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REVIEW



# Acute myeloid leukemia: therapeutic targeting of stem cells

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**ABSTRACT**

**Introduction:** Despite advances in the treatment of acute myeloid leukemia (AML), long-term survival remains low. In 1994, it was proposed that leukemic stem cells (LSCs) played a key role in relapsed and refractory disease. LSCs are capable of self-renewal, proliferation, differentiation, immune evasion, and drug resistance through several unique mechanisms. More recent leukemia drug development initiatives have included efforts to target LSCs. With LSCs, the challenge with such drug design is finding a way to selectively target LSCs while sparing normal hematopoietic stem cells (HSCs).

**Areas covered:** In this review, we explore the evolving knowledge of the unique LSC biology and physiology in the scientific literature, while noting the several agents that have been designed throughout the years to target this subgroup of leukemic cells. Our review includes discussion on chimeric antigen receptor T cells, monoclonal antibodies, antibody-drug conjugates against cell surface markers, signaling pathway targets, pro-apoptotic agents, epigenetic regulators, and more.

**Expert opinion:** As our understanding of the intricate pathophysiology of LSCs continues to grow, it is clear that targeting such heterogenous cells successfully will require a thoughtful and multi-modal approach.

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Leukemic stem cell; acute myeloid leukemia; targeted therapy; hematopoiesis

## 1. Introduction

Historically, the treatment of AML has consisted of induction chemotherapy with an anthracycline and cytarabine. Despite the advent of targeted therapies, relapse and progression of AML continue to be a challenge. Specifically, the five-year survival remains lower than desired at 29.5% [1]. Traditionally, AML relapse has been associated with residual disease following induction therapy [2–6]. Our knowledge regarding this subgroup of resistant residual cells continues to evolve. In 1994, Dick et al. characterized these cells as leukemic stem cells (LSCs) [7]. Dick and colleagues would later demonstrate that AML is organized in a hierarchical fashion, being driven by LSCs, similar to the normal hierarchy of hematopoiesis described by Metcalf in the 1960s [8,9]. Furthermore, LSCs were found to masquerade with a similar immunophenotype (CD34+ CD38-) as that of normal adult hematopoietic stem cells (HSCs), but with the potential of propagating malignant cells [10]. LSCs possess the ability for self-renewal, proliferation, differentiation, and drug evasion (Figure 1) [11]. At time of relapse, there is a 10-to-100-fold increase in LSCs [12]. As such, LSCs can give rise to recurrent AML and thus cure should ideally entail treatment capable of eradication of LSCs while sparing HSCs [13]. The extent to which LSCs influence leukemogenesis continues to be a controversial and highly studied topic. The notion that LSCs may play an important role in relapse and refractory disease has guided the direction of AML research to include the search for a deeper understanding of the LSC and development of therapeutic strategies targeting these residual leukemic cells. The shared features of LSC with normal HSCs or other blood cell markers have largely impeded the progress in targeted therapeutics. In this review, we expand on the variety of agents being developed for the treatment of AML with the specific intent of eliminating the LSC population. Importantly, we will focus on targets that are relevant for LSC interaction with their niche. Furthermore, we will address some of the purported metabolic vulnerabilities that may distinguish LSCs from normal or non-LSC leukemic cells.

## 2. Targeted therapeutics

### 2.1. Extracellular proteins and the bone marrow niche

LSCs overexpress several unique markers on their cell surface that provide attractive targets for drug design. This section will focus on the introduction of several LSC-specific markers, leading up to the discussion on CAR-T and BiTE therapies, perhaps some of the most promising anti-LSC therapeutics in present day.

In 2010, Saito et al. discovered that CD32 (Fcγ receptor II) and CD25 (IL-2 receptor) were highly expressed on LSCs [14]. Majeti would then go on to demonstrate that monoclonal antibody therapy directed against these markers did not impact normal hematopoiesis [15]. This would pave the way for cell surface antigen targeted therapies.

CD33 is a notable extracellular marker observed on a subset of LSCs. In the case of LSCs, CD33 plays a role in evasion of normal immune functions. A frequently used anti-leukemic agent, gemtuzumab ozogamicin (GO), utilizes an anti-CD33 antibody conjugated to an antitumor antibiotic that has

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**Article highlights**

- Effective treatment of acute myeloid leukemia (AML) requires successful targeting of leukemic stem cells (LSCs).
- From the emerging therapies, Chimeric antigen receptor (CAR) T cells and bispecific T-cell engager molecules (BiTEs), targeting LSC antigens such as Tim3, CLL-1, CD123 and CD70, seem the most promising.
- Additional agents exploit the relationship between LSCs and the bone marrow niche to prevent adhesion.
- Intracellular pathways vital for LSC survival serve as areas for further drug development, including JAK-STAT, NF-kB, PI3K/Akt/Nrf2, Notch, Hedgehog, and Wnt-β-catenin signaling.
- LSC dependence on oxidative phosphorylation functions as an additional target with specific attention to mitochondrial enzymes.

