CHAPTER 6

REPROGRAMMING ACUTE MYELOID LEUKEMIA INTO SENSITIVITY FOR RETINOIC-ACID-DRIVEN DIFFERENTIATION

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Published in Experimental Hematology, 52, 12-23 (2017)
ABSTRACT
The success of all-trans retinoic acid (ATRA) therapy for acute promyelocytic leukemia (APL) provides a rationale for using retinoic acid (RA)-based therapy for other subtypes of acute myeloid leukemia (AML). Recently, several studies showed that ATRA may drive leukemic cells efficiently into differentiation and/or apoptosis in a subset of AML patients with an NPM1 mutation, a FLT3-ITD, an IDH1 mutation, and patients overexpressing EVI-1. Because not all patients within these molecular subgroups respond to ATRA and clinical trials that tested ATRA response in non-APL AML patients have had disappointing results, the identification of additional biomarkers may help to identify patients who strongly respond to ATRA-based therapy. Searching for response biomarkers might also reveal novel RA-based combination therapies with an efficient differentiation/apoptosis-inducing effect in non-APL AML patients. Preliminary studies suggest that the epigenetic or transcriptional state of leukemia cells determines their susceptibility to ATRA. We hypothesize that reprogramming by inhibitors of epigenetic-modifying enzymes or by modulation of microRNA expression might sensitize non-APL AML cells for RA-based therapy. AML relapse is caused by a subpopulation of leukemia cells, named leukemic stem cells (LSCs), which are in a different epigenetic state than the total bulk of the AML. The survival of LSCs after therapy is the main cause of the poor prognosis of AML patients, and novel differentiation therapies should drive these LSCs into maturity. In this review, we summarize the current knowledge on the epigenetic aspects of susceptibility to RA-induced differentiation in APL and non-APL AML.
Acute myeloid leukemia (AML) is a heterogeneous disease characterized by the accumulation of immature myeloid cells with abnormal proliferation, differentiation, and survival capacity\(^1,2\). Different subtypes of AML can be identified based on morphology, immunophenotypic characteristics, cytogenetic aberrations, alterations in gene expression, DNA methylation profiles, and molecular abnormalities\(^3,4\). Classification based on the integration of karyotype and mutations allows stratification of patients by recurrence risk and survival. For the past 4 decades, treatment of AML with combination chemotherapy consisting of cytosine arabinoside (Ara-C) and an anthracycline (e.g., daunorubicin or idarubicin) has been unchanged. Favorable-risk patients are treated only with chemotherapy, and adverse-risk patients often receive allogenic stem cell transplantation when they have reached a first remission. Despite initial high complete remission rates, the 5-year overall survival rate of adult AML patients is less than 40%\(^2,8\). This very poor treatment outcome is mainly due to chemotherapy resistance.

The only exception is treatment of acute promyelocytic leukemia (APL) with all-trans retinoic acid (ATRA) differentiation therapy. Expression of the fusion gene encoding chimeric PML-retinoic acid receptor alpha (RARA) in the leukemic cells of APL patients allows successful application of ATRA\(^9,10\). ATRA-based therapy has significantly improved long-term clinical outcomes for APL patients and converted this AML subtype from a poor prognostic leukemia to a curable one\(^11,12\). Although ATRA-based differentiation therapy is successful in the treatment of APL, its clinical effectiveness in other AML subtypes is limited.

Previous preclinical studies have provided evidence that specific molecular abnormalities can unlock ATRA-driven responses in non-APL AML patient cells; that is, AML with aberrant expression of ecotropic viral integration site 1 (EVI-1)\(^13\), mutant isocitrate dehydrogenase 1 (IDH1)\(^14\), mutant nucleophosmin gene (NPM1)\(^15-19\), and AML with an FMS-like tyrosine kinase 3/internal tandem duplication (FLT3-ITD)\(^20\). Together, these studies indicate that a subset of EVI-1-positive, IDH1 mutant, NPM1 mutant, and FLT3-ITD positive AML cases are sensitive for ATRA-induced differentiation (Table 1). Because inhibition of the demethylase LSD1 (also called KDM1A) has been shown to induce susceptibility to ATRA [21 (Table 2), we hypothesize that AML cells need to have a particular epigenetic and transcriptional state to be susceptible to retinoic acid (RA)-induced differentiation. Therefore, the application of epigenetic-modifying drugs might represent an effective therapeutic strategy for increasing sensitivity to ATRA-induced differentiation. To date, a limited number of preclinical studies have combined epigenetic-modifying drugs with RA-based therapies to test susceptibility to ATRA-induced differentiation in non-APL AML patient samples. Moreover, there is a lack of knowledge of the epigenetic mechanisms underlying ATRA unresponsiveness and of the identity of biomarkers that can predict the response to ATRA. Because the epigenetic state of the leukemic cells might be the key to successful administration of ATRA-based differentiation therapy in non-APL AML, research should focus on elucidation of molecular and epigenetic mechanisms by which susceptibility to ATRA is accomplished\(^22\). In this review, we discuss the current understanding of transcriptional and epigenetic regulation involved in mediating ATRA-driven myeloid differentiation responses in APL and non-APL AML subtypes.
Table 1. Primary human AML with sensitivity to ATRA

