

Differentiation therapy of acute myeloid leukemia: past, present and future

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Purpose of review

Since the 1970s, the concept of differentiation therapy has been viewed as a promising and revolutionary approach for the treatment of acute myeloid leukemia (AML) and other cancers. However, the successful clinical application of differentiation therapy has only been realized since the late 1980s and only in one subtype of AML, acute promyelocytic leukemia (APL). The use of all-*trans*-retinoic acid (ATRA) and arsenic trioxide, both of which induce degradation of the progressive multifocal leukoencephalopathy/retinoic acid receptor α oncoprotein, in combination with chemotherapy is currently the accepted treatment of APL, presenting a potential paradigm for differentiation therapy in clinical oncology.

Recent findings

We have begun to understand why ATRA fails to induce differentiation in AML. The underlying reasons identified thus far are associated with an inability to target the removal of leukemogenic fusion proteins, aberrant epigenetic regulation of genes involved in the ATRA signaling pathway and the presence of factors that interfere with proper retinoic acid receptor α function.

Summary

Here, we examine the reasons why the exquisite sensitivity of APL to ATRA-based differentiation therapy has not been extended to other of AML subtypes. Current differentiation-based combinatorial approaches to target AML will also be analyzed. Finally, we will evaluate the potential of novel strategies, high-throughput screening, and functional genomics to uncover new differentiation-based therapies for AML.

Keywords

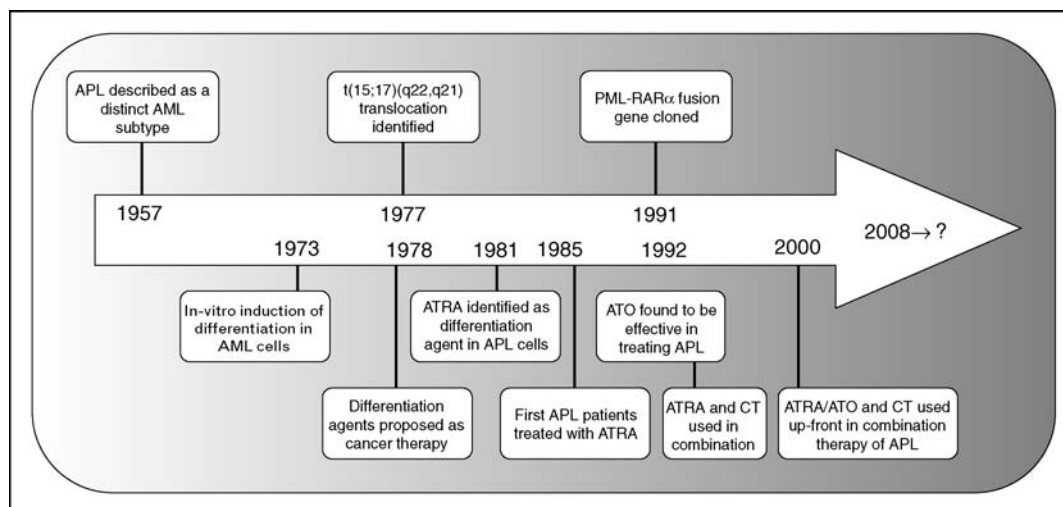
acute myeloid leukemia, all-*trans*-retinoic acid, differentiation therapy, retinoic acid receptor α

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Introduction

First described as a distinct subtype of acute myeloid leukemia (AML) over 50 years ago, [1] acute promyelocytic leukemia (APL) has now been transformed by differentiation therapy from a fatal disease into one that can be considered essentially curable [2^{••}]. The first step on this path can be traced back to the early 1970s when research demonstrated that AML cells could be induced to undergo terminal differentiation [3]. Following on from these studies, it was recognized that this process could form a basis for anticancer therapy [4], but it was not until 1980 that various compounds, among them the retinoid all-*trans*-retinoic acid (ATRA), were found to induce differentiation in an AML cell line and APL (but not other AML subtype) patient samples [5,6]. Around the same time, a research group based in Shanghai and New York [7] had started to screen for differentiation inducers (including ATRA) and this led to the first ATRA-based treatments in 1985 for APL patients har-