shown promise in the treatment of AML. While initially withdrawn from the market because of toxicities, it was reapproved by the FDA in 2017 at lower doses for relapsed/refractory or new cases of CD33+ AML and is also used in high-risk APL [16]. GO has a particular efficacy in core-binding factor leukemia (CBF) where CD33 is overexpressed and its addition to intensive-therapy backbone has increased the estimated five-year survival in this subgroup from 50% to 75% [17,18]. While this notes a marked improvement in survival, the remaining group with persistent disease despite GO suggests that targeting CD33 is insufficient in eradicating the LSC population. Another CD33-directed therapy designed was Vadastuximab talirine (SGN-33A), which conjugated the DNA binding agent pyrrolobenzodiazepine to CD33. This was under clinical study in combination with azacitadine or decitabine (NCT02785900), but trial was terminated early due to side effect profile, specifically higher rate of deaths secondary to infections.

CD123 was the first marker believed to be LSC-specific. It comprises the alpha chain of the interleukin-3 receptor (IL-3Rα) and regulates cell growth, proliferation, cell survival, and differentiation of HSC. Jordan and colleagues identified a strong presence of CD123 in the CD34+/CD38- LSC population in comparison to virtually no CD123 in the CD34+/CD38- HSC population [19]. Several anti-IL-3 receptor antibodies, bispecific immunoglobulins, and even diphtheria toxin fusion proteins have been developed in efforts to target CD123. Talacotuzumab (a humanized anti-CD123 monoclonal antibody) in combination with decitabine was compared to decitabine alone in patients with AML ineligible for intensive chemotherapy, with no statistically significant difference in overall survival [20]. Studies would later reveal that CD123 is actually expressed in various hematopoietic malignancies including AML, B cell acute lymphocytic leukemia (B-ALL), and in blastic plasmacytoid dendritic cell neoplasm (BPDCN) [21]. Testa and colleagues would later evaluate CD123 expression in AML, noting that up to 45% of patients in their study population overexpressed CD123 [22]. As such, this was largely abandoned as an LSC-specific target.

In addition to the above, other studied extracellular LSC markers include CD44, CD47, TIM3, CD96, CD99, CLL-1, CD32, CD25, IL1RAP, GPR56, and CD93 [23]. Similar to HSCs, LSCs require cellular interaction with the bone marrow niche to sustain its stem cell properties. Tavor et al. demonstrated the delicate interplay between CXCR4 and SDF-1α for growth of LSCs within trabecular bone [24]. It was described that interactions between CXCR4 and CXCL12 were key players in the retention of these progenitor cells. Additionally, chemokine interactions through CXCL12 upregulated vascular cell adhesion molecule 1 (VCAM-1) and very late antigen 4 (VLA-4) to further stabilize the LSCs within the bone marrow niche [25–27]. Thus,

