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Rational Targeting in Acute Promyelocytic Leukemia

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Abstract. *Acute promyelocytic leukemia (ARL) is characterized by the nearly homogeneous expression of the fusion oncogenic protein PML-RARα and the testis-specific cyclin A1 protein, which are implicated in its pathogenesis. PML-RARα binds all-trans retinoic acid with high affinity inducing granulocytic differentiation and remission. Current approaches with high doses of single or combined all-trans retinoic acid and chemotherapeutic agents, though relatively efficacious in the beginning, are highly toxic with severe side-effects (retinoic acid syndrome) and are followed by relapse in a high proportion of patients. Here it is proposed that targeting APL with low levels of all-trans retinoic acid combined with small molecule inhibitors of cyclin-dependent kinases may have the potential to be equally or more efficacious as any of the current single or combined agent approaches, affording reduced toxicity and relapse rates.*

Acute promyelocytic leukemia (APL) is a common variant of acute myeloid leukemia (AML) comprising 10 to 15% of all cases and is characterized by molecular features that render it amenable to combined molecular targeting therapy (1). First, chromosomal rearrangements that generate oncogenic fusion proteins are present in the majority of cases (2). For example, up to 95% of APL patients specifically harbor the t(15;17) (22;q11.2-12) balanced translocation and express the fusion protein PML-RARα (3, 4). Less than 1% of the other translocations fuse RARα with the promyelocytic leukemia zinc finger protein (PLZF), nuclear mitotic apparatus (NUMA), nucleophosmin (NPM) or signal transducer and activator of transcription 5B (STAT5B) (reviewed in 2, 5). In addition to expressing the fusion proteins, APL cells at the promyelocyte and myeloblast stages (6, 7) also constitutively express the male germ cell-restricted cyclin A1 protein in virtually all cases (Figure 1) (7, 8), and notably, evidence suggests that PML-RARα induces the expression of cyclin A1 in APL (9). APL-derived patient cells and cell lines are characterized by proliferative self-renewal and arrest of differentiation (Figure 1) at the promyelocytic stage (10-13). APL is responsive to pharmacological ($\geq 10^{-6}$ M) levels of all-*trans* retinoic acid (ATRA), which causes terminal differentiation of patient leukemic cells and of cell lines into granulocytes with concomitant expression of granulocyte-associated proteins such as CD11b and distinct morphological changes (13, 14), affording clinical remission for some patients and thus providing the first significant proof of principle that differentiation therapy can be effective in human cancer. Generally, the chromosomal translocations characteristic of APL occur in immature precursors of blood cells (promyelocytes) that have the capacity to self-renew perhaps even before overt clinical manifestation (8), thus raising the possibility that early chemopreventive strategies that target pathways regulated by the fusion oncogenic protein and cyclin A1 might be applicable as in other paradigms (Figure 2).

APL is unique because of the overwhelming prevalence of PML-RARα and cyclin A1, which make it an attractive experimental model both *in vitro* (using NB4 and other APL cell lines that express the fusion protein and cyclin A1) and *in vivo* in transgenic mice. The presence of these two aberrantly expressed proteins has provided a potentially exploitable attribute, and the rationale, for combined pharmacological intervention. First, the low

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level expression of PML-RARα is critical for the development of APL (7). Second, PML-RARα mediates the differentiation effects of ATRA. Third, PML-RARα induces expression of cyclin A1 and therefore inhibiting cyclin A1 function coupled with ATRA/PML-RARα-mediated forced transcriptional activation of a granulocytic differentiation pathway can provide an effective therapeutic window. Fourth, as newer findings suggest, and in spite of its counter-intuitiveness, it might be advantageous to inhibit the ATRA-induced degradation of the fusion protein because its presence would enhance differentiation and also because its degradation does not appear to be correlated to the response when ATRA is given alone. In clinical ATRA trials, optimal differentiation of cells derived from patients (defined as >50% differentiation at day three with 0.1 M ATRA alone) strongly correlates both with event-free survival (p=0.05) and with overall survival (p=0.10) (13).

Early attempts to test the *in vivo* role of PML-RARα in the pathogenesis of APL met with limited success as its ubiquitous overexpression in mice results in embryonic lethality (14) and its targeting to the early hematopoietic compartment (through the cFes promoter) or the differentiated myeloid compartment (through the CD11b promoter) fails to give rise to leukemia (15). Restricted expression to the myeloid or promyelocytic compartments was achieved by the use of human cathepsin-G (hCG) (16) and the hMRP8 promoters (17). While virtually all the mice exhibited a myeloproliferative disorder, only 15 to 20% developed APL-like disease (low penetrance) with a 6- to 14- month latent period, suggesting that the fusion protein is by itself insufficient to cause APL. These results led to the hypothesis that the fusion protein has a more restricted role in APL and depends on the translocation occurring in target cells that are at specific developmental stages (7, 18).

