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Biology and Clinical Relevance of Acute Myeloid Leukemia Stem Cells

Andreas Reinisch^{1,*}, Steven M. Chan^{1,*}, Daniel Thomas^{1,*}, and Ravindra Majeti¹

¹Department of Medicine, Division of Hematology, Cancer Institute, and Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine

Abstract

Evidence for the cancer stem cell model was first demonstrated in xenotransplanted blood and bone marrow samples from patients with acute myeloid leukemia (AML) almost two decades ago, supporting the concept that a rare clonal and mutated leukemic stem cell (LSC) population is sufficient to drive leukemic growth. The inability to eliminate LSCs with conventional therapies is thought to be the primary cause of disease relapse in AML patients, and as such, novel therapies with the ability to target this population are required to improve patient outcomes. An important step towards this goal is the identification of common immunophenotypic surface markers and biological properties that distinguish LSCs from normal hematopoietic stem and progenitor cells (HSPCs) across AML patients. This work has resulted in the development of a large number of potential LSC-selective therapies that target cell-surface molecules, intracellular signaling pathways, and the bone marrow microenvironment. Here, we will review the basic biology, immunophenotypic detection, and clinical relevance of LSCs, as well as emerging biological and small-molecule strategies that either directly target LSCs or indirectly target these cells through modulation of their microenvironment.

Introduction

Acute myeloid leukemia (AML) is an aggressive malignancy of the hematopoietic system associated with a relatively poor outcome, which has not improved significantly for the past three decades, with long-term overall survival rates for younger patients ranging from 40 to 50% [1]. Recently, high-throughput sequencing technology and DNA methylation profiling helped to characterize the genomic and epigenomic landscape of this disease. The process of leukemic transformation is driven by a series of somatically acquired mutations and chromosomal aberrations, which appear to determine many of the biological and clinical aspects of the disease at presentation [2]. Chromosomal abnormalities detected through

Correspondence should be addressed to: Ravindra Majeti MD, PhD, Stanford Institute for Stem Cell Biology and Regenerative Medicine, Lokey Stem Cell Building, 265 Campus Drive, Stanford, CA 94305, rmajeti@stanford.edu.

*authors contributed equally

Conflict of Interest Statement

The authors have no conflicts to declare.

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conventional cytogenetics are present in more than half of adult AML samples [3, 4] and somatically acquired recurrent mutations have been identified in a number of genes including *NPM1*, *FLT3*, *CEBPA*, *MLL*, *NRAS*, *WT1*, *KIT*, *RUNX1*, *TET2*, *IDH1*, *IDH2* and others [5].

Similar to many (but not all) other human malignancies, many cases of AML display evidence of a “hierarchical” cellular organization, with a minor fraction of self-renewing cancer stem cells (CSCs) at the apex of this hierarchy that maintain the disease. CSCs are defined as cells that are capable of re-initiating the disease if transplanted into immunodeficient animals and differentiating into all the cells comprising the malignancy. The earliest conceptual idea of leukemia being organized in a hierarchical manner traces back to studies performed to identify clonogenic AML progenitors *in vitro* [6, 7]. Dick and colleagues later demonstrated that AML is organized in this hierarchical fashion *in vivo*, similar to normal hematopoiesis, and is therefore replenished by leukemia stem cells (LSCs) [8–10]. From a clinical perspective, the cancer stem cell model implies that in order to eradicate the disease and achieve long-term cure for patients, the treatment also has to eliminate the LSC population. As a neoplastic cellular reservoir that likely contributes to chemotherapy resistance, subclone generation, persistent residual disease, and eventual relapse, LSCs are an attractive target for the development of new anti-leukemic therapies. Their properties of relative quiescence, resistance to apoptosis, self-renewal, and increased drug efflux mandate a modified approach from that of conventional therapies aimed at the bulk, rapidly-dividing disease. In this review, we aim to review emerging therapeutics specifically targeting AML-LSCs including biological and small molecule therapeutics, as well as hematopoietic niche-related therapy approaches.