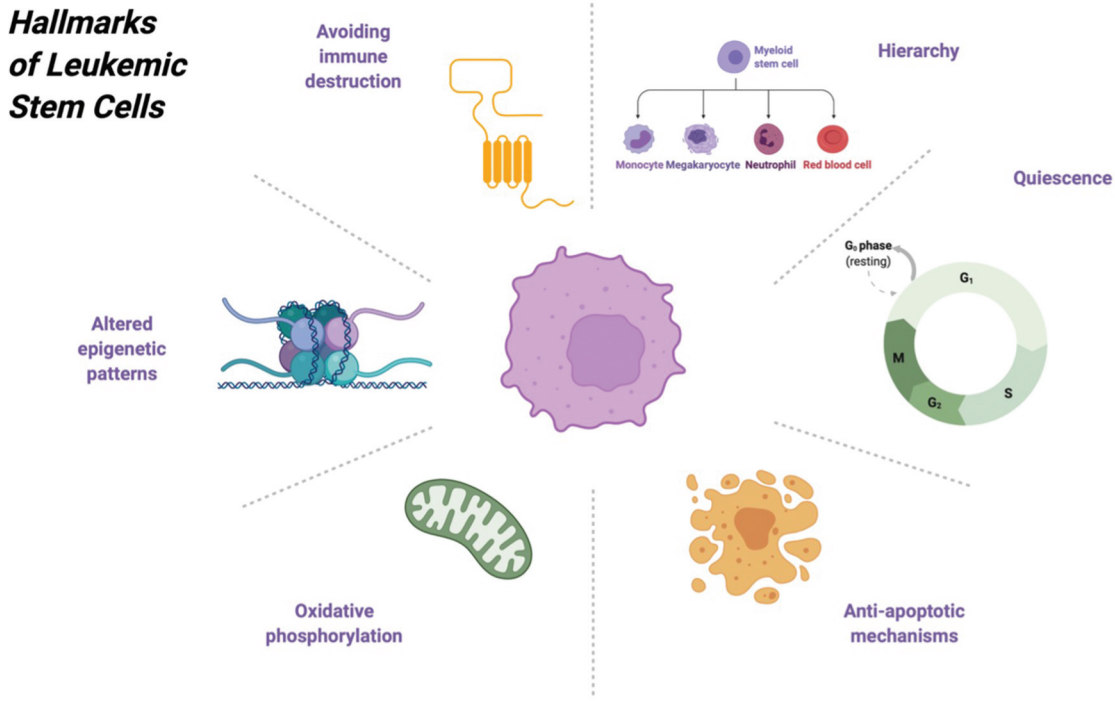


Figure 1. Leukemic stem cell properties.

the interaction of the LSC with their niche is crucial for their support and propagation. Not surprisingly, targeting some of these proteins that may disrupt the LSC-niche interaction is an area of ongoing investigation.

More recently, CLL-1C-type lectic domain family 12 (also known as CLL-1 and CLEC12A) has been noted in pre-clinical studies to contribute to high tumorigenicity [28]. LSCs and blasts express CLL-1, whereas normal HSCs do not [29]. Van Rhenen and colleagues have found that CLL-1 can be identified and quantified on LSC in patients at diagnosis and following chemotherapy, which makes it ideal to serve as a marker of minimal residual stem cell disease (MRD) [13,30]. The group demonstrates that high LSC frequency at diagnosis is correlated with high MRD frequency after chemotherapy and poor survival. Jiang et al. reported that CLL1-antibody-drug conjugate (CLL1-ADC) could become an effective agent in the targeting of LSCs in AML [31]. Other transmembrane proteins being evaluated for potential drug development in AML include CD96, CD70 and CD99. A common theme in these markers is their role in cell survival and mobilization, suggesting potential off-target effects if such proteins coexist in healthy HSCs.

CD44 is another transmembrane protein that has been highlighted as key for LSC adhesion and interaction with the bone marrow niche [27]. Certain variants of CD44, such as CD44v6, have been associated with poor prognosis in AML [32]. Activating CD44-specific monoclonal antibody H90 was studied in non-SCID mice with AML in 2004, revealing inhibition of LSCs in a dose-dependent manner [33]. Recombinant anti-CD44 RG7356 was trialed in phase 1 studies for relapsed/refractory AML and while generally safe and well tolerated, did not reveal substantial benefit in progression-free survival [34]. More recently, Yu and colleagues demonstrated that CD44 inhibition led to increased sensitivity to venetoclax and eradicating LSCs [35]. Further studies are needed to demonstrate the anti-leukemic efficacy of anti-CD44 agents especially in combination with venetoclax-based therapy may reveal better results than single-agent anti-CD44 therapy.

LSCs' capability of evading chemotherapeutic agents has been explored in the context of membrane proteins such as ATP-binding cassette transporters as well as in drug resistance genes such as ABCC1/LRP. Adjunctive agents such as salinomycin have been trialed in traditional cytotoxic regimens to help overcome chemoresistance in LSCs with early success and further studies warranted to determine clinical effectiveness [36].

Immune evasion by overexpression of checkpoint inhibitors has also been described in LSCs. For instance, T-cell immunoglobulin mucin-3 (TIM3), a marker on innate immune cells, are highly expressed on LSCs than HSCs, serving as a negative regulator of Th1 T cell immunity [37]. TIM3 expression is even higher in AML associated with core-binding factor translocations or mutations in CEBPA which suggest a potential role in chemoresistance; further investigation is required to fully understand the role of TIM3 in leukemogenesis [38].

Chimeric antigen receptor (CAR) T cells have been designed as a further means of targeting frequently seen epitopes on LSCs such as CD123. In 2013 Mardiros et al. demonstrated that CD123 CAR T cells were able to eliminate cells expressing CD123 in vitro through multiple pathways as evidenced by upregulation of CD107a and production of both IFN-γ and TNF-α [39]. Further leukemic epitopes that may be of interest in the design of CAR T cells include CD33, MUC1, folate receptor β, and TIM-3. However, careful attention must be directed toward off-target effects on cells that may also express such epitopes such as CD33 in hepatic Kupffer cells and T cells [39,40]. Further work has been completed to reduce such unwanted effects on non-leukemic cells. In 2021, El Khawanky et al. developed a third-generation anti-CD123 CAR T cell with humanized CSL362-based recombinant antibody and a CD28-OX40-CD3ζ intracellular signaling domains which was effective in anti-leukemic effects without impacting healthy cells [41]. Interestingly, this group also demonstrated that hypomethylator 5'-Azacitadine increases anti-CD123 CAR T cell numbers, potentiating cytotoxic effects and suggesting potential synergy between a subset of hypomethylating agents and CAR T cells in the treatment of leukemia [41].