Westervelt and colleagues (19) tested this hypothesis in mouse knock-in models by re-targeting the original bcr-1-derived PML-RARα cDNA into the 5' untranslated region of the endogenous cathepsin G locus, but without the original PGK-neo cassette, resulting in mice that expressed the fusion protein at even lower levels (less than 3%) than those in the transgenic models. Significantly, more than 90% of knock-in mice developed APL-like disease. These data were consistent with the hypothesis that the observed high penetrance arises because the lower levels of the fusion protein not only prevent the death of affected early progenitor cells (20), but also they likely facilitate a gain-of-function influence by selecting for a transformable pool of myeloid precursors, in addition to functioning as a dominant negative transcriptional repressor and silencing genes that are instrumental for the orderly differentiation stages towards terminal granulopoiesis.

As the hallmark of APL is the accumulation of differentiation-arrested promyelocytes (Figure 2), it seemed reasonable to hypothesize that some feature of promyelocytes might be crucial in APL development. This hypothesis was tested by transiently expressing PML-RARα in myeloid and non-myeloid cell lines and it was found that it is cleaved in the early myeloid line U937 but not in the erythroleukemia cell line K256. Significantly, the same pattern of cleavage was generated when *in vitro* translated fusion protein was incubated with murine bone marrow extracts. Further analysis showed that the proteolytic activity is due to neutrophil elastase (NE), which is encoded by the *Ela2* gene and is maximally active during the early promyelocytic stage. The crucial role (21) NE-mediated cleavage of PML-RARα plays in APL development was demonstrated in NE-deficient mice. In these mice, more than 90% of the fusion protein remains uncleaved, and as a result mice do not develop APL, suggesting that although the presence of intact fusion protein may be necessary for some aspects of differentiation arrest in early promyelocytes that are mediated through its transcriptional repressor properties, NE-mediated cleavage is a critical determinant of its leukemogenic potential. Transient expression of PML-RARα at low levels, followed by assessment of viability clearly demonstrates that the fusion protein not only confers higher proliferation rates on early myeloid cells, but also it causes delayed differentiation in an NE-dependent manner (22).

The Dual Role of PML-RARα in APL Pathogenesis and Therapy

Expression of PML-RARα protein at low levels (<3%) is not only important in the pathogenesis of APL but also, and one might add surprisingly, may directly mediate the differentiation response induced by ATRA in leukemic, perhaps even in pre-leukemic blasts (5, 19). The key model of APL pathogenesis proposed that the ability of PML-RARα to repress transcription in a negative dominant fashion coupled to its unresponsiveness to physiological levels of ATRA (10^{-9} to 10^{-8} M) were critical, if not the major, oncogenic mechanisms (23, 24). ATRA interacts with PML-RARα with an affinity that is comparable to that with the wild-type RARα. Additionally, PML-RARα binds retinoic acid response elements (RAREs) as homodimers or as heterodimers complexed with RXR (25, 26).

Interference of PML-RARα with differentiation, even in the presence of physiological levels of ATRA, is associated with the ability of PML-RARα to interact with and to recruit a protein complex that contains the nuclear receptor co-repressors SMRT or N-CoR, co-repressors mSin3A and B, and histone diacetylases, with an affinity that is higher

than that of the wild-type RAR receptor, leading to transcriptional repression of RARE-containing promoters of target genes (24, 27). This explains why pharmacological levels of ATRA (>10^{-7} to 10^{-6} M) are required to dissociate the co-repressor complexes and to convert PML-RARα into a RARE element-bound activator (28, 29). Functionally, physiological concentrations of ATRA fail to prevent fusion protein-mediated blocking of ATRA-regulated activation of reporters in several different cell lines, and consequently fail to induce granulocytic differentiation, for example of HL-60 cells (30), or monocytic differentiation of U937 cells (31). On the other hand, strong experimental evidence suggests that removal of PML-RARα is not absolutely required for induction of differentiation. First, *in vitro* studies indicate that ATRA-induced differentiation is accompanied by proteolysis of the fusion protein, a potential mechanism for alleviating transcriptional repression and differentiation block (32-34). Notably in this case, PML-RARα is processed into ΔPML-RARα, an 85 kDa species (33), which is distinct from the 60 and 50 kDa species resulting from NE-mediated cleavage (19). Second, in several ATRA-resistant NB4 sub-clones PML-RARα is degraded by arsenic treatment without concomitant differentiation and third, several ATRA-resistant cell lines exhibit constitutive degradation of the fusion protein (35, 36) without concomitant induction of differentiation making it unlikely that degradation by either agent is sufficient to contribute to differentiation and by extension to clinical remission.