Detection of AML Stem and Progenitor Cells

In the original studies using fluorescence-activated cell-sorting (FACS) from a variety of AML subtypes, LSC were shown to be negative for expression of lineage markers (Lin⁻), positive for expression of CD34, and negative for expression of CD38 as demonstrated by engraftment in NOD/SCID mice [10] and long-term culture-initiating activity *in vitro* [11, 12]. Further work by the Dick laboratory and others [13, 14], using both intrafemoral and intravenous tail-vein injection and a more permissive NOD/SCID/interleukin 2 receptor gamma^{null} (NSG) mouse model, has shown that in virtually all cases leukemia-initiating cells reside in the CD34⁺/CD38⁻ fraction [15]. In at least half of the samples, LSCs are also present in at least one other fraction (usually the CD34⁺/CD38⁺ fraction and sometimes in the CD34⁻ fraction), although a fraction devoid of leukemia initiating activity always exists but cannot be identified based on current methods [15]. Furthermore, in certain genetically defined cases such as AML with mutation in nucleophosmin 1 (NPM1c), LSCs have been found predominantly within the CD34⁻ fraction in half of the cases [16].

More recently, CD34⁺ LSC populations have been further refined to show the coexistence of two distinct LSC populations studied in patients with CD34⁺ AML (which is detectable on the bulk population in the majority of cases) [17]. These two populations resemble normal lymphoid-primed multi-potent progenitors (LMPP-like LSCs) (Lin⁻/CD34⁺/CD38⁻/CD90⁻/CD45RA⁺) and granulocyte-macrophage progenitors (GMP-like LSCs) (Lin⁻/CD34⁺/

CD38⁺/CD123⁺/CD45RA⁺) and are consistent with a progenitor acquiring self-renewal properties rather than a direct HSC origin for AML [17]. Further improvements of LSC detection using more sensitive methods such as humanized microenvironments [18] and integration of genetic mutation data are required to find superior immunophenotypic markers for LSCs.

Separation of leukemic cells based on drug efflux (so called “side” population cells which exclude Hoechst stain via ATP-binding cassette transporter G2) [19, 20] and high aldehyde dehydrogenase 1 activity (using a cell permeable fluorescent substrate) [21] has also been demonstrated to enrich for leukemia-initiating activity, analogous to studies with normal hematopoietic cells.

Detection of Pre-Leukemic Stem Cells

Gene expression profiling identified TIM3, a cell-surface mucin-domain containing molecule, upregulated at the mRNA and protein level in CD34⁺/CD38[−] LSCs but not CD34⁺/CD38[−] HSCs [22]. This was found to be an informative flow-cytometry marker to prospectively separate residual HSCs from leukemic cells (including LSCs) in blood and bone marrow (BM) samples at the time of diagnosis. DNA sequencing of these residual HSCs has found that many patients harbor a high proportion of mutated “pre-leukemic” stem cells bearing some, but not all, of the mutations present in the bulk AML. These residual HSCs can give rise to normal lymphoid and myeloid engraftment when transplanted into NSG mice. Similar to colon cancer, the discovery of pre-leukemic HSCs supports a step-wise progression in the clonal evolution of AML and suggests that relapse could possibly occur not only from leukemic clonal or sub-clonal outgrowth, but also from further evolution of pre-leukemic mutated clones [23]. Many of the mutations found in Lin[−]/CD34⁺/CD38[−]/TIM3[−]/CD99[−] HSCs are in epigenetic modifiers such *TET2*, *DNMT3A*, and *IDH1/2*, rather than proliferative mutations, underscoring a role for epigenetic dysregulation in establishing a pro-leukemogenic state [24]. Recently, the existence of clonal hematopoiesis carrying the same mutations was confirmed in two large cohort studies performed in elderly individuals with no hematologic malignancies [25, 26].

