partners other than PML are not discussed here. For a description of the pathogenetic role and response to therapy of the various non-PML/RARα RARA fusion variants the reader is addressed to some recent reviews on this topic (1).

Acute promyelocytic leukemia (APL) is a distinct subset of acute myeloid leukemia (AML) associated with consistent clinical, morphologic and immunophenotypic features and a unique genetic abnormality requiring specific treatment approach. This leukemia was discovered in 1957 and since then dramatic progress has been made in understanding the biology of APL and in its treatment.

At the molecular level, this leukemia is characterized by an unique and specific chromosome translocation t(15;17) which involves the retinoic receptor-alpha gene (RARA) on chromosome 17 and the promyelocytic leukemia gene (PML) on chromosome 15, resulting in the generation of
the chimeric oncogene PML/RARA (2).

RARα is a nuclear hormone receptor that heterodimerizes with retinoid X receptor (RXR), resulting in the recruitment of transcription corepressor complexes (CoCs) and, consequently, in gene transcription repression. The binding of retinoic acid to RARα determines a conformational change in the RAR/RXR complexes, resulting in increased DNA binding, release of corepressors and recruitment of coactivator complexes, and, finally, transcriptional activation of target genes. In APL, PML/RARα fusion protein actively recruits CoCs, thus inducing a sustained transcriptional inhibition and resulting at functional level in a block of myeloid differentiation and increased self-renewal of leukemic progenitors (3,4). As a consequence of these biologic properties PML/RARα acts as a potent transcriptional repressor of retinoic acid α (RARα) binds to the RARA domain of the fusion protein PML/RARα and promotes the dissociation of CoCs from this molecule and the subsequent recruitment of transcription coactivators. The final result of these molecular changes induced by ATRA consists in an epigenetic reprogramming, with transcriptional derepression and activation of the myeloid differentiation program. Consequently, treatment of APL patients with ATRA induces terminal granulocytic differentiation of APL blasts and, at clinical level, transient remissions.

Arsenic trioxide (ATO) binds to the PML moiety of the PML/RARα fusion molecule and promotes its degradation, inducing apoptosis, in vivo partial differentiation of these cells and inhibition of leukemic progenitor self-replication (6). As a consequence of these remarkable effects, arsenic compounds have been introduced in clinical use and were shown to cure a significant proportion of APL patients.

**ATRA and LICs in APLs**

A large number of studies support the view that ATRA therapy is able to induce the differentiation of APL blasts to induce the differentiation of APL blasts into functional granulocytes (7,8). However, when administered alone, ATRA is usually unable to induce a long-term cure of the disease. Various lines of evidence support this view. First treatment of APL patients with ATRA induces clinical complete remissions, but does not induce complete molecular remissions, because the fusion transcripts PML/RARα remain detectable (9). Second, Zheng and coworkers, using various models of APL leukemic stem cells (purified populations of murine HSCs retrovirally transduced with PML/RARα, leukemic stem cells purified from mice with PML/RARα leukemia and the side population of the NB4 APL cell line) demonstrated that ATRA was unable to abolish self-renewal or engraftment potential of leukemia-initiating cells; paradoxically, ATRA enhanced the proliferation of PML/RARα+ LSCs (10). Third, Nasr and coworkers using an in vivo model of murine APL (based on the transplantation of murine APL cells derived from PML/RARα transgenic mice to syngeneic normal recipients) have shown that low doses of ATRA are sufficient to induce terminal differentiation of APL cells, but are unable to induce the killing of leukemia initiating cells (LICs) and, thus, to cure the disease; interestingly, higher ATRA doses are able to partially inhibit LICs and to decrease their frequency, but are unable to completely eradicate these cells and to cure the animals; cAMP synergizes with low-dose ATRA for LIC eradication (11).