boring the t(15;17)(q22;q21) translocation that encodes the promyelocytic leukaemia (PML)/retinoic acid receptor α (RAR α) fusion oncoprotein [8,9]. Although ATRA, in unprecedented fashion in oncology, was able to induce complete remission as a single agent, all cases eventually relapsed [2^{••}]. In contrast, rarely occurring t(11;17)-associated APL, which expresses the promyelocytic leukaemia zinc finger (PLZF)/RAR α fusion oncoprotein, is resistant to ATRA-induced differentiation therapy due to unabated transcriptional repression [10,11]. Subsequent incorporation of induction chemotherapy into the treatment strategy significantly improved long-term patient survival [2^{••}]. In 1992, it was reported that Ailing-1, a traditional Chinese medicine containing high levels of arsenic trioxide (ATO), induced dramatic remissions in APL patients, even those that had relapsed and were resistant to ATRA treatment [12]. Differentiation therapy of APL has subsequently undergone further refinements and results from 2007 show that up-front use of ATRA/ATO plus induction chemotherapy leads to

Figure 1 A timeline of differentiation therapy in AML

Selected milestones in the history of differentiation therapy of acute myeloid leukemia (AML), and in particular its application in the treatment of acute promyelocytic leukemia (APL). Indicated on top are some events associated with the characterization of APL. Shown below are milestones associated with differentiation therapy. Interestingly, identification of all-*trans*-retinoic acid (ATRA) as a differentiation agent in APL preceded the discovery of its receptor and its role in disease pathogenesis. ATO, arsenic trioxide; CT, chemotherapy.

complete remission rates in excess of 93% with these patients achieving 5-year overall survival rates approaching 100% [2**]. Thus, we can see that it has taken a considerable period of time for differentiation therapy to reach its full potential in APL (see Fig. 1). The majority of AML cases are characterized by specific single chromosomal alterations encoding leukemogenic proteins that function as constitutive transcriptional repressors of differentiation and programmed cell death and, in common with APL, are therapeutic targets. However, from the late 1980s until recent years, clinical studies were focused on APL, and it therefore seems unsurprising that this success still remains to be reproduced in the other subtypes of AML.

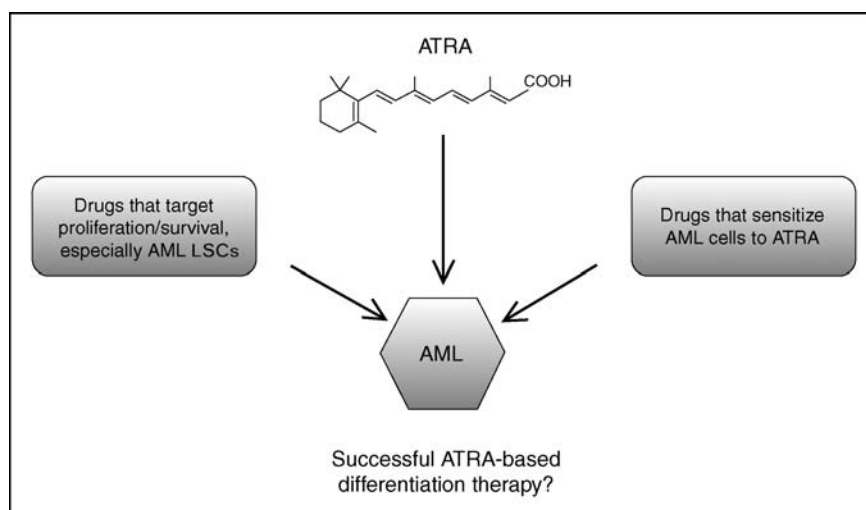
Can all-*trans*-retinoic acid form a basis for differentiation therapy in non-acute promyelocytic leukemia acute myeloid leukemia?