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<tr>
<th>Molecular aberrancies</th>
<th>Frequency</th>
<th>Response to ATRA</th>
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<tbody>
<tr>
<td>EVI-1 positive</td>
<td>10–11%</td>
<td>Induction of blast differentiation and apoptosis; reduction of leukemic clonogenic capacity in nine of 13 AML cases; reduction of myeloid engraftment in two of three primary EVI-1-positive AML cases</td>
</tr>
<tr>
<td>IDH1-R312H mutant</td>
<td>6–7%</td>
<td>Induction of nuclear lobulation and associated neutrophilic differentiation in eight of 25 samples; induction of granulo-monocytic differentiation in 11 of 25 samples</td>
</tr>
<tr>
<td>FLT3-ITD positive</td>
<td>24%</td>
<td>Decreased clonogenic capacity in three of three FLT3-ITD patients’ samples; reduced myeloid engraftment in two of two patient samples (first transplantation)</td>
</tr>
<tr>
<td>NPM1 mutant</td>
<td>30–35%</td>
<td>Inhibition of cell growth in four of 11 primary AML samples and induction of apoptosis due to selective proteasomal degradation of mutant NPM1 protein in three of these 11 samples and in one patient harboring the NPM1 mutation without FLT3-ITD; ATRA + ATO combination therapy: significant downregulation of NPM1 mutant protein and induction of differentiation, cell-cycle arrest, and apoptosis in two of three patient samples</td>
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RA THERAPY IN APL

Retinoids are a class of signaling molecules derived naturally from vitamin A (or retinol) and involved in regulating multiple biological processes, including embryonic development and hematopoietic cell growth and differentiation. After uptake by intestinal mucosa cells, dietary-derived retinoids are oxidized into the active compounds retinal and RA, which can then bind to and activate one or more nuclear RA receptors (RARs) and rexinoid receptors (RXRs) to mediate gene expression. For both the RAR and RXR receptor families, three subtypes—characterized as α, β, and γ—act as ligand-inducible transcription factors that bind predominantly as either homodimers or heterodimers to retinoid acid response elements (RAREs) in the promoter regions of their target genes. In the absence of agonists, complex formation of RXR–RAR heterodimers with nuclear receptor corepressors, including N-CoR, and silencing mediator for retinoid and thyroid hormone receptors actively repress gene transcription via recruitment of histone deacetylases (HDACs) and subsequent chromatin condensation (Fig. 1A). To date, the endogenous high-affinity ligand of RXR, 9-cis–RA, and the physiological active ligand of RAR, ATRA, are identified as the main activators of RARs. Upon binding of these agonists, conformational changes in the ligand-binding domains of the receptors result in destabilization of the corepressor complex and recruitment of transcriptional coactivators, including histone acetyltransferases (HATs), such as CREB-binding protein (CBP) or p300, and the SRB mediator-containing complex. This leads to transcriptional activation of retinoid target genes through decondensation of the chromatin (Fig. 1A).

In contrast to RARB, RARA and RARY are widely expressed in hematopoietic cells, including myeloid cells. Several in vitro and in vivo studies have focused on the effect of knocking out RARA or RARY in normal hematopoietic cells. Although hematopoietic stem cells (HSCs) highly express
Table 2. Preclinical in vitro studies involving combination therapy with ATRA in non-APL AML. Table continuous on next page

<table>
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<tr>
<th>Potential combination therapy with ATRA</th>
<th>Cell type</th>
<th>Experimental outcome</th>
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<tr>
<td>Combinations involving molecular aberrancies in AML</td>
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</table>
|FLT3-ITD+                                | Blasts derived from AML patients        | Reduction in cell viability of leukemic blasts in vitro; monocytic differentiation, reduction of leukemic cells, and improved survival in mouse AML xenograft models  