The implications at the clinical level of these observations involve the need to associate ATRA administration with either anti-leukemic chemotherapy or ATO to obtain a complete eradication of LICs in APL and to cure the disease. Interestingly, Werner and co-workers have analyzed the effects of chemotherapy and ATRA administration on the leukemic stem cell compartment and have developed a mathematical model, based on the data observed in the context of a clinical trial (12). According to this analysis it was concluded that chemotherapy alone induces a significant clinical response, but was unable to induce a curative effect, being unable to completely eradicate leukemic stem cells; ATRA administration together with chemotherapy resulted in an improvement in the number of the long-term surviving patients, but at least 1 year administration of ATRA in the maintenance period was required to reduce the number of relapses (12). This long-term need for ATRA administration was related to the capacity of this drug to slowly decrease the proliferation of the leukemic stem cell compartment and to progressively eliminate these cells over the course of approximately 1 year of treatment (12).
A recent study by Ablain and coworkers contributed to understand why ATRA is a potent inducer of APL differentiation, but only a moderately active cleaner of APL LICs. In fact, it was well established that at molecular level ATRA is able to trigger transcriptional activation of PML/RARα and initiates also to promote its proteasome-mediated degradation. Ablain and coworkers, using synthetic retinoids that potently activated PML/RARα-dependent transcription but failed to promote PML/RARα degradation, reached the important conclusion that PML/RARα activation was required to induce APL differentiation, while PML/RARα degradation was required to impair leukemia-initiating activity of APL (13). According to these observations it was concluded that induction of APL cell differentiation is insufficient for APL eradication, while PML/RARα loss is strictly required to eliminate APL LICs (13). In keeping with these findings, clinical studies had showed that APL patients in long-term remission and presumably cured have no PCR-detectable PML/RARA transcripts in their marrow cells (14). In a recent study Ablain and coworkers have used a mouse model of APL to explore in detail the mechanism of anti-leukemic effect of ATRA. In this model three ATRA doses, low, intermediate and high, were used: all these three doses induced cell differentiation, but only high-dose elicited a curative effect (15). The same experiments were carried out in a mouse model of PLZF/RARα leukemia: in this model all three ATRA doses induced cell differentiation, but none of them was able to induce a significant anti-leukemic effect in term of mice survival (15). These observations again support an uncoupling of cell differentiation and inhibition of leukemia-initiating activity. The curative effect observed with high-dose ATRA treatment was associated with an inhibitory effect on leukemic cell cycling (15). Analysis of the genes selectively induced by high-dose ATRA in PML/RARα mice indicated that a significant part of them pertain to the family of p53-regulated genes, thus suggesting that high-dose ATRA treatment activates p53 signaling, determining cell cycle arrest and cellular senescence (15). In line with this interpretation, high-dose ATRA was found to activate p53, through protein stabilization; importantly, p53 deficiency resulted in a decrease of the inhibitory effect of high-dose ATRA on APL self-renewal (15). Using this experimental system it was then possible to determine the contribution of PML to the ATRA-induced loss of self-renewal: PML silencing resulted in a blockade of the ATRA-mediated inhibition of APL self-renewal (15). These observations led to the important conclusion that the inhibitory effect of ATRA on APL-initiating activity requires PML, nuclear body reformation and p53 activation (15).

**Arsenic and LICs in APL**

At variance with ATRA, Arsenic does not directly act on RARA-dependent pathways, but seems to alter PML nuclear bodies (NBs) biogenesis. PML is localized at the level of peculiar intranuclear domains, called NBs: this protein acts as key organizer of these nuclear domains, where a consistent number of regulatory proteins are recruited and accumulated. The PML/RARα fusion protein disrupts these domains (16,17). Two mechanisms have been proposed to explain how As₂O₃ triggers PML sumoylation. A first mechanism implies that PML/RARα oxidation is a key event required for the sumoylation and degradation of this molecule. Thus, it was shown that As₂O₃ exposure determines an increased production of reactive oxygen species (ROS), due to the capacity of As₂O₃ to bind vicinal cysteines; ROSs in turn determine the formation of disulphide-linked PML or PML/RARα multimers that become sumoylated and then degraded (18). This mechanism is supported by the observation that non-arsenical oxidants are able to mimic As₂O₃ and then to induce PML/RARα multimerization, NB-association, degradation and trigger regression of PML/RARα-driven murine leukemias (18). The involvement of ROS/oxidation in degradation of PML/RARα is also supported by another study, which showed that α-Tocopheryl succinate (α-TOS) inhibits the mitochondrial respiratory chain, leading to accumulation of ROSs and partial degradation of PML/RARα: in consequence of these effects, alpha-TOS was as effective as ATO against APL cells *in vivo* (19). A second mechanism implies an important role of the SUMO E3-ligase inhibitor PIAS1 in the sumoylation of PML and PML/RARα proteins (20). PIAS1-mediated sumoylation of PML and PML/RARα promotes the CK2 interaction and ubiquitin/proteasome-mediated degradation; importantly, PIAS1-mediated sumoylation of PML/RARα is essential for induction of its degradation by As₂O₃ (20). This observation is in line with other studies showing that the ubiquitin E3-ligase RNF4 is essential for As₂O₃-induced PML/RARα degradation for its capacity to recognize poly-sumoylated PML and PML/RARα (21,22).

