Differentiation Therapy of AML: Past, Present and Future

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Purpose of review

Since the 1970's, the concept of differentiation therapy has been viewed as 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 1980's and only in one sub-type on AML, acute promyelocytic leukemia (APL). The use of all-*trans*-retinoic acid (ATRA) and arsenic trioxide (ATO), both of which induce degradation of the PML/RARα oncoprotein, in combination with chemotherapy is currently the accepted treatment for 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 thus far identified 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 RARα 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, differentiation therapy, all-trans-retinoic acid, retinoic acid receptor α

Abbreviations

AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ATRA	all-*trans*-retinoic acid
DNMTi	DNA methyltransferase inhibitor
HDACi	histone deacetylase inhibitor
VPA	valproic acid

Introduction

Since it was first described as a distinct sub-type of acute myeloid leukemia (AML) over 50 years ago, [1] differentiation therapy of acute promyelocytic leukemia (APL) has transformed 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 seventies 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 anti-cancer 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 sub-type) 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 harboring the t(15;17)(q22;q21) translocation that encodes the PML/RARα 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 express the PLZF/RARα fusion oncoprotein, are 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 Ailing-1, a traditional Chinese medicine containing high levels of 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 complete remission rates in excess of 93% with these patients achieving five-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 Figure 1). The majority of AML 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 1980's 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 AML.

Can ATRA form a basis for differentiation therapy in non-APL AML?

Given the poor results of differentiation therapy with ATRA in non-APL AML, the seemingly selective effectiveness of this drug in PML/RARα-associated APL poses an important question as to whether the presence this fusion protein renders this sub-type 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/RARα and is classified as a variant M2 subtype of AML (APL is classified as M3). Furthermore, clinical studies with ATRA in previously untreated older patients with AML have yielded some encouraging results and several clinical trials have indicated ATRA effectiveness when used in combination with other agents such as conventional chemotherapy [13,14] or more rationally derived combinations with epi-drugs such as inhibitors of histone deacetylases (HDACi) and/or DNA methyltransferases (DNMTi) [15-18•,19]. However, while 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 anti-AML therapy remain to be

resolved. ATRA displays a lack of specificity for either RARα, -β, or -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 cross-talk between RARs and cytokine receptor signaling is deregulated in AML.

AMLs are a heterogeneous group of diseases with different underlying molecular genetic abnormalities but they can in all cases be considered to comprise distinct abnormalities that confer two properties to the leukemic cells: impaired differentiation (for example due to expression of PML/RARα or AML1/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 while a single mutation may impair hematopoietic 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 (LSC) and obtaining molecular remission in patients (Figure 2).

Recent research has shed light on why the ATRA/ATO combination induces and maintains complete molecular remission in APL, while 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 since it fails to eradicate PML/RARα-positive LSC and may actually promote their proliferation [24]••. A key step in the process by which ATO specifically targets PML/RARα-positive LSC in APL has also recently been elucidated. It is a well-established fact that ATO-induced proteasomal degradation of PML/RARα is sumoylation dependent and we now know that upon ATO treatment, PML and PML/RARα 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 chemotherapy) fulfills the criteria set out above in terms of also eradicating LSC and remains a paradigm for treating AML.

Impairment of the ATRA signaling pathway in AML

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

RARα binds its cognate response element as a heterodimer with RXRα, and in the absence of ATRA RARα associates with co-repressors that repress promoter activity. However, ATRA binding causes a conformational change leading to co-regulator exchange and recruitment of positively acting factors that promote gene transcription [37]. In addition to potentially promoting the aberrant DNA methylation of RARα target genes, AML1/ETO may also interfere directly with RARα function by binding to the receptor in a ligand independent manner, thus blocking the ability of ATRA to mediate co-regulator 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 worse prognosis and a shorter survival in AML patients with a normal karyotype [40]. MN1 is a co-factor 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/transplantation experiments in mice have shown that MN1 overexpression causes myeloproliferative disease, and combined expression in mouse bone marrow of MN1 and CBFβ/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 (Figure 2).

Current state of research into differentiation therapy for AML

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/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 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 in order to inhibit DNMTs and induce DNA demethylation. Unfortunately, in addition to relieving gene silencing associated with aberrant promoter hypermethylation, these drugs also exert non-specific 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 non-histone 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 [15-18•]. Furthermore, a note of caution has been recently introduced regarding the use of VPA and potentially other HDACi in anti-AML therapy. While therapeutic concentrations of VPA killed mature leukemic cells, this HDACi 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 non-specific HDACi.