With the advent of CAR-T cells, CAR-NK cells have also been explored in the setting of hematologic malignancies. MD Anderson Cancer Center pioneered the first-phase I/IIa study using cord blood-derived CAR-NK cells targeting CD19, resulting in 73% response rate in patients with relapsed/refractory non-Hodgkin's lymphoma and chronic lymphocytic leukemia, prompting further investigation of CAR-NK cells in other malignancies such as AML. Multiple CAR-NK preclinical and clinical studies are underway targeting various LSC-antigens such as CD33, CLL-1, TIM-3, and CD123.

In addition to CAR-T cells for immune-based therapy to target LSC, there has also been much promise in bispecific T-cell engager molecules (BiTEs), which promote T cell destruction of tumor cells through recognition of specific cell-surface tumor antigen. Success with BiTE therapy has been evidenced with Blinatumumab in B-cell acute lymphoblastic leukemia [42]. In 2022, Arruda and colleagues demonstrated that CD34-specific BiTE can result in CD-34 dependent T cell activation, targeting and eliminating CD34+ blasts and LSCs in a dose-dependent manner [43]. AMG-330 is a CD33-specific BiTE antibody that has shown promising results in vitro and in mouse models [44,45]. This has expanded into a multicenter phase 1 study in patients with relapsed and refractory AML with results pending. While CD123 was noted above to not be specific to LSCs, the acknowledgment of its presence in several hematologic malignancies has led to interest in the development of BiTEs targeting CD123. Such agents include XmAb14045, JNJ-63709178, and dual-affinity retargeting (DART) molecule flotetuzumab (MGD006) [46]. In ESMO 2017, it was presented that flotetuzumab has anti-leukemic activity in patients with relapsed/refractory AML. Subsequently, in 2021, results from the first phase 1/2 flotetuzumab study indicated that this BiTE served as an efficacious and novel treatment option for primary induction failure and/or early relapse AML, with complete remission (with or without hematologic recovery) rates of 27% and ORR of 30% [47]. Early data suggests BiTE-based therapy may be a feasible and quite promising tool in eradicating leukemic cells, and as such this continues to be an exciting and developing field within leukemia research.

### 2.2. Signaling

Similar to CD44, CXC chemokine ligand 12 (CXCL12, also known as stromal cell-derived factor-1) and its receptor CXCR4 were thought to be exciting targets for inhibiting the LSC interaction with the niche [48]. However, further investigation of CXCL12/CXCR4 interactions revealed that the likely mechanism by which it contributed most to leukomogenesis was through activation of signaling pathways that are key to cell survival, including MEK/ERK, JAK-STAT, and PI3K/AKT [49]. Bortezomib, a proteasome inhibitor used for multiple myeloma and mantle cell lymphoma, has been shown to decrease CXCL12 production in a dose-dependent manner, inhibiting the migration of AML blasts to CXC12 [50]. Unfortunately, phase II studies with bortezomib in combination with other cytotoxic regimens for AML did not show promising results [51]. Another CXCR4-directed agent, AMD3465 revealed enhanced apoptotic-effects on FLT-3 mutated AML when combined with a FLT-3 inhibitor in murine studies [49]. By blocking CXCR4, AMD3465 also contributed to reduced PI3K/AKT and MAPK survival pathways, both of which mediate the mobilization of LSCs to a more chemosensitive state within the microenvironment [52]. Plerixafor (AMD3100), another CXCR4 antagonist, was explored in phase I and II clinical trials with limited success [53]. Borthakur and colleagues investigated plerixafor with addition of sorafenib and G-CSF, hypothesizing modifications in the bone marrow microenvironment that would produce a heightened disease response to the CXCR4 inhibitor [54]. They found an improvement in response, with 36% of patients with relapsed/refractory FLT3-ITD-mutant AML achieving complete and/or partial response. Such findings emphasize the role that extracellular processes such as the tumor microenvironment may also play in response to therapy.

Nevertheless, intracellular pathways continue to be a large focus in the development of agents targeting LSCs. JAK-STAT signaling activates STAT proteins that are important in the transcription of genes, cell division, and cell survival [55]. In LSCs, JAK-STAT activates STAT3, allowing for uncontrolled malignant cell proliferation [56]. OPB-111077 is a novel STAT3 inhibitor that has been clinically tested on a variety of cancers with ongoing trials in AML (NCT03197714; NCT03063944). STAT5 phosphorylation has also been noted in leukemic stem cells and may serve as an additional target for investigation [57]. Within the JAK family, JAK2 inhibitors have also been proposed as potential therapeutics for AML with the theory that it targets leukemic stem cells as such directed therapies have specifically shown benefit in a subset of patients with relapsed/refractory AML [58].