A plethora of observations suggests that PML-RARα mediates the *in vivo* and *in vitro* response to pharmacological levels of ATRA in APL cells. The fusion protein retains virtually all of the functional domains of RARα and PML, including both the ligand- and DNA-binding domains of wild-type RARα. Thus, ectopic expression of the PML-RARα protein in U937 myeloid cells, a cell line that lacks endogenous expression, enhances their responsiveness to ATRA *in vitro* or in ATRA-treated transgenic mice that express the fusion oncogene (37, 38). In fact, the differentiation response of NB4 cells, the prototypical APL cell line that has been extensively used to study molecular aspects of the disease, depends on the presence of the fusion protein (39). More importantly, relapsed patients and ATRA-resistant cell lines (40, 41) harbor PML-RARα proteins bearing mutations in their ligand-binding domain but not in wild-type RARα receptor, or lack expression of the protein altogether (36, 42). These data suggest that the ATRA-induced differentiation of APL cells is mediated directly by the fusion protein through signal transduction pathways that lead to activation of growth inhibitory genes such as *p21*, or genes that lead to caspase-mediated degradation of the fusion protein (43) and finally to granulocytic differentiation-specific genes such as type II transglutaminase (37) or to *C/EBPε* (43, 44). Although it was originally suggested that elimination of PML-RARα by eventual ATRA-induced degradation contributed to its therapeutic effect (reviewed in (45)), the fact that promyelocytic differentiation could occur in the presence of the intact fusion protein (46) suggested that its transcriptional repressor properties and ATRA-induced activation functions could be dissociated and furthermore that they could be experimentally manipulated. For example, in Zn-inducible PR9 cells, a U937 derivative that expresses full length PML-RARα and is therefore ATRA responsive, it was demonstrated that commitment to differentiation occurred 24 hours post-ATRA treatment and notably differentiation correlated with persistence of the fusion protein.

Retinoic Acid in APL Cell Differentiation and Therapy

Unlike other forms of AML, APL is characterized by its responsiveness (Figure 1) to pharmacological (≥10^{-6} M) levels of ATRA, which induces patient leukemic cells or cell lines to differentiate into granulocytes with concomitant expression of granulocyte-associated proteins such as CD11b and distinct morphological changes, affording clinical remission for some patients and thus providing the first significant proof of principle that differentiation therapy can be effective in human cancer (7). Retinoic acid is the first and most significant therapeutic agent to induce differentiation of human cancer cells and remission in patients whose application exploited a specific molecular defect of APL, namely the expression of PML-RARα (47). Despite its initial success, long-term application leads to attenuation of its clinical efficacy, which results from the emergence of resistance (reviewed in (48)) and from its toxicity due to the high pharmacological levels employed (49). Differentiation of cells requires that they exit the cell cycle and generally agents that inhibit cell cycle traverse, for example through inhibition of cyclin-dependent kinase function, can facilitate differentiation programs. Thus, rational approaches that exploit the inhibition of the CDKs in defined experimental systems may be promising approaches in targetable systems such as APL (reviewed in (50, 51). The constitutive expression of cyclin A1 in human AML cell lines (53) in peripheral blood samples of patients with myeloid leukemia (54) in nearly 99% of all cases suggests that such an approach may be successful. Activation of cyclin A1 transcription in U937 cells by ectopically expressed PML-RARα and down-regulation of its expression by ATRA (7) raises the possibility that it may be critical in the etiology of myeloid leukemia. That this is the case was demonstrated by its overexpression in the myeloid compartment of transgenic mice, which