To date one genuinely specific HDACi (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 co-treatment with tubacin and 17-AAG (which targets the chaperone activity of hsp90 by inhibiting ATP binding) diminishes the viability of primary AML cells [46]. A Class I HDAC-selective inhibitor has been recently developed, MGCD0103, which potently targets HDAC1 but also has inhibitory activity against HDACs 2, 3 and 11 [47]. While MGCD0103 has not yet been tested in combination with ATRA, it has undergone a Phase I trial with high-risk AML and MDS patients with some encouraging preliminary results [48]. Looking to the future as the biological activities of individual HDACs are uncovered and novel, more specific, HDACi 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 RARα-mediated transcription activation. However, aberrant signal transduction also plays an important role in AML and APL by promoting proliferation/survival of LSC – as mentioned earlier, kinase signaling is required for ATO targeting of PML/ RARα 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 reseach showing that acting, at least in part, via the MAP kinase pathway, GFs enhance ATRA-dependent activation of RARα 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-AML differentiation therapies

While 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 RARα to induce differentiation [20,21] whereas its effects via RARγ are anti-differentiative 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 recent 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 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 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/RARα degradation by ATRA and ATO in APL, the diterpenoid analogues Eriocalyxin 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 since 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 is also a target of ATO, plays a vital role in maintaining the survival of LSC in chronic myeloid leukemia (CML) [65]. ATO treatment of LSC 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 semi-metal 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/RARα 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 indentified benzodithiophenes as facilitating the removal of RARα repressor complexes by lowering the threshold for ligand-mediated corepressor/coactivator exchange with RARα 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 CBFβ, 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 sub-type, or possibly take advantage of characteristics shared by different AMLs. Progress towards 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.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- ● of special interest
- ● ● of outstanding interest

● ● [2] This is a comprehensive review charting the history of differentiation therapy in APL.

● [18] This clinical study indicates that combining ATRA with decitabine and valproic acid can be beneficial the treatment of non-APL AML.

● ● [24] This study shows that arsenic trioxide but not ATRA can overcome the aberrant stem cell capacity of PML/RARα-positive leukemic stem cells.

● ● [25,26] These two studies identify PML as an *in vivo* target of the RING finger ubiquitin E3 ligase RNF4, which specifically binds polysumoylated PML via four tandem SUMO interaction motifs and is essential for the arsenic-induced catabolism of both PML and PML-RARα.

● ● [29] This study suggests that the retinoic acid signaling pathway is blocked by AML1/ETO. This is because the fusion oncoprotein recruits negatively acting epigenetic factors to RARα target gene promoters and offers an explanation as to why AML1/ETO-associated AML does not respond to ATRA.

● ● [30] This study demonstrates that loss of *RARA* expression in primary AML cells is not associated with promoter hypermethylation, but rather diminished levels of histone H3 acetylation and lysine 4 methylation. These results highlight for the first time existence of DNA methylation independent and histone code driven gene-silencing mechanisms in cancer pathogenesis.

● [31] This study demonstrates that H3K27 trimethylation is associated with DNA methylation-independent gene silencing in prostate cancer.

● [32] JMJD3 functions as a trimethyl H3K27 demethylase and is ATRA-regulated during neural cell differentiation. This indicates that H3K27 methylation plays a role in the myeloid differentiation and possibly AML.

● [38] The MN1-TEL myeloid leukemia-associated fusion protein inhibits RAR-RXR-mediated transcription in response to ATRA signaling.

● [41] MN1 can interfere with the ATRA pathway by blocking expression of ATRA target genes. MN1 is overexpressed some AML subtypes and this may contribute to the lack of ATRA responsiveness in these cases.

● [44] This study highlights a potential problem with use of valproic acid and possibly other non-selective histone deacetylase inhibitors in the treatment AML as it causes proliferation of leukemic progenitor cells.

● [59] This is the first example of the use of functional genomics to screen for factors that influence ATRA-induced differentiation.

● [60] This study carried out a small molecule differentiation screen with over 6000 compounds and identified 6-benzylthioinosine as an agent that can induce differentiation in AML.