In AML, activating mutations of FLT3 have been associated with a worse prognosis. Both, mutated and wildtype FLT3 can activate MAPK and PI3K signaling pathways, adding to cell proliferation. Mutated FLT3-ITD additional can lead to abnormal activation of STAT5, a key protein in leukemic stem cell proliferation and oncogenesis [57]. Gilteritinib is the first FLT3 inhibitor approved for relapsed/refractory AML. Several other FLT3 tyrosine kinase inhibitors are presently in trial. It is proposed that FLT3 inhibitors specifically induce leukemic stem cell differentiation, thus expelling them from their quiescent state and allowing for a more vulnerable target [59]. Thus far, these agents have shown to be most effective in FLT3-ITD but not FLT3-TKD AML. This poses the question as to whether the TKD mutation contributes to chemoresistance in leukemic stem cells, an area of active research.

### 2.3. Metabolism

It has been well studied that LSCs preferably rely on oxidative phosphorylation, thriving in an environment with low oxygen tension [60–62]. This has been coined as the 'Reverse Warburg Effect.' [63] Additionally, it has been recently reported that leukemia cells halt the activation and metabolism of T cells, essentially paralyzing T cells in close proximity, so as to promote oncogenesis while evading the immune system. Such feat is achieved through the release of lactic acid [64]. In such conditions, the cell cycle is halted in the G0/G1 phase, with upregulation of p27 and decrease of the S phase, granting LSCs their quiescent quality that allows them to evade traditional cytotoxic regimens [65]. Within the oxygen-deprived environment, LSCs release several cytokines and altered intracellular signaling mechanisms with activated PI3K/AKT, activated MEK-ERK, and increased expression of XIAP that contribute to anti-apoptotic effects. Angiopoietin-1 (ANG1) and the receptor tyrosine kinase Tie2 contribute further to LSC quiescence and serve as protection against myelotoxic stressors [66]. These peculiar findings of LSC metabolism suggest that LSCs may harbor metabolic vulnerabilities that can be leveraged to overcome relapse. Thus, pharmacologic targeting of tricellular signaling pathways has been focused on disruption of mitochondrial activity to result in LSC-specific damage [67]. Dihydroorotate dehydrogenase (DHODH) is within the inner mitochondrial membrane and required for DNA synthesis [68,69]. Preclinical studies with Brequinar sodium (BRQ), a DHODH-inhibitor, showed selective reduction in LSCs [68]. Several DHODH-directed therapeutics are actively being studied in phase I and phase II trials (ASLAN003 – phase II NCT03451084; BAY2402234 – phase I NCT03404726, and PTC299 – phase 1 NCT03761069). Neprolysin, a mitochondrial enzyme known to be overexpressed in stem cells with oncogenic properties, is also under current investigation [70]. Beyond mitochondrial oxidative phosphorylation, leukemic stem cells thrive through fatty acid oxidation as well [71,72]. This has been proposed as a mechanism for drug resistance and provides an opportunity for future areas of drug development specific to LSC survival [73,74].

### 2.4. Quiescence and apoptosisa

Leading hypothesis as to why LSCs are chemoresistant is their predominantly quiescent state [75]. Protein kinase CK2, which is overexpressed in LSCs, regulates PI3K/Akt, JAK/STAT, and NF-kB signaling and inhibits apoptotic pathways to maintain tumor growth [76]. CK2 inhibitor CX4945 forces LSCs out of their quiescent G0 state and induces cell cycle arrest in the late S/G2/M phases [76,77]. While only preclinical research of CK2 inhibitors in AML exist so far, the notion of an agent that targets multiple pathways critical to LSC survival shows much

promise in the development of AML therapeutics [76]. Additional agents that may potentially force LSCs out of quiescence include inhibitors of X-linked IAP such as dequalinium chloride. Such inhibitors are able to induce blockage of cell S-phase entry and preferentially promote the differentiation of LSCs, suggesting an opportunity to expose them to cytotoxic regimens more readily [78].

S1PR3, a receptor for the bioactive lipid sphingosine-1-phosphate, serves to regulate myeloid differentiation through potentiation of TNFα via NF-κB pathways and has been linked with chemosensitivity [79]. S1P signaling has been highlighted as a potential therapeutic target in the treatment of LSCs. Such an agent, S1PR3OE, has been shown to induce differentiation in HSCs, which may in turn promote LSC differentiation and escape from quiescent state, exposing them more readily to cytotoxic regimens [79,80].

A third factor that contributes to LSC quiescence is the TNF receptor superfamily member lymphotoxin-B receptor (LTBR) and its LIGHT ligand that function to regulate quiescence and self-renewal of LSCs [81]. The deficiency of LTBR reduces the number of LSCs and has become an active area of research for drug development in the past year.