Clinical Outcome and Minimal Residual Disease

The number of LSCs in a patient’s BM, either defined immunophenotypically or by xenograft transplantation, correlates strongly with both prognosis and ultimate clinical outcome. A high proportion of CD34⁺/CD38[−] cells at the time of AML diagnosis is associated with LSC engraftment in NOD/SCID mice, high minimal residual disease after chemotherapy, and poor patient survival [27]. Similarly, ALDH bright cells in primary samples correlated with leukemic blast persistence after induction chemotherapy and adverse outcome [28]. In addition, patients whose leukemic cells are able to initiate leukemia in the NOD/SCID assay had a significant shorter overall survival than those with leukemia cells that did not engraft, indicating that standard leukemia-initiating cell assays have prognostic meaning [29]. More recently, a detailed study of CD34⁺ sub-compartments in patients receiving epigenetic therapies azacitidine and sodium valproate showed

persistence of LMPP and GMP-like LSCs even in patients that achieved a complete remission (CR), highlighting the need for more effective therapy active against LSCs [30].

Biological Therapies Targeting LSC

To date, a major focus of research has been the identification of cell surface markers that are differentially upregulated on LSCs but not on normal human HSCs in the majority of patients. These have been difficult to identify, and thus far no one unique marker has been discovered that is universally expressed on CD34⁺/CD38⁻ LSCs (or other rigorously demonstrated fraction) across AML patients, but not on bulk blasts or normal HSPCs. This challenge is partially due to the intrinsic heterogeneity of AML (both between patients and within a major clone) and the limitations of the *in vivo* assays, but also arises from the fact that LSCs in many respects closely resemble early myeloid hematopoietic progenitor populations as discussed above.

Despite these difficulties, a number of cell surface markers have been identified that are up-regulated on CD34⁺/CD38⁻ LSCs compared to normal CD34⁺/CD38⁻ HSCs and enrich for leukemia-initiating cells in mice including CD123 [31], CD47 [32, 33], TIM3 [22, 34], CD96 [35], CLL-1 [19, 36], CD32 [37], CD25 [37], and IL-1 receptor accessory protein (IL1RAP) [38] (Table 1). Monoclonal antibodies that bind and/or block the function of these antigens have been demonstrated to reduce leukemia initiating activity in pre-clinical models of human AML, (for comprehensive review see Majeti, 2011 [39]). Translation to the clinic has progressed rapidly for a humanized monoclonal IgG2 antibody (CSL360) that binds the interleukin-3 receptor alpha chain (CD123) with high affinity and blocks interleukin-3 mediated survival and proliferation of LSCs. In a phase I trial of relapsed AML, intravenous administration of CSL360 was demonstrated to be safe with clinical responses obtained in 2 out of 16 patients as monotherapy. An engineered version with superior NK cell CD16 Fc-mediated binding affinity (CSL362) is now in phase I/II trial [40].

The inhibitory SIRPα ligand, CD47, was found to be up-regulated in LSCs compared to normal HSCs by gene expression profiling [33]. CD47 is ubiquitously expressed on all tissues including red blood cells comprising a negative signal to prevent homeostatic phagocytosis, and its increased expression on LSCs likely reflects one of several innate immune escape mechanisms utilized by CSC. Clinical development of CD47 blocking monoclonal antibodies and engineered high affinity SIRPα variants is currently in progress.

While the myeloid marker CD33 is expressed at low levels on CD34⁺/CD38⁻ LSCs as originally defined, its expression can be found in many sub-populations that have demonstrated leukemia-initiating activity making it a potential target. Multiple clinical trials with gemtuzumab ozogamicin, an anti-CD33 monoclonal antibody conjugated to chaliceamicin, removed from the market due to toxicity, have demonstrated increased remission in combination with chemotherapy in young adults [41] but whether it has a major role in eradicating LSCs has not been shown. A new antibody drug conjugate, SGN-CD33A, engineered to have more predictable pharmacokinetics and drug delivery with a better toxicity profile is currently under clinical investigation [42].