<p>| Sorafenib                               |                                        |                                                                                                                                                      |
|                                        | MOLM13 and MV4-11 cells                | Significant increase of antiproliferative activity and induction of apoptosis                        |
|                                        | PKC421                                  | Dose-dependent inhibition of cellular proliferation and induction of apoptosis                        |
|                                        | NPM1 mutant                            |                                                                                                                                                      |
|                                        | NSC348884                               | Sensitization of ATRA-induced apoptosis in three primary AML patient samples                                           |
| Combination with epidrugs               |                                        |                                                                                                                                                      |
|LSD inhibitor                            | TEX, KG1a, and HL60 cell lines and primary AML patient samples | <em>In vitro</em> induction of myeloid differentiation, nuclear lobulation and neutrophilic differentiation, and post-differentiation apoptosis; induction of differentiation-associated formation of granulocytes in primary AML samples; reduced engraftment of primary AML samples in NSG mice; Phase I clinical trial of ATRA-TCP in patients with AML and MDS (NCT02273102) and Phase I/II clinical trial in patients with relapsed or refractory AML (NCT02261779) are currently ongoing |
|TCP                                     | KOLC-48 cell line                      | Increased cell-cycle arrest and induction of myeloid differentiation in MLL-AF4-positive cells                                           |
|HDAC inhibitor                           | Kasumi-1 cell line and non-APL AML patient samples | Synergistic effect on differentiation partly due to restored RARB2 expression; <em>ex vivo</em> induction of differentiation and enhanced growth inhibition, but no increase in apoptosis in primary blasts from three of three AML patients |</p>
<table>
<thead>
<tr>
<th>Potential combination therapy with ATRA</th>
<th>Cell type</th>
<th>Experimental outcome</th>
</tr>
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<tbody>
<tr>
<td>VPA</td>
<td>OCI-AML2 cell line and non-APL AML patient samples</td>
<td>Induction of cell-cycle arrest associated with upregulated p21 expression <em>in vitro</em>; cell-cycle arrest and promotion of apoptosis in four of six primary patient samples(^{26}); <em>in vivo</em> induction of cell proliferation and differentiation in blasts of seven of eight patients caused by histone H3 and H4 hyperacetylation at the RARA regulatory site(^{27})</td>
</tr>
<tr>
<td>TSA</td>
<td>MOLM14 cell line Non-APL AML patient samples</td>
<td>Induction of growth inhibition and enhanced differentiation(^{28}) Activation of myeloid differentiation in leukemic blasts of 23 of 23 patient samples(^{29})</td>
</tr>
<tr>
<td>DNMT inhibitor 5-Aza</td>
<td>THP1 cell line</td>
<td>Additive induction of myeloid differentiation, inhibition of cell proliferation, and induction of apoptosis compared with ATRA monotherapy(^{30})</td>
</tr>
<tr>
<td></td>
<td>SNI and KOCL33 cell lines</td>
<td>Induction of growth inhibition and granulocytic differentiation in MLL-rearranged AML cell lines(^{31})</td>
</tr>
</tbody>
</table>
RARA, its disruption does not cause abnormalities in hematopoiesis\textsuperscript{35}. Conversely, mice deficient in RARY showed reduced HSCs, significantly increased granulocyte/macrophage progenitors and granulocytes in the bone marrow, peripheral blood, and spleen, and developed a myeloproliferative syndrome (MPS)\textsuperscript{38,40}. This development of MPS was not hematopoietic cell intrinsic but caused by loss of RARY in the microenvironment, suggesting a function of niche-expressed RARY in regulating the balance between HSC proliferation, differentiation, and self-renewal\textsuperscript{40}. The enhanced repopulation potential of HSCs after ATRA treatment might also be regulated via niche-expressed RARY\textsuperscript{38}. HSCs that are dissociated from their microenvironment undergo differentiation. However, inhibition of RA signaling maintained their primitive phenotype and function, suggesting that HSCs are intrinsically programmed to undergo RA-mediated differentiation unless prevented from doing so by the bone marrow niche\textsuperscript{41}. These knockout studies indicate that RAR activity is regulated

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**Figure 1. Mechanism of transcriptional regulation by RARs and PML–RARA before and after ligand binding.**

**A** Heterodimers of RAR and RXR receptors bind to RARE-binding sites in the promoter regions of their target genes. In absence of ligands, a corepressor complex including N-CoR (green), silencing mediator for retinoid and thyroid hormone receptors (pink), and HDACs (orange) is formed, resulting in chromatin condensation. Upon RA binding (red triangle), the corepressor complex is dissociated and transcriptional coactivators are recruited, such as histone methyltransferases (yellow), HATs (purple), and the SRB mediator-containing complex (blue), which leads to transcriptional activation of target genes through chromatin decondensation.

**B** Suppression of PML–RARA target gene transcription and induction of a differentiation block in APL cells. PML–RARA fusion proteins induce aberrant recruitment of repressive epigenetic modifiers, including SUV39H1 (green), PRC1 and 2 (yellow), and TopoII (blue). Upon RA treatment (i.e., ATRA), the corepressor complex is dissociated, miRs are up-regulated, and transcriptional coactivators, including phosphorylated PHF8 (orange), are recruited. H3 acetylation of most of PML–RARA DNA-binding sites is increased, which is also associated with the binding of RNA polymerase II (RNApol II; green) close to or at these sites. Transcription of PML–RARA target genes is activated.
differentially during various stages of normal hematopoiesis and that ATRA treatment can enhance HSC self-renewal capacity.