Given the poor results of differentiation therapy with ATRA in non-APL AML, the seemingly selective effectiveness of this drug in PML/RAR α -associated APL poses an important question as to whether the presence of this fusion protein renders this subtype of AML uniquely susceptible to ATRA treatment. A compelling argument against such a view is that from a historical perspective ATRA effectiveness in AML has been observed in the HL-60 cell line, which lacks PML/RAR α and is classified as a variant M2 subtype of AML (APL is classified as M3). Furthermore, clinical studies with ATRA in previously untreated older AML patients have

yielded some encouraging results with several clinical trials indicating ATRA effectiveness when used in conjunction with other agents such as conventional chemotherapy [13,14], or more rationally derived combinations with epi-drugs such as inhibitors of histone deacetylases (HDACi) or DNA methyltransferase inhibitors (DNMTi), or combinations of both [15–17,18*,19]. However, although it has been shown that ATRA signaling plays an important role in myelomonocytic differentiation [20,21] and should be a good target for anti-AML therapy [22], some key problems associated with the use of ATRA in the treatment of AML remain to be resolved. ATRA displays a lack of specificity for either RAR α , RAR β , or RAR γ and we still have an incomplete picture of the functional specificities of individual RARs and their isoforms in AML. We also have a poor understanding of the mechanisms by which aberrant epigenetics functionally affect ATRA signaling pathways in AML. Lastly, we have yet to define how normal crosstalk between RARs and cytokine receptor signaling is deregulated in AML.

AMLs are a heterogeneous group of diseases with different underlying molecular genetic aberrations but they can in all cases be considered to comprise distinct abnormalities that confer two properties to the leukemic cells: impaired differentiation [e.g., due to expression of PML/RAR α or AML1/Eight-Twenty-One (ETO) fusion proteins] and enhanced proliferation/survival (such as activating mutations to FLT3, RAS, or KIT) [23]. Mouse models of AML, including APL, have demonstrated that though a single mutation may impair hematopoietic

Figure 2 For ATRA-based differentiation therapy to find success in non-APL AML, treatments will need to be combined to effectively target leukemic cell proliferation/survival and restore the ATRA signaling pathway



AML, acute myelocytic leukemia; APL, acute promyelocytic leukemia; ATRA, all-*trans*-retinoic acid; LSC, leukemic stem cell.

development, contribute to expansion of the stem cell pool or lead to myeloproliferation, this is not sufficient to cause AML [23]. It therefore follows that any differentiation-based therapy that fails to contain a component dealing with leukemic cell proliferation/survival will not be effective. This problem is of critical significance in relation to targeting AML leukemia stem cells (LSCs) and obtaining molecular remission in patients (Fig. 2).

Recent research has shed light on why the ATRA/ATO combination induces and maintains complete molecular remission in APL, whereas ATRA treatment alone induces complete hematological remission, but with eventual relapse. It has become clear that in contrast to ATO, ATRA does not target APL stem cells in a therapeutically useful manner because it fails to eradicate PML/RAR α -positive LSCs and may actually promote their proliferation [24^{••}]. A key step in the process by which ATO specifically targets PML/RAR α -positive LSCs in APL has also been recently elucidated. It is a well established fact that ATO-induced proteasomal degradation of PML/RAR α is sumoylation dependent, and we now know that upon ATO treatment, PML and PML/RAR α are bound by RNF4, a ubiquitin E3 ligase that specifically interacts with polysumoylated PML via four tandem SUMO interaction motifs [25^{••},26^{••}]. The primary events through which arsenic directs this process remain to be uncovered but evidence suggests that ATO-induced PML phosphorylation plays a role, possibly as the trigger for an interaction with a SUMO E3 ligase [27]. Thus, we can see that ATRA-based differentiation therapy incorporating ATO (and induction chemother-

apy) fulfills the criteria set out above in terms of also eradicating LSCs, and remains a paradigm for treating AML.

Impairment of the all-*trans*-retinoic acid signaling pathway in acute myeloid leukemia