Growing evidence indicates that these nuclear domains are veritable cell factories whose biogenesis is controlled
by interferons and oxidative stress through protein sumoylation (23). Interferons, as well as arsenic compounds, are potent inducers of PML expression and, hence, of NB formation (23). Stress induces oxidation-mediated PML aggregation at the level of NB. Oxidized PML recruits UBC9, favoring PML sumoylation (interaction with SUMO proteins); sumoylated PML drives the recruitment of numerous biochemical partners, resulting in turn in their sumoylation and inducing other post-translational modifications of these proteins, such as acetylation or poly-ubiquitination, ultimately resulting in partner degradation, inactivation or activation (23). Thus, three main biological functions have been ascribed to PML-NBs: (I) they operate as molecular platforms able to promote the assembling of various proteins; (II) NBs form molecular platforms acting as catalytic surfaces promoting molecular modifications of proteins, such as sumoylation, acetylation, ubiquitination and phosphorylation; (III) NBs act as active sites of transcriptional regulation and heterochromatin formation.

Increasing evidence indicates that PML acts as a tumor suppressor and as an important regulator of hematopoietic stem cells. PML acts as a potent inhibitor of the PI3K/AKT/mTOR pathway and, through this activity, like PTEN, acts as a tumor suppressor. PML exerts its tumor suppressor function by inactivating pAKT inside the nucleus (24). Furthermore, under hypoxic conditions, PML exerts an inhibitory effect on mTOR activity (25). Finally, PML-NBs exert an additional inhibitory effect on PI3K/AKT by promoting the accumulation of mono-ubiquitinated PTEN in the nucleus (26). Given these effects of PML, it is not surprising that the biological effects of PML are important for HSCs, as supported by various lines of evidence: (I) PML expression is high in HSCs and declines when these cells differentiate; (II) the number of HSCs decreases in the time in PML-/- mice due to progressive exhaustion of these cells occurring as a consequence of their reduced quiescence; (III) PML was highly expressed in blasts of chronic myeloid leukemia patients and high expression of this protein correlates with a negative clinical outcome; (IV) inhibition of PML by ATO resulted in an inhibition of LIC maintenance and combined administration of ATO and chemotherapy resulted in curative effects (27).

Recent studies in part clarified the molecular mechanisms responsible for the effect of ATO on PML NBs. These studies have shown that ATO promotes PML/RARα degradation in APL cells through a mechanism involving its direct binding with PML (28). In fact, ATO promotes PML and PML/RARα degradation by their sumoylation through a mechanism dependent on the direct binding of arsenic to cysteine residues located within the RBCC domain of PML/RARα and PML; arsenic binding induces PML oligomerization and increases its capacity to interact with the SUMO-conjugating enzyme UBC9, resulting in increased sumoylation and consequent protein degradation (28,29).

Given this capacity of ATO to efficiently induce PML/RARα degradation, it is not surprising that this compound definitely cures a substantial proportion of APL patients when used as a single agent (30,31).