The role of retinoids in cancer has been studied predominantly in APL. APL, defined by the French–American–British classification as the M3 subtype of AML, accounts for 10% of adult AML patients and is characterized by a differentiation arrest at the promyelocytic stage. APL is associated with the presence of a specific chromosomal translocation involving the RARA gene on chromosome 17 and the promyelocytic leukemia (PML) gene on chromosome 15. In the 1980s, several studies demonstrated that leukemic cell lines and primary APL cells could effectively undergo granulocytic differentiation in response to ATRA. A pharmacological dose of ATRA overcomes the PML–RARA-mediated differentiation block and restores RA target gene transcription and differentiation. In a follow-up to these studies, a clinical trial showed that ATRA induces complete remission in the majority of APL patients. Subsequently, the use of combinations of ATRA with anthracycline-based chemotherapy or arsenic trioxide (ATO), which induces PML–RARA degradation, oxidative stress, differentiation, and apoptosis of APL blasts, resulted in remission in more than 90% of patients. Nevertheless, about 15% of APL patients encounter an early death or relapse after ATRA/ATO treatment. Mutations in epigenetic modifiers in addition to the PML–RARA fusion gene impose a poor prognosis on APL under treatment, indicating that the epigenetic landscape of the APL cells influences the response to RA therapy. Recent clinical trials reported the therapeutic effectiveness of another synthetic retinoid, tamibarotene, in patients with relapsed or refractory APL after treatment with ATRA and ATO. Tamibarotene has a strong differentiation-inducing activity on APL cells and is expected to be more effective and to have less toxicity than ATRA.

**Epigenetics involved in the RA treatment response of APL cells**

In most APL cases, expression of the PML–RARA protein induces susceptibility to ATRA. However, there is still a small subset of APL patients who are unresponsive to ATRA or who relapse following treatment. This lack of response to ATRA might be due to the particular epigenetic and transcriptional states of the unresponsive APL cells. A better understanding of the transcriptional mechanisms associated with ATRA susceptibility might help in the development of therapeutic strategies that can overcome ATRA resistance in APL, which might also be applicable to other AML subtypes. PML–RARA fusion proteins aberrantly recruit multiple repressive epigenetic modifiers, such as HDACs, DNA methyltransferases (DNMTs), the lysine methyltransferase SUV39H1, and polycomb repressive complexes 1 and 2, to downregulate the expression of target genes (e.g., RARB) and to induce a differentiation block (Fig. 1B). RA treatment induces epigenetic modifications at the PML–RARA target loci and dissociation of corepressors, leading to the recruitment of transcriptional coactivators and transcription of target genes. The ATRA-susceptible state of APL cells is dependent on the expression, function, and localization of several of these coactivators and epigenetic proteins as well as the transcriptional program induced by the PML–RARA fusion.

DNA methylation and histone modifications play key roles in regulation of gene expression linked to ATRA responsiveness. DNA methylation is controlled by DNMTs, which catalyze the transfer of methyl groups to cytosine in the cytosine–guanine dinucleotide islands found in
promoter regions. PML–RARA binds to DNMT1 and DNMT3a, promoting DNA hypermethylation and repression of RA target genes\(^5\). RA treatment decreases the expression and activity levels of DNMTs, which correlates with demethylation at specific sites in the RARB promoter and the ability of APL blasts to undergo myeloid differentiation. In an ATRA-resistant APL cell line, in which PML–RARA is no longer able to interact with ATRA, none of these events occurred\(^6\).

Several studies identified PML–RARA DNA-binding sites and demonstrated that these are significantly different from those of wild type RAR\(^6\)\(^7\)\(^9\). Wang et al. showed that the majority of the PML–RARA-binding sites are atypical RARE sites in close proximity to consensus binding sites for PU.1, ETS, and AP-1, suggesting that PML–RARA fusion proteins collaborate with these transcription factors. In addition, PML–RARA interacts directly with PU.1\(^9\). PML–RARA influences RAR signaling by regulating RARA and RARB expression and by binding to classical RAR/RXR target genes. Moreover, PML–RARA binds to genes important for normal hematopoietic differentiation such as PU.1, GFI1, and RUNX1. Using chromatin immunoprecipitation sequencing, Martens et al. showed that only a small number of PML–RARA-binding sites have changes in DNA methylation after expression of the PML–RARA fusion protein. Treatment of PML–RARA-positive leukemia cells with ATRA did not result in significant changes in DNA methylation of the majority of the PML–RARA-binding sites\(^6\).