A key barrier to the implementation of successful differentiation therapy in AML is that, in contrast to APL, the ATRA signaling pathway in AML fails to respond to pharmacological doses of ATRA. Strategies rationally designed to overcome this problem will require a detailed picture of the underlying molecular mechanisms involved. Unfortunately, our understanding of these processes remains poor, although some progress has been made in the last few years. Aberrant epigenetics have been widely demonstrated to play an important role in cancer, including AML [28], and we now know that this can impact upon the ATRA signaling pathway through the activities of AML1/ETO, which has been found to induce abnormal DNA methylation of *RARB2*, a model ATRA target gene promoter [29^{••}]. In this example AML1/ETO recruited an array of negatively-acting epigenetic factors to *RARB2* via direct interactions with RAR. Recent research has also shown that expression of the *RARA* gene is diminished in AML in a DNA methylation-independent manner and may be due, at least in part, to a decrease in histone H3 acetylation and Lys4 (H3K4) methylation [30^{••}]. H3K27 trimethylation has also recently been linked with DNA methylation-independent gene silencing in prostate cancer [31[•]]. Although a role for the aberrant H3K27 trimethylation-associated silencing of

genes important for myeloid differentiation has yet to be established in AML, it is interesting to note that removal of this repressive mark by the JMJD3 histone demethylase occurs during ATRA-induced differentiation of neural stem cells [32*].

Aberrant epigenetics is likely to also play an important role in the ATRA insensitivity of PLZF/RAR α -associated APL. Although the treatment with a therapeutic concentration of ATRA induces degradation of PLZF/RAR α in t(11;17) cells, this is not accompanied by complete clinical remission [33]. In this case, PLZF/RAR α may recruit negatively-acting epigenetic factors that silence RAR α target genes without further requirement for the presence of the fusion protein. This notion is supported by the finding that HDACi relieve PLZF/RAR α -associated repression of RAR α target genes [34,35]. However, the role of the reciprocal fusion protein generated as a result of t(11;17), RAR α /PLZF, which upregulates expression of PLZF target genes including *CRABP1* (involved in ATRA catabolism) cannot be ruled out [36].

RAR α binds its cognate response element as a heterodimer with RXR α and, in the absence of ATRA, RAR α associates with corepressors that repress promoter activity. However, ATRA binding causes a conformational change leading to coregulator exchange and recruitment of positively acting factors that promote gene transcription [37]. In addition to potentially promoting the aberrant DNA methylation of RAR α target genes, AML1/ETO may also interfere directly with RAR α function by binding to the receptor in a ligand independent manner, thus blocking the ability of ATRA to mediate coregulator exchange and preventing activation of *RARB2* transcription [29**]. This is consistent with the finding that another AML-associated fusion protein, MN1/TEL, blocks RAR/RXR-mediated transcription by preventing the recruitment of coactivator complexes [38*]. Overexpression of MN1 is associated with some AML subtypes including inv(16) AML [39] and is also linked with a poorer prognosis and shorter survival in AML patients with a normal karyotype [40]. MN1 is a cofactor of RAR/RXR-mediated transcription and a recent study has found that MN1 can both stimulate and inhibit ATRA-induced transcription [41*]. MN1 overexpression abrogated ATRA-induced expression of a number of genes including *DHRS9*, which is involved in ATRA synthesis from vitamin A. Consistent with the notion of impaired ATRA responsiveness as a feature of AML, bone marrow transduction or transplantation experiments in mice have shown that MN1 overexpression causes myeloproliferative disease and combined expression in mouse bone marrow of MN1 and CBF β /MYH11 [the product of inv(16), which causes a differentiation block in transgenic mice] resulted in rapid development of AML [39].

Future research will surely identify other AML-associated factors that impair ATRA signaling, either through direct interactions with RAR/RXR or by affecting other components of the pathway. Restoration of ATRA signaling in AML should allow for an effective therapeutic response to this agent when used in conjunction with other targeted drugs or conventional chemotherapy (Fig. 2).

Current state of research into differentiation therapy for acute myeloid leukemia

In contrast to genetic abnormalities, which are irreversible, aberrant epigenetic modifications can be reversed pharmacologically. Therefore, epi-drugs such as DNMTi, HDACi, and inhibitors of histone methyltransferases or demethylases, have a strong therapeutic potential and these classes of enzymes represent *bone fide* targets for anti-AML drug development. There has been some progress in recent years with US Food and Drug Administration (FDA) approval granted for the demethylation agents azacitidine (Vidaza) and decitabine (Dacogen), used in the treatment of myelodysplastic syndrome, and the HDAC inhibitor SAHA (Zolinza) for therapy of cutaneous T-cell lymphoma. However, it also has to be acknowledged that from a clinical perspective, up till now epi-drugs have not yet met the early expectations placed upon them. Nucleoside analogs such as Vidaza and Dacogen need to be incorporated into genomic DNA to inhibit DNMTs and induce DNA demethylation. Unfortunately, in addition to relieving gene silencing associated with aberrant promoter hypermethylation, these drugs also exert nonspecific cytotoxic effects [42].