Beyond quiescence, further chemoresistance of LSCs is supported by its anti-apoptotic abilities. Several pro-apoptotic agents have been recently introduced into pre-clinical studies of AML, summarized in Table 1. A pro-apoptotic agent with special recognition is venetoclax, a BH3 mimetic that selectively targets BCL-2 [82]. BCL-2 inhibition impedes oxidative phosphorylation, promoting apoptosis of LSCs [83,84]. In a phase Ib trial, newly diagnosed AML patients at least 65 years old were given demethylation agents combined with venetoclax. The patients experienced good tolerance of this combination with 67% of them achieving CR and/or CR with incomplete count recovery (CRi) (NCT02203773) [85]. Further, dose escalation studies of venetoclax in combination with azacitadine or decitabine in newly diagnosed AML have shown that this regimen is well tolerated and has superior response rates to alternative therapies. Furthermore, those with IDH mutations have been especially responsive to BCL-2 inhibition, suggesting a subgroup more likely to benefit from such targeted treatment. DiNardo and colleagues have cautioned, however, that such benefit with venetoclax is not as clear in the relapsed/refractory setting [86,87]. Additionally, recent work demonstrated the venetoclax resistance is associated with metabolic re-wiring of LSC [74,88,89].

Table 1. Pro-apoptotic agents.

| Drug | Pro-apoptotic mechanism |
|---|---|
| Triptolide | ROS generation, downregulation of Nrf2 pathway and HIF1α pathways |
| Cantharidin | Regulates the expression of SLUG, NFIL3 and c-myc, thereby inducing p53 and mitochondrial-caspase cascade |
| Cyclopamine | Neutralizes Hh ligands, inducing apoptosis in CD34+ cell lines as well as sensitizing them to cytarabine |
| 17-AAG | Degrades mutant p53; activates macrophage autophagy |
| Avocatin B | Induces ROS-dependent, mitochondria-mediated apoptosis in AML |
| Flavinoids | Inhibits CDK9 and induces repression of transcription of Mcl-1, myc and cyclin D1 |
| Phenols | Decreases STAT3 and BCL-XL in KG1 cells; also inhibit the Hedgehog and Notch pathways |

NF-kB is an important transcription factor in cell survival, proliferation and differentiation that is constitutively activated in LSCs, preventing cell destruction [90]. Pathenolide and Dimethylaminoparthenolide (DMAPT), two NF-kB inhibitors, are each in clinical trials at the moment. Resveratrol, which inhibits interleukin-1b-induced activation of NF-kB, is also being investigated for its ability to disrupt the anti-apoptotic ability of LSCs [91]. Pre-clinical studies of Resveratrol suggests multiple mechanisms of action in the treatment of AML including STAT3 inhibition, downregulation of BCL-2, and potential reversal of chemoresistance by regulation PI3K/Akt/Nrf2 signaling [92,93].

Notch, Hedgehog, and Wnt-β-catenin signaling pathways in cancer are well-studied resistance mechanisms to various cancer treatment modalities [74]. Not surprisingly, LSC growth, maintenance and chemotherapy-resistance are supported by these pathways. In AML initiating cells, activation of Notch was found to inhibit AML growth and survival in vitro and in vivo, which involved caspase-mediated apoptosis driven by Bcl-2 and p53 in response to induction of Notch signaling [94]. More is needed to be understood about the specific crosstalk between Hedgehog, Notch and Wnt-β-catenin in hematological disorders before drug development is underway.

### 2.5. Epigenetic modifications

Within the LSCs, non-coding microRNA (miR) has received recognition for its role in AML epigenetic regulation. Wang et al. specifically identified miR-34a, miR-126, miR-21, miR-196b, and miR-17-92 as important regulators of LSCs [95]. Long non-coding RNA (lncRNA) has also been found to contribute to tumor propagation through a variety of mechanisms. HOX antisense intergenic RNA (HOTAIR), for instance, is an lncRNA that promotes drug resistance in a variety of conditions including anthracycline resistance in leukemia [96–99]. Zhou and colleagues were able to demonstrate that HOTAIR doxorubicin resistance in AML was achieved through upregulation of DNMT3b-dependent pathways that subsequently suppressed PTEN (multi-functional tumor suppressor) [96]. Pre-clinical studies are ongoing to further understand the role that non-coding RNA has in the LSC development into AML [100]. In 2019, Bill and colleagues identified a signature of 111 lncRNAs strongly associated with LSCs, which included lncRNA DANCR, which is particularly upregulated in LSC-enriched populations [101]. Murine models with knock-down DANCR were found to have fewer LSCs in quiescence, suggesting that LSCs have distinct lncRNAs that may serve as future targets in drug development.