Although these results suggest that DNA methylation is not a major contributor to the repressive effect of PML–RARA on transcription, the fusion proteins bind to DNMTs, and methylation at a few PML–RARA-binding sites might be sufficient to induce ATRA susceptibility. Recently, it was shown that DNMT3A is required for PML–RARA-induced self-renewal in myeloid progenitor cells and for the initiation of APL in vivo\(^7\)\(^0\).

After ATRA treatment of APL cells, there is an increase in histone 3 (H3) acetylation in most of the PML–RARA DNA-binding sites, whereas H3Lysine(K)27methylation(me)3 and H3K9me3 levels remain predominantly unaltered\(^6\). This increase in H3 acetylation is observed only in APL cells that are responsive to ATRA. Moreover, there is an association between H3 acetylation and the binding of RNA polymerase II close to or at PML–RARA-binding sites. Together, these results suggest that histone acetylation and recruitment of HDACs by PML–RARA is a key factor in determining ATRA responsiveness (Fig. 1B). These results also suggest that targeting HDACs might be a successful strategy for enhancing the sensitivity of APL cells for ATRA. In the past two decades, several attempts to overcome ATRA resistance or enhance ATRA sensitivity using HDAC inhibitors have been made\(^7\)\(^9\)-\(^7\)\(^3\). The HDAC inhibitor valproic acid (VPA) induces differentiation of APL blasts and prolonged disease latency in APL mouse models. However, these inhibitors aggravated the early stage of disease due to their effect on expansion of a subpopulation of leukemic cells that is able to reinitiate the tumor (so-called LSCs)\(^9\). Failure to remove APL LSCs by VPA was also observed in a study by Leiva et al\(^7\). Inhibition of HDACs by trichostatin A (TSA) induces specific changes in the chromatin state at RA target sites, increases the effect of RA on promoter activity, and induces expression of PML–RARA target genes and differentiation\(^6\), indicating potential for the combination of HDAC inhibitors and ATRA to resensitize resistant APL cells.

Moreover, the HAT p300/CREB-binding protein associated factor (P/CAF) has recently been shown to be associated with ATRA-induced differentiation of APL cells. Knockdown of P/CAF inhibited ATRA-induced differentiation in APL cell lines and primary cells, whereas overexpression
induced differentiation. Treatment with ATRA elevated the expression of P/CAF and H3 acetylation on promoters of ATRA target genes, resulting in transcriptional activation of genes associated with differentiation.

Furthermore, recruitment of the histone demethylase PHF8 (also called KDM7B) to RARA fusions is needed for an active ATRA response in APL cells. The JmjC domain containing PHF8 demethylase acts preferentially on H3K9me1/me2 and is associated with transcriptional activation and susceptibility for ATRA-induced differentiation. Binding of PHF8 to promoter regions of multiple PML–RARA targets, including RARB, TGM2, and IDH1, is increased upon ATRA treatment, whereas its binding to other PHF8-associated promoters is reduced. This PHF8-induced switch of promoter occupancy upon ATRA treatment is thought to enhance the expression of PML–RARA target genes (Fig. 1B). In ATRA-resistant APL cells, PHF8 is downregulated, but its enforced expression reactivates the ATRA-induced differentiation program. Moreover, regulation of PHF8 activity provides a pharmacological way to overcome ATRA resistance in APL, as inhibition of PHF8 dephosphorylation by okadaic acid sensitizes ATRA-resistant APL cells for ATRA-induced differentiation.

The formation of aberrant repression complexes at promoter regions of RAR target genes, which are not readily dissociated by treatment with ATRA, might be responsible for resistance to ATRA in APL. For instance, topoisomerase II beta (TopoII) binds to PML–RARA and modulates RARA transcriptional activity. Enhanced levels of TopoII cause resistance to ATRA in APL cell lines, and subsequent downregulation of TopoII can reverse this resistance. Chromatin immunoprecipitation showed that TopoII is bound to a RA response element. Decreasing the TopoII levels reactivated the ATRA response, leading to an increase in H3K9ac and transcriptional activation.

MicroRNAs (miRs) can regulate hundreds of genes at the same time, inducing changes in the transcriptional state of cells. Because several miRs are repressed transcriptionally by the PML–RAR oncogene and re-expressed after treatment with ATRA, miR expression might be crucial to obtain ATRA susceptibility in APL cells. miR-210 and miR-23a and let-7a, let-7c, and let-7d are repressed by PML–RARA complexes in APL cells, and ATRA stimulation results in the increased expression of miR-23a, miR-210, miR-223, and miR-181a and upregulation of let-7a, let-7c, and let-7d.