With regard to HDACi, a somewhat disappointing characteristic of the vast majority of inhibitors that have been developed thus far is an overall lack of selectivity towards individual HDAC family members. A potential reason for this is the finding that the region surrounding the catalytic pocket of HDAC8 actually undergoes conformational changes to accommodate structurally different HDACi [43]. This malleability, if it extends to other family members, may at least in part account for the ability of these enzymes to deacetylate diverse target proteins. There also still remains much to learn with respect to specific histone and nonhistone substrates of individual family members and target genes that they may act upon.

The HDACi valproic acid (VPA), in combination with ATRA and various other drugs including DNMTi, has been studied in several clinical trials with AML patients but this strategy has thus far had limited success [15–17,18*]. Furthermore, a note of caution has been recently introduced regarding the use of VPA and potentially other HDACi in anti-AML therapy. Although

therapeutic concentrations of VPA killed mature leukemic cells, this HDAC inhibitor enhanced the maintenance and clonogenic capacity of both normal CD34+ progenitors and also, worryingly, AML CD34+ leukemic progenitor cells [44[•]]. Although these data remain to be evaluated *in vivo* and the study has yet to be extended to other HDACi, this issue raises concerns regarding the treatment of AML with nonspecific HDACi.

To date, one genuinely specific HDAC inhibitor (tubacin), which targets HDAC6, has been identified and it may prove to have therapeutic potential in AML. Tubacin should not actually be considered an epi-drug, though, since HDAC6 does not associate with chromatin and histones are not its *in-vivo* substrate. HDAC6 can deacetylate hsp90, which leads to inhibition of its chaperone function and proteasomal degradation of hsp90 client proteins, which include Bcr-Abl and FLT3 [45]. From the perspective of novel ATRA-based combination therapies of AML, it is noteworthy that recent research has found that cotreatment with tubacin and 17-AAG (which targets the chaperone activity of hsp90 by inhibiting ATP binding) diminishes the viability of primary AML cells [46]. Encouragingly, a Class I HDAC-selective inhibitor (MGCD0103) that potently targets HDAC1 but also has inhibitory activity against HDACs 2, 3 and 11 has recently been developed [47]. Although MGCD0103 has not yet been tested in combination with ATRA, it has undergone a phase I trial with high-risk AML and myelodysplastic syndrome patients with some encouraging preliminary results [48]. Looking to the future as the biological activities of individual HDACs are uncovered and novel HDACi with improved specificity continue to be developed, these agents have the potential to be successfully used combinatorially in anti-AML differentiation therapy.

The potential roles of histone methyltransferases and demethylases in AML are still poorly understood but, in contrast to HDACs, these enzymes display a high degree of substrate specificity making them ideal candidates for drug development [49]. To date, relatively few compounds have been identified but include inhibitors that target enzymes responsible for H3K27 methylation [50,51] and H3K4 demethylation [52–54].

So far in this review, we have focused on factors that affect the ATRA signaling pathway on the genomic level in terms of epigenetic dysregulation of ATRA target genes and impairment of RAR α -mediated transcription activation. However, aberrant signal transduction also plays an important role in AML and APL by promoting proliferation or survival of LSCs; as mentioned earlier, kinase signaling is required for ATO targeting of PML/RAR α in APL. For an up-to-date review of this topic see Scholl *et al.* [55]. Signal transduction pathways activated by ATRA

have also been found to play an important role in modulating its effects on differentiation in APL cells [56]. There is also significant, although incompletely understood, cross-talk between ATRA and myelomonocytic growth factors (GFs) with recent research showing that acting, at least in part, via the MAP kinase pathway, GFs enhance ATRA-dependent activation of RAR α and maturation of APL and non-APL AML primary cells [20]. These results suggest that combinatorial use of these agents may be effective in differentiation therapy of non-APL AML.