The study of LSCs have also revealed variations in methylation patterns in comparison to HSCs. Specifically, LSCs have demonstrated significantly reduced methylation confirming little-to-no efficacy with agents such as azacitadine or decitabine whose mechanism of action is to reduce methylation patterns [102]. One leading hypothesis as to why LSCs have reduced methylation is due to its overexpression of lysine-specific demethylase (LSD1). LSD1 inhibitors have shown to promote differentiation and apoptosis [103,104]. A phase II trial of Iadademstat + azacitidine is ongoing (EudraCT No.: 2018–000482-36) with encouraging preliminary results (ORR

77%) [105]. Also, Bernt et al. identified MLL gene rearrangements in AML where the H3K79 methyltransferase DOT1L plays a key role in leukemogenesis [106]. In 2020, Wang and colleagues revealed that lncRNA LAMP5-AS1 results in higher degrees of methylation by DOT1L, resulting in increased stem cell properties in patients with MLL-rearranged AML [107]. This self-renewal capacity is likely a large contributor to the aggressive nature of MLL-rearranged AML [106,108–111]. Given the off-target challenges that direct MLL inhibition may pose, both DOT1L or LAMP5-AS1 are of particular interest for further investigation in this subgroup of leukemia. Inhibitors of DOT1L are already in clinical trials and are hypothesized to show benefit in AML subtypes with MN1 overexpression, DNMT3 mutations, IDH1/2 mutations, and/or MLL rearrangements [106]. Pinometostat (EPZ-5676) is the most clinically advanced DOT1L inhibitor. Phase I studies have revealed good tolerability with some complete responses, though the challenge of administration over 28 days presents a logistical challenge. Phase II studies are ongoing.

Additionally, *IDH1* or *IDH2* mutations have demonstrated metabolic dysregulation through increased expression of the oncometabolite 2-hydroxyglutarate, which favors the leukemic phenotype [112]. The presence of IDH mutations early in AML suggest that this epigenetic mechanism serves as a driver for LSCs [113]. As such, clinical trials of IDH inhibitors combined with standard of care for the treatment of relapsed/refractory AML or newly diagnosed AML are in process.

### 2.6. Immune evasion

In 2018, Mathew et al. explored a subset of individuals with relapsed AML with FLT3-ITD mutations, noting an improved response when treated with sorafenib, a multikinase inhibitor [114]. Their in vitro studies demonstrated that in those with FLT3-ITD mutations, sorafenib therapy was associated with increased mitochondrial and glycolytic capacities of CD8 + T cells and pro-immunomodulatory cytokines, theoretically leading to reprogramming of the immune system response to leukemic cells [114]. Such graft-versus-leukemia effects have been further reviewed by Zhang et al., noting that specific tyrosine kinase inhibition of FLT3 produces similar anti-leukemic effects and may play a role in prevention LSC immune evasion [115].

Additional key proteins in immunoregulatory roles have allowed for the maintenance of LSCs without host recognition. CD47, an integrin-associated signal transducer that prevents phagocytosis of host cells, serves as an escape mechanism for LSCs. Anti-CD47 monoclonal antibody Hu5F9-G4 is in phase I studies treating patients with relapsed/refractory AML. TTI-621, another CD47-directed agent, is being evaluated in a multicenter, open-label, phase 1a/1b trial alone or in addition to Rituximab or Nivolumab in several solid and hematologic malignancies.

In 2019, Paczulla et al. demonstrated that NKG2D, a ligand that alerts cytotoxic lymphocytes to foreign invaders, is often present in AML cells while absent on LSCs, providing further immune evasion in this subgroup [116]. Poly-ADP-ribose polymerase 1 (PARP1) traditionally represses NKG2D ligand expression. As such, PARP1 inhibition may theoretically serve as a treatment for relapsing and/or therapy-resistant LSCs with the hypothesis that it would induce expression of the NKG2D ligand leading toward immune destruction.

## 3. Expert opinion

Targeting LSCs remains a critical goal in the management of AML and in the prevention of recurrence. However, targeting LSCs has been hampered by several challenges. LSCs are thought to be rare bone marrow residing cells at a frequency of one in 250,000 [117]. Further, distinguishing LSC from early progenitor or more differentiated AML cells requires functional validation in transplantation studies. Importantly, the shared targets of LSCs and normal hematopoietic stem cell confer another layer of complexity to delineate LSC from healthy stem cells. Further, the mere expression of certain targets in LSCs does not necessarily translate into a therapeutic functional target that can lead to LSC eradication. For instance, the quiescent nature of LSC makes these cells less likely to be eradicated by conventional chemotherapy. Even when LSCs are potentially targeted via venetoclax-based therapies, LSCs undergo metabolic rewiring and are able to adapt to therapy and lead to relapses. Hence, understanding the biology of LSC is crucial to overcome these barriers, and applying immune-based therapy that can recognize aberrant cells would be crucial. One such approach is activating macrophages (via CD47/SIRP1a pathway) or via T-cell checkpoint inhibition (PD-1, TIM3, CTLA4, etc.). Another approach would be to generate BiTEs or conjugated antibodies that leverage the T cell function and target markers on LSCs, such as CD123, CLL1 or CD70. While a large number of clinical studies are leveraging these approaches, none of these studies have translated into FDA approvals, yet. With the advance of single-cell transcriptomics, CyTOF, and single-cell proteomics, we anticipate that we will be able to identify LSC-specific targets that are otherwise not expressed in HSCs and could translate into novel therapeutic options. Until we are able to eradicate LSCs, the risk of relapse among AML patients will continue to loom over the long-term outcomes of AML patients.