**RA-based therapy in non-APL AML**

For more than 40 years, AML patients without PML–RARA translocations have been treated with combination therapy consisting of cytarabine and an anthracycline. All leukemic blasts residing within the bone marrow of an AML patient show an arrest at the early stage of differentiation. This general characteristic of AML cells is the basis for the hypothesis that promoting differentiation and overruling the differentiation block can be an attractive therapeutic strategy for AML patients. Currently, only APL patients are treated successfully with differentiation therapy consisting of the combination of ATRA and ATO. However, RA-based differentiation therapy might be applicable to other subgroups of AML patients. In APL, induction of myeloid differentiation by ATRA is regulated via activation of the RARα gene, and one of the reasons that non-APL AML cases are blocked for ATRA-induced differentiation is low expression of RARα, which is regulated via H3K4me2 levels in the RARA promoter region.
Several studies have explored the efficiency of ATRA and liposomal ATRA in combination with chemotherapy to induce differentiation and apoptosis or to reduce clonogenic capacity in non-APL AML cell lines\textsuperscript{86,87} and primary AML patient cells\textsuperscript{88,89,90}. Moreover, some clinical trials showed the effectiveness of ATRA in combination with chemotherapy for AML patients\textsuperscript{91,92}, whereas others had disappointing results\textsuperscript{93-96}. In one of the trials, combination treatment with a low dose of cytarabine and ATRA resulted in prolonged survival of elderly patients compared with chemotherapy alone\textsuperscript{92}. A clinical trial performed by the AML Study Group Ulm showed also an advantage of receiving ATRA in conjunction with chemotherapy\textsuperscript{91}.

Recently, two research groups showed that the combination of ATRA and ATO synergistically induces proteosomal degradation of mutant NPM1, leading to growth arrest, differentiation, and apoptosis\textsuperscript{16,17} (Fig. 2; Table 1). El Hajj et al.\textsuperscript{17} treated five elderly, NPM1-positive patients who were ineligible for chemotherapy with ATRA/ATO and observed reductions in the number of blasts in three of the patients, suggesting that the ATRA/ATO strategy may represent a viable option for NPM1 mutant AML. However, only some of the NPM1 mutant patients responded to ATRA, and clinical trials studying the efficacy of the combination of chemotherapy and ATRA in NPM1-positive patients showed contrasting results. In one trial, the presence of an NPM1 mutation without the FLT3-ITD mutation seemed predictive for ATRA sensitivity in elderly AML patients\textsuperscript{97}. However, other clinical trials showed no significant effect of ATRA on treatment outcome of AML patients with an NPM1 mutation\textsuperscript{94-96}. In all of these studies, a combination of ATRA and chemotherapy, rather than ATO, was used, and therefore, these results are not completely comparable to the successful ATRA/ATO treatment strategy used with APL patients.

In AML cell lines and primary patient samples containing the FLT3 mutation (FLT3-ITD+ AML), the combination of ATRA and FLT3 tyrosine kinase inhibitors showed synergistic activity in induction of apoptosis, lowering the FLT3-ITD+ LSC population, reducing AML engraftment, and prolonging the survival of leukemic mice\textsuperscript{20,22,23} (Fig. 2; Table 2). It is unclear whether this combination is also effective for AML cases with wild-type FLT3. In a study by Ma et al.\textsuperscript{20}, ATRA monotherapy induces differentiation in FLT3-ITD+ AML cell lines and decreases engraftment of primary AML cells. However, this response might not be as strong as that observed in APL cases since Balusu et al. showed that the presence of an FLT3-ITD in NPM1-mutated AML decreased sensitivity for ATRA-induced differentiation\textsuperscript{19}.

In addition to the mutation status of AML cells, low or high expression of certain genes might affect the susceptibility of leukemic cells to ATRA. For instance, a member of the RAR-associated co-repressor complex preferentially expressed antigen in melanoma (PRAME) is overexpressed in AML\textsuperscript{98}, and high levels of PRAME are associated with responsiveness to ATRA\textsuperscript{99}.