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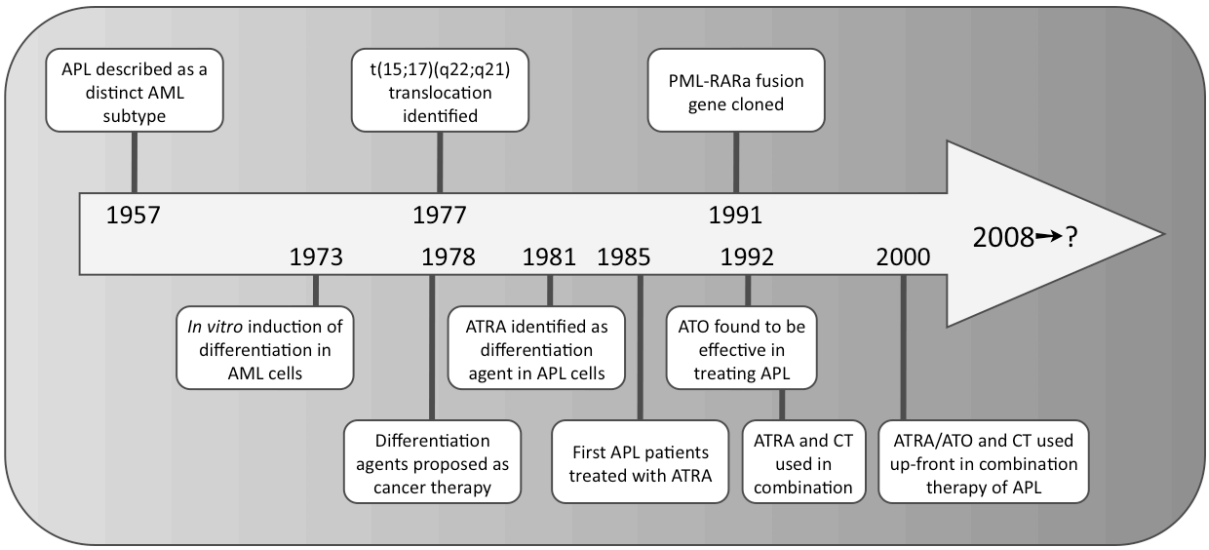

Figure 1. A timeline of differentiation therapy in AML. Selected milestones in the history of differention therapy of AML, and in particular its application in the treatment of APL. Indicated on top are some events associated with the characterization of APL. Shown below are milestones associated with differentiation therapy. Interestingly, identification of ATRA as a differentiation agent in APL preceeded the discovery of its receptor and its role in disease pathogenesis. ATO, arsenic trioxide; CT, chemotherapy.

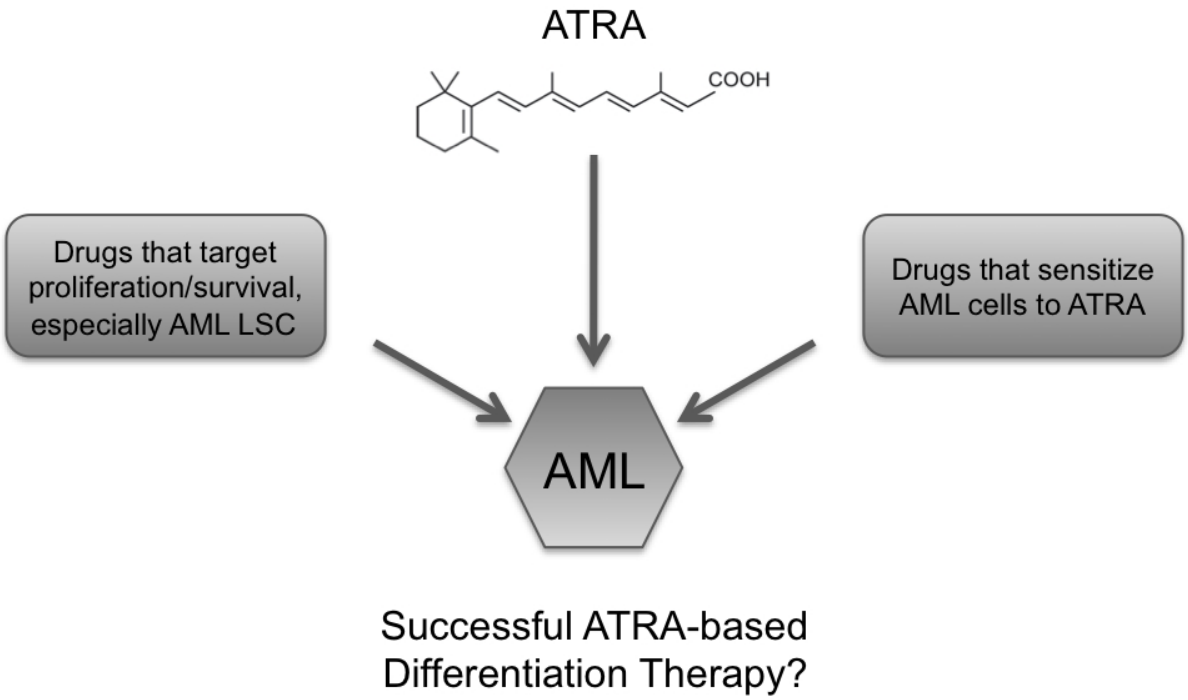

Figure 2. For ATRA-based differentiation therapy to find success in non-APL AML, treatments will need to be combined to effectively target leukemic cell proliferation/survival and restore the ATRA signaling pathway. LSC, leukemic stem cell.