## 4. Conclusions

The reconstitution of AML by transplanting CD34+ CD38- cells into immune-deficient mice over 28 years ago confirmed the existence of leukemia stem cells at a frequency of approximately one in 250,000 cells [117]. Since then, ample research culminated into a deeper understanding of the LSC cell architecture, biology, intracellular signaling pathways, and immunomodulatory functions that allow for its survival and pathogenesis in AML. Molecular analysis of the AML LSC population has specifically shown that survival requires several signaling functions via NF-κB, STAT, PI3 kinase pathways, self-renewal by means of regulatory pathways such as Wnt-β-catenin, Hedgehog and Notch, and evasion of apoptosis (Figure 2) [118].

Drug design and development is a time-consuming process that requires in vitro and pre-clinical studies prior to clinical application. The repurposing of drugs may allow for faster approval of anti-leukemic agents if found to be effective against LSCs and should be strongly considered in ongoing

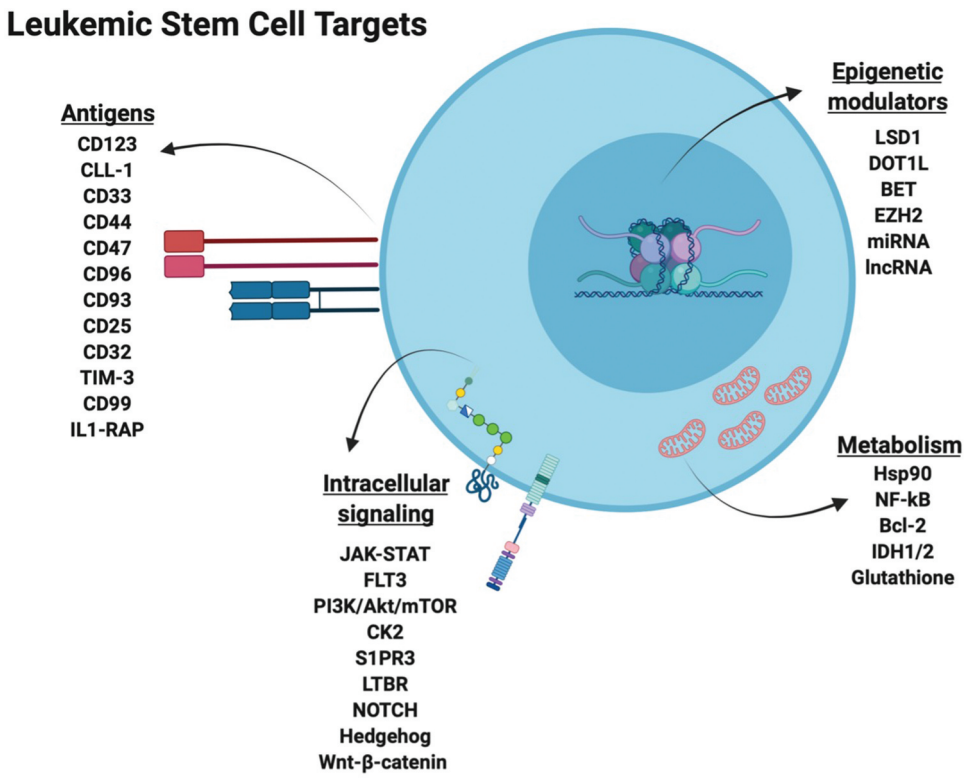


Figure 2. Leukemic stem cell targets.

investigations for LSC targets. Additionally, other technologies and strategies need to be developed to further identify targets unique to LSCs that spare HSCs. Joint single-cell transcriptomics, clonal tracking approach (MutaSeq and mitoClone), and iPSC-derived LSC models offer new opportunities to understand the biological properties of LSCs.

While many targeted therapies are under investigation, it is important to note that AML is quite a heterogenous and dynamic disease. As such, we will likely require a multitargeted approach, considering both the cell-intrinsic and the cell-extrinsic mechanisms that support LSC growth. Furthermore, studies suggest that upfront targeting of LSCs will ultimately lead to highest chance of cure. As such, clinical investigations should consider these novel agents in the front-line in combination with current standard of care for successful treatment strategies that reduce or prevent relapse.

## Declaration of interest

M Konopleva has Consultancy roles with (includes expert testimony): AbbVie, Genentech, F.

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