**Epigenetics involved in the RA treatment response in non-APL AML**

Identification of biomarkers that indicate whether non-APL AML cells are sensitive to RA-induced differentiation therapy and the identification of successful ATRA-based combination therapies for non-APL AML cases might lead to improvements in patient outcome. Several studies have suggested that the lack of an effective ATRA response in non-APL AML is due to aberrant epigenetics and/or transcription. Somatic mutations and proteins aberrantly expressed in AML affect the transcriptome.
and might significantly affect susceptibility to ATRA. Epigenetic reprogramming to change the transcriptional landscape in one that is linked to RA sensitivity might be the key to successful administration of ATRA differentiation therapy. Aberrant epigenetics in ATRA-resistant AML cells may silence the promoters of RAR target genes or modify expression of repressors, activators, and transcription factors, thereby protecting leukemic blasts from induction of differentiation and apoptosis upon ATRA treatment. Conversely, aberrantly expressed or mutated proteins might induce a transcriptional program that actively blocks differentiation and induces susceptibility for RA-induced differentiation. Examples of this are the PML–RARA fusion protein and overexpression of the EVI-1 protein. Enhanced expression of EVI-1 occurs in about 10% of AML patients and is associated with a very poor disease outcome. Recently, we observed that a substantial part of EVI-
1-positive AML cases are susceptible to ATRA-induced differentiation\(^\text{13}\) (Table 1). EVI-1-positive AML cases respond to ATRA by induction of differentiation and decreased clonogenic capacity of myeloid blasts. Most importantly, a significant reduction in leukemic engraftment of EVI-1-positive AML was shown upon treatment with ATRA \textit{in vivo}. EVI-1 is a nuclear zinc finger transcriptional repressor essential to the proliferation and maintenance of HSCs\(^\text{101}\). The expression of EVI-1 in leukemic cells imposes an epigenetic state and transcriptional program that is linked to susceptibility to ATRA-induced differentiation\(^\text{13,99}\). This ATRA sensitivity might be due to the interaction of EVI-1 with several epigenetic modifiers and transcription factors. EVI-1 interacts with the co-repressor carboxyl-terminal-binding protein 1 (CtBP1) and P/CAF, both of which have histone acetyltransferase activity. Interaction with either CBP or P/CAF leads to acetylation of EVI1 and its localization into nuclear speckles\(^\text{102}\), which likely regulate transcription. EVI-1 binds directly to DNA as well as to GATA2\(^\text{103}\), PBX1\(^\text{104}\), and PML\(^\text{105}\), thereby modifying their expression and/or activity. Expression of EVI-1 can induce epigenetic changes by binding to the PCG proteins EZH2, SUZ12, EED, BMI1, RING1, RING2, and HHP2, thereby enhancing H3K27me3 marks on the PTEN locus\(^\text{106}\). Moreover, EVI-1 interacts with the H3K9 methyltransferases SUV39H1 and G9a, which silence gene expression\(^\text{107}\). Knockdown of SUV39H1 or G9a reduced the colony-forming activity of EVI-1-transduced bone marrow cells, suggesting that H3K9 methyltransferases are actively involved in EVI-1-induced transformation.

In addition to its involvement in oncogenic functions, the interaction of EVI-1 with epigenetic modifiers, such as histone methyltransferases, might be involved in sensitivity to ATRA. Like PML–RARA, EVI-1 interacts with DNMT3A and DNMT3B and is presumably responsible for the distinctive DNA methylation profile observed in EVI-1-positive AML compared with normal bone marrow cells\(^\text{108}\). Moreover, EVI-1 also interacts with PU.1\(^\text{109}\), suggesting that PU.1 is involved in EVI-1-induced ATRA responsiveness.

As many as 15% of AML patients exhibit mutations in the isocitrate dehydrogenase 1 and 2 (\textit{IDH1/2}) genes. These \textit{IDH} mutations lead to production of the oncometabolite (R)-2-hydroxyglutarate (2-HG) and a block in differentiation\(^\text{110}\). Tumors with \textit{IDH1/2} mutations exhibit distinctive profiles of 2-HG-dependent DNA methylation and histone hypermethylation that alter the transcriptome\(^\text{111}\). Because these transcriptomes consist of several genes involved in RA metabolism and signaling\(^\text{112,113}\), Boutzen et al\(^\text{14}\) investigated the sensitivity of leukemic cells with the \textit{IDH1-R132H} mutation for ATRA. \textit{IDH1-R132H}-positive AML blasts showed an increase in H3K4me3 at the CCAAT/enhancer-binding protein alpha (\textit{CEBPA}) gene promoter as well as enhanced expression of \textit{CEBPA} and an increase in expression of \textit{CEBPA} target genes as compared to wild-type AML cells. In addition, RA-responsive genes are deregulated in cells with the \textit{IDH1-R132H} mutation. Epigenetic activation of \textit{CEBPA} is proposed to be involved in the regulation of these RA-associated genes. Importantly, the results of this study showed that the \textit{IDH1-R132}-mutated protein and its product, 2-HG, sensitize AML cell lines and primary patient AML cells for ATRA-induced granulocytic differentiation and apoptosis\(^\text{14}\) (Fig. 2; Table 1).