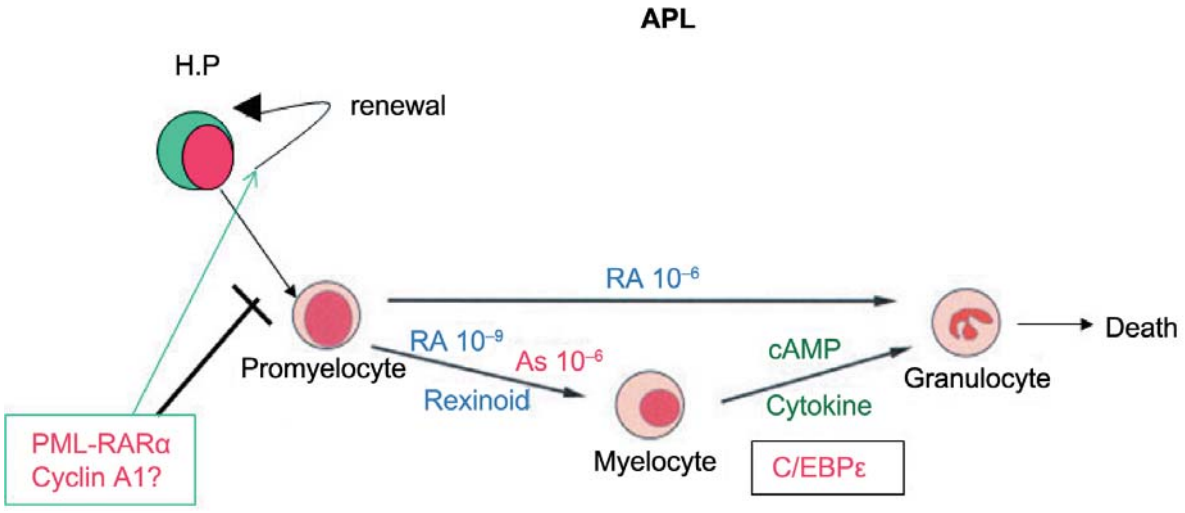

Figure 1. *General model of APL: Schematic depiction of APL stages where PML-RARα and cyclin A1 are thought to act. Reciprocal chromosomal translocation of RARα with PML generates the fusion protein PML-RARα that activates expression of cyclin A1, a putative cancer testis gene. Cyclin A1 contributes to cell cycle traverse of leukemic blasts, and represses transcription of other genes that are required for granulocytic differentiation. All-trans retinoic acid (ATRA) is a ligand of PML-RARα and mediates the differentiation response of leukemic blasts at high and often toxic levels. Evidence suggests that several mechanisms mediate the effects of ATRA (57, 58). APL: Acute promyelocytic leukemia; RA: retinoic acid; As: arsenic; cAMP: cyclic adenosine monophosphate.*

exhibited long latency, low penetrance and blockage of early myeloid cell differentiation, all reminiscent of the phenotypes obtained with the fusion protein in transgenic mice (55). cyclin A1 functionally interacts with CDK2 and with Rb, a central regulator of mammalian cell cycles, and is also expressed in NB4 cells. These results clearly suggest that cyclin A1 might be critical for APL development and inhibition of its expression might contribute to establishing preventive and, in the future, possibly therapeutic measures.

Conclusion and Prospects

Established therapeutic approaches for APL relying either on single-agent administration or combination of agents have delivered substantial therapeutic benefits. Anthracycline and ara-C were the first agents found to be effective for AML and APL and have remained standard therapy for more than 30 years. Since the introduction of ATRA in the 1980s administered singly or in combination with anthracycline-based chemotherapy of arsenic trioxide (ATO), the cure rates have been improved primarily due to synergism; however, relapse and serious side-effects resulting from high pharmacological levels of ATRA are still a major problem. Although ATRA plus chemotherapy is the most beneficial, approximately 10% of patients die early and 20-30% of patients relapse (56). Administration of ATO to patients gives an overall survival probability of

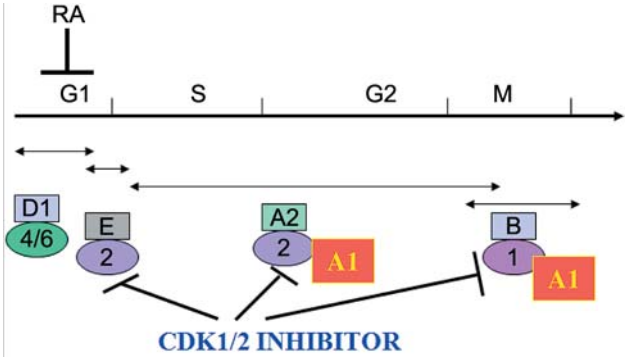

Figure 2. *Model for molecular mechanism-based targeting in APL. A rational approach that exploits (a) the nearly homogeneous expression of PML-RARα (~95%) and its ligand binding properties for ATRA, and (b) the homogeneous expression of cyclin A1 (~99%) in APL cells. This approach also exploits the requirement for cell cycle exit or arrest at the G_0/G_1 stages for induction of differentiation of proliferating cells. Note that cyclin A1 and cyclin A2 are associated with CDK1/CDK2 complexes at different times and stages of the cell cycle. A1: Cyclin A1; A2: cyclin A2; B: cyclin B; D1: cyclin D1; E: cyclin E; 1: cyclin-dependent kinase 1; 2: cyclin-dependent kinase 2; 4, 6: cyclin-dependent kinase 4/6.*

77% at 3 years compared to only 47% for patients given combined ATRA/chemotherapy regimens. The ATO/ATRA combination is more effective than either alone, however, relapse remains a problem, probably stemming from the lack of target specificity and the uniformly high

pharmacological levels of ATRA. Combining low, sub-pharmacological levels of ATRA with a cell cycle inhibitor of cyclin A1/CDκ1/CDK2 function (Figure 2) can potentially be equally or even more effective and therefore less toxic since (a) nearly 99% of APL cells express cyclin A1, (b) nearly 95% of all cases express the fusion oncogenic protein and (c) inhibition of cell cycle progression is known to be required for induction of differentiation (57, 58), especially when cells are at the G_0/G_1 stage of the cell cycle.

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