Perspectives on the future development of anti-acute myeloid leukemia differentiation therapies

Although this review has focused on the prospects for differentiation therapy in AML utilizing ATRA as the differentiating agent, it should be noted that another likely problem underlying the lack of success with ATRA in AML is that this retinoid is not RAR isotype-selective. Studies from human cell lines and mouse models clearly demonstrate that ATRA acts through RAR α to induce differentiation [20,21], whereas its effects via RAR γ are antidifferentiative and expand hematopoietic stem cells [57,58]. Therefore, the use of RAR isotype-selective synthetic retinoids, both agonists and antagonists, could lead to improved clinical results [22].

Functional genomic strategies and high-throughput small compound screening will play a critical role in the discovery of novel differentiation-based therapies for AML. For example, a functional genomic RNAi screen using a library of 8500 shRNAs identified a ubiquitin-conjugating enzyme (UBE2D3) as a mediator of ATRA-induced growth arrest in NB4 APL cells [59[•]]. Also, a high-throughput study that screened around 6000 compounds for their ability to induce differentiation in HL-60 cells recently identified 6-benzylthioinosine as a candidate drug [60[•]]. Rather than targeting the ATRA-mediated differentiation pathway, this agent may act to induce growth arrest and differentiation through depletion of cellular ATP stores and, promisingly, impairs tumor growth in mice. Rationally targeted small-scale drug screens can also yield results and in another recent publication this approach identified an inhibitor of glycogen synthase kinase 3 (SB216763) as an agent active against *MLL* leukemia cells [61]. SB216763 was found to induce G1 arrest in both B cell and myeloid progenitors transformed by *MLL* oncogenes. In the future, these types of study could also identify small molecules that sensitize AML cells to the effects of ATRA or retinoids.

In analogy to the specific induction of PML/RAR α degradation by ATRA and ATO in APL, the diterpenoid analogues ericalyxin B and oridonin have recently been found to specifically degrade AML1-ETO [62,63]. ATO

itself may have applications in the treatment of non-APL AML because it can also induce the targeted degradation of the AML1/MDS1/EVI1 (AME) oncoprotein [64]. Also of note is the finding that wild-type PML, which like PML/RAR α is a target of ATO, plays a vital role in maintaining the survival of LSCs in chronic myeloid leukemia (CML) [65]. ATO treatment of LSCs in a mouse model of CML significantly diminished the capacity of these cells to recapitulate the disease when transplanted into recipient mice. These results, along with the development of novel organic arsenic compounds [66], could see the emergence of applications for this semimetal in AML.

Another exciting line of investigation that has potential for development as an anti-AML therapy in combination with ATRA utilizes peptides or small molecules that block specific interactions between oncoproteins and factors required for their leukemic activity. For example, in APL, peptides targeting the interface between PML/RAR α and NCoR or SMRT have been found to restore ATRA sensitivity to differentiation-resistant NB4 cells [67]. Also, a screen for compounds that enhance ATRA-induced differentiation of leukemic cells identified benzodithiophenes as facilitating the removal of RAR α repressor complexes by lowering the threshold for ligand-mediated corepressor or coactivator exchange with RAR α and enhancing changes in ATRA-regulated gene expression [68,69]. There has also been development of small molecule inhibitors that disrupt the interaction between AML1 and CBF β , thus enabling AML1/ETO-positive Kasumi and SKNO-1 cells to differentiate in response to ATRA [70,71]. Probably the best known inhibitor of protein–protein interactions is nutlin-3, which binds MDM-2 and prevents it from interacting with p53, releasing it from negative control by MDM-2 and leading to effective p53 stabilization and activation [72]. Treatment of AML patient samples with nutlin-3 induces both apoptosis and differentiation [73] and while nutlin-3 potentiates the effects of TRAIL, it has not yet been tested with ATRA or other retinoids.

In summary, this review has underlined the importance of developing new and better differentiation-based combinatorial therapies that can be targeted against specific abnormalities underlying the pathogenesis of a given AML subtype, or possibly take advantage of characteristics shared by different AMLs. Progress toward achieving these ends is going to come from both high-throughput techniques and rationally-designed research based on improved knowledge of the biology of AML.

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