\textit{MLL}-positive AML is characterized by fusion of the \textit{MLL} gene (also called \textit{KMT2A}) with a variety of other chromosomes. \textit{MLL} is a lysine methyl transferase, and its fusion partners are subunits of the super-elongation complex and/or DOT1L complex. As a demethylating agent and a HDAC inhibitor enhance ATRA-induced differentiation in \textit{MLL}-positive AML cells\(^\text{28,30}\), the epigenetic
landscape of these AML cells may be important for their sensitivity to ATRA. Indeed, Sakamoto et al. showed that, in MLL-AF9-positive AML cell lines, high levels of H3K4me2 in the RARA promoter region, the PLU.1 regulatory regions, or the RUNX1 enhancer are associated with sensitivity to ATRA\textsuperscript{24}.

The potential of reversing chromatin modifications in AML cases to induce or enhance sensitivity to ATRA has been investigated by several research groups (Table 2). Treatment of 23 AML patient samples with the combination of the pan-HDAC inhibitor TSA and ATRA demonstrated that targeting HDACs can restore RA signaling and differentiation in non-APL AML cases\textsuperscript{29}. Ferrara et al. also showed that the AML-ETO translocation is an HDAC-dependent repressor of RA signaling. The HDAC inhibitor VPA has been shown to restore the ability to respond to ATRA in an AML cell line and in six primary AML cases\textsuperscript{26}. ATRA-based combination therapy with VPA or the HDAC inhibitor Entinostat enhanced histone acetylation levels on RA target sites and increased the expression of genes involved in differentiation\textsuperscript{26,27,28}. Despite these results, several clinical trials with AML patients treated with a combination of ATRA and HDAC inhibitors, including VPA, showed limited effect on the overall survival of the patients\textsuperscript{27,114-116}. By reversing DNA methylation using the DNA methyltransferase inhibitor 5-azacytidine (5-Aza), sensitivity for ATRA could also be induced or enhanced\textsuperscript{117} (Table 2). An increase in the expression of transcription factors involved in myeloid differentiation, including CEBPA and PLU.1, likely is involved in this 5-Aza-induced increase in the ATRA response in non-APL AML cells\textsuperscript{24,30}.

In non-APL AML, several RA target genes have low occupancy of methylated H3K4. The lysine demethylase LSD1 demethylates mono- and dimethylated H3K4, thereby repressing gene expression. LSD1 is overexpressed in AML patients (http://www.proteinatlas.org/), and the inhibition of LSD1 using tranylcypromine (TCP) resensitizes non-APL AML cells for ATRA-induced differentiation\textsuperscript{21} (Table 2). TCP was able to unlock ATRA-driven gene expression and induce apoptosis and differentiation in a subset of non-APL AML cases\textsuperscript{21}. This was accompanied by an increase in H3K4me2 near the transcriptional RARA start site. Interestingly, treatment with ATRA and TCP significantly reduced the engraftment of primary AML cells in a xenograft mouse model, and the investigators have started a clinical trial (NCT02273102) to test whether this combination suppresses LSC survival and restores differentiation programs in AML patients. The results of this Phase I clinical trial are expected at the end of 2017. In an earlier preclinical study, an increase in ATRA sensitivity upon treatment with TCP was observed in an ATRA-resistant MLL-AF4-positive AML cell line\textsuperscript{24}.

**CONCLUSIONS**

Because only a subset of non-APL AML subgroups respond preclinically to ATRA and only a few patients have improved outcome upon combination treatment with ATRA and standard chemotherapy in clinical trials, the optimal biomarker or combination of biomarkers predicting ATRA sensitivity of AML cells has not yet been identified. Given the complexity of the ATRA response and the involvement of numerous candidate biomarkers (Fig. 2), it is unlikely that a single biomarker will be sufficient to identify patients who are likely to benefit from ATRA differentiation
therapy. Rather, the integration of multiple parameters, such as protein expression, (epi)genomics, and transcriptomics, may be necessary for an accurate prediction of clinical benefit using ATRA as a monotherapy. Identification of biomarkers of response might also lead to the identification of efficient ATRA-based combination therapies. Combining ATRA/ATO with chromatin-modifying agents might be a promising possibility to enhance the efficacy of ATRA-driven differentiation and apoptosis in non-APL AML cells. Moreover, it remains to be established whether ATRA-based therapies target LSCs and induce differentiation and apoptosis in relapse-initiating cells.
REFERENCES


22. Scholl, S., Müller, R., Clement, J.H., Loncarevic, I.F., Böhmer, F.D., and Höffken, K. ATRA can enhance apoptosis that is induced by Flt3 tyrosine kinase inhibition in Flt3-ITD positive cells. Leuk Res. 2006; 30: 633–642


differentiation and promotes survival of myeloid precursor cells. Cell. 1993; 74: 423–431


77. Feng, W., Yonezawa, M., Ye, J., Jenuwein, T., and Grummt, I. PHF8 activates transcription of rRNA genes through H3K4me3 binding and H3K9me1/2 demethylation. Nat Struct Mol Biol. 2010; 17: 445–450