**Anti-leukemic synergism between ATRA and arsenic**

Several experimental studies have provided evidence about the existence of a pronounced synergy between ATRA and ATO for APL eradication. An initial study carried out on APL leukemic cell lines and on fresh APL blasts showed that treatment *in vitro* of these cells with ATO was unable to induce phenotypic differentiation and reduced the level of differentiation induced by ATRA (32). In spite this differentiating effect antagonism between ATRA and ATO observed *in vitro*, various studies carried out in mouse models showed a consistent synergism between these two agents in their capacity to eradicate the leukemic disease (11,33-35). Biochemical studies on induction of PML/RARα degradation have shown the capacity of ATRA and ATO to target different domains of the PML/RARα fusion molecule and the absence of cross resistance of the two different degradation pathways, thus offering a molecular explanation for the observed synergism between the two drugs (36). These observations have provided the preclinical experimental support for the development of clinical trials evaluating the combination of the two drugs as frontline therapy in patients with newly diagnosed APL. Thus, a first clinical trial of combination therapy was reported in 2004. This study implied APL patient randomization into three induction therapy arms. These included ATRA alone, ATO alone or the ATRA-ATO combination followed in each arm by chemotherapy consolidation. The time to achieve complete remission was accelerated in the combination drug group compared to single-treatment groups; furthermore, during an initial follow-up of 18 months all cases in the combination group remained in complete remission, while about 20% of patients relapsed in the mono-therapy groups (37). A subsequent 5-year survival analysis in the same study suggested a definitive cure of all
patients in the combinatorial group (38). Clinical studies carried out at the MD Anderson Cancer Center confirmed the excellent response of APL patients to frontline treatment based on the combined administration of ATO and ATRA: in these studies no chemotherapy consolidation was administered and only patients experiencing treatment-related hyperleukocytosis received additional treatment with gemtuzumab ozogamicin (39). About 92% of these APL patients achieved CR and after 3 years 85% of them survived to treatment (40). A meta-analysis was recently performed on a total of 415 patients treated with ATRA alone, ATO alone, or both drugs (41). The analysis of these data unequivocally showed that double drug treatment significantly improved the complete remission rate and decreased the cutaneous reactions compared with ATRA alone (41). However, the incidence of liver toxicity was higher in the ATO + ATRA and ATO groups than in the ATRA-alone group (41).

The impact of the combined ATRA+ATO therapy in the frontline treatment of patients with low-intermediate risk APL was recently assessed in a large phase III study, comparing ATRA plus ATO with ATRA + chemotherapy in a large group of low/intermediate-risk APL patients. All patients in the ATRA + ATO group achieved a complete remission, compared to 95% in the ATRA + chemotherapy group; the 2-year event-free survival rates (primary objective of the study) were 97% and 86% in the ATRA+ATO and ATRA + chemotherapy groups, respectively; overall survival was also significantly better in the ATRA+ATO group compared to the ATRA + chemotherapy group; finally, compared to ATRA + chemotherapy, ATRA + ATO treatment was associated with considerably reduced hematologic toxicity and fewer infections (42). This study suggested a new standard in treatment of APL and provided the first paradigm of curability of an AML subtype by targeted therapy, without chemotherapeutic agents. However, a longer follow-up of this clinical trial and independent confirmatory studies are required to definitely prove the curability of APL with ATRA + arsenic and establish this strategy as a new standard of care. The health-related quality-of-life (HRQOL) results of this trial were recently reported. The analysis of HRQOL results favored the ATRA + ATO group, the largest difference between the two groups of patients being observed for fatigue severity at the end of the induction treatment (43). These observations further support the use of ATRA + ATO as preferred first line treatment of patients with low-or intermediate-risk APL.

While ATO is given as an intravenous formulation, the therapeutic impact of an oral arsenic tetra-sulfide formula was investigated in China. In a randomized non-inferiority trial conducted in newly diagnosed APL, Zhu et al. recently compared ATO vs. the oral tetra-sulfide formulation and showed similar outcome results in both groups as well as no significant differences in toxicity (44). Using this oral preparation of arsenic, these authors most recently reported the results of a pilot study on 20 non-high risk APL patients receiving prolonged As$_4$S$_4$ + ATRA without chemotherapy consolidation: all patients achieved complete remission and experienced an acceptable toxicity and a nearly normal quality of life (45). About 50% of these patients completed the induction therapy without need for hospitalization (45).

Future important developments will imply the extension of the ATRA + arsenic therapy to other categories of APL patients, including high-risk, elderly and pediatric patients (46).

**ATRA and arsenic resistance**

In spite the high efficacy of ATRA to obtain a rapid and efficient control of the disease, some APL relapsing/refractory patients show resistance to ATRA treatment. From a clinical point of view two different types of ATRA resistance have been identified: primary ATRA resistance, only very rarely observed; and secondary, acquired ATRA resistance, which is more frequently observed (47).

Various mechanisms have been proposed as molecular determinants of ATRA resistance, including occurrence of mutations at the level of the Ligand Binding Domain (LBD, zones I, UU and III) of RARA (PRα/LBD$^+$), increased catabolism of ATRA, expression of cytoplasmic retinoic acid binding protein (CRABP) and impaired ATRA delivery at the level of the cell nucleus [reviewed in (48)]. ATRA mediated by PRα/LBD mutants is due to their decreased binding affinity for ATRA and impaired ligand-dependent co-repressor release and co-activator recruitment. Only the first of these mechanisms was documented through molecular studies in APL patients relapsing under ATRA treatment. In this context, Gallagher and coworkers analysed 45 APL patients relapsing from the chemotherapy/ATRA arm of the US Intergroup protocol C9710 and showed that 40% of these patients harbored PRα/LBD$^+$ mutations: 65% of these PRα/LBD$^+$ patients relapsed during ATRA treatment, while 49% of them relapsed after cessation of ATRA therapy (49). This observation is very important because it indicates that in most instances the involvement of the PRα/LBD$^+$ cells
in relapse clone is related to the capacity of PRα/LBD+ cells to be resistant to ATRA-induced differentiation, resulting in their passive selection following the progressive elimination of ATRA-sensitive APL cells displaying WT PML/RARα; however, the high percentage (39%) of PRα/LBD+ APL patients who relapsed after the cessation of ATRA therapy suggests that PRα/LBD+ mutants may have an active, ATRA-independent role in APL relapse (49). A potential mechanism by which PRα/LBD+ could exert this active role is through a potentiation of the self-renewal-promoting effect of PML/RARα. This hypothesis is supported by the observation that APL cells expressing mutant PRα/LBD+ induce the formation of more aggressive leukemias than those expressing WT PML/RARα in a mouse transgenic model (50).

The association between LBD mutational status and additional genetic abnormalities showed some interesting findings: The majority of PRα/LBD+ patients relapsing on ATRA are FLT3-ITD+, while the majority of PRα/LBD+ patients relapsing off ATRA have additional chromosomal abnormalities (49). The positive association between FLT3-ITD and PRα/LBD+ patients relapsing on ATRA therapy suggests a complementarity in the interaction between these two mutants. On the other hand, the association between PRα/LBD+ and additional chromosomal abnormalities in APL relapsing off-ATRA may suggest the existence of an intrinsic chromosome instability in these patients (49).

In addition to ATRA resistance, some studies have described the occurrence of As2O3-resistant PML/RARα mutants. Particularly, Goto and coworkers described two APL patients exhibiting As2O3 resistance after treatment with ATRA + chemotherapy: arsenic resistance was related to the occurrence of missense mutations determining the substitution of amino acids at the level of the PML-B2 domain of the PML/RARα molecule (51). Seemingly, these mutations determine a reduced binding of As2O3 to PML/RARα, due to conformational changes in arsenic binding sites (51). In this context, it is very important to point out the clinical data on the follow-up of the APL patients treated with the combined administration of ATO and ATRA indicate the absence of disease relapse due to ATRA or Arsenic resistance.

Conclusions

During the last three decades fundamental progresses have been achieved in the understanding of the cellular and molecular bases of APL. PML/RARα was identified as the main leukemia-initiating event, responsible in large part for APL pathogenesis. The targeting of this molecule at the level of its RARα domain by ATRA or of its PML domain by ATO allows to obtain a reversal of the inappropriate activity of PML/RAR as a transcriptional modulator and to induce the degradation of this fusion protein. A curative treatment of this leukemia requires the complete eradication of leukemia-initiating cells. At the clinical level the introduction in therapy of ATRA and ATO has dramatically improved the prognosis of APL and results of recent clinical trials indicate that the combined administration of ATRA and ATO induces a very high rate of durable remissions in low/intermediate risk APLs.

Although the overall survival of APL has been dramatically improved by the introduction of ATRA and ATO, some issues in the treatment of this leukemia remain still unresolved, such as the development of a curative treatment also for high-risk APL patients (i.e., patients with leucocyte count above 10x10⁹/L at diagnosis) and the reduction of early disease-related mortality.

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Footnote

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