Targeting of LSCs Using Small Molecule Agents

Ever since the discovery of LSCs in AML, there has been a tremendous amount of interest in the development of therapies that exploit the inherent biological differences between LSCs and normal HSPCs. In contrast to antibody-based therapies as discussed in the previous section, therapies based on small molecule agents have the ability to target intracellular proteins and pathways. These therapies act by inhibiting signaling pathways (e.g. NF- κ B) or cellular functions (e.g. oxidative phosphorylation) that are uniquely required for LSC survival and/or maintenance. Other therapies act by inducing stresses (e.g. oxidative stress) that preferentially compromise LSC activity. Many of the agents have pleiotropic effects. Here, we present a comprehensive list of small molecule agents that have previously been reported to have anti-LSC activity (Table 2) and focus our discussion on some of the most promising approaches.

Inhibition of nuclear factor (NF)- κ B

NF- κ B is a dimeric transcription factor complex that controls the expression of a vast number of genes in cellular responses to stimuli [43]. In the unstimulated state, the complex is sequestered in the cytoplasm by a family of inhibitors, called I κ Bs [43]. Guzman *et al.* initially reported that NF- κ B is constitutively active in AML cells enriched for LSCs (defined immunophenotypically as CD34⁺/CD38⁻/CD123⁺) as determined by electrophoretic mobility shift assay (EMSA) and gene expression analysis [44]. In contrast, NF- κ B activity was not detectable in unstimulated normal CD34⁺ HSPCs. Treatment of primary AML samples with the proteasome inhibitor MG-132 which inhibits NF- κ B activity by preventing degradation of I κ B α , resulted in rapid induction of cell death in AML LSCs but not normal CD34⁺ HSPCs, providing evidence for the importance of NF- κ B signaling in supporting LSC survival.

The underlying basis for constitutive NF- κ B activity in AML cells is likely due to aberrant FLT3, RAS and/or AKT/PI3K signaling which are known to induce strong activation of the NF- κ B pathway [45, 46]. Autocrine TNF α secretion has also been shown to promote constitutive NF- κ B activation [47]. Transforming growth factor- β activated kinase 1 (TAK1) which forms a kinase complex required for NF- κ B activation was recently found to be upregulated in CD34⁺ AML cells compared with CD34⁺ normal bone marrow cells, providing another potential mechanism for upregulation of NF- κ B activity in LSCs [48].

In addition to proteasome inhibitors like MG-132, other compounds with inhibitory activity against NF- κ B including parthenolide (PTL) [49], AR-42 [50], and niclosamide [51] have also been reported to target primitive AML cells. PTL is the major active compound in the herbal medicine Feverfew (*Tanacetum parthenium*) and is one of the first compounds shown to selectively ablate LSCs without significant toxicity to normal HSPCs [49, 52]. Treatment of primary AML samples *ex vivo* with PTL preferentially reduced the viability of CD34⁺CD38⁻ AML cells and their engraftment potential in NOD/SCID mice [49, 52]. Although PTL clearly inhibits the activity of NF- κ B, it also increases the level of reactive oxygen species (ROS) and activates p53 signaling. These processes contribute to the induction of apoptosis in LSCs along with NF- κ B inactivation [49, 52]. Indeed, expression

of a degradation-resistant form of I κ B α which specifically downregulates NF- κ B activity was found to be insufficient for the rapid and extensive apoptosis of LSCs seen with PTL [53]. Thus, NF- κ B inhibitors will most likely be used in combination with conventional chemotherapeutic or other novel targeted agents to achieve a synergistic effect. Potential agents include JNK inhibitors [54], histone deacetylase (HDAC) inhibitors [55], and idarubicin [53], which have all been shown to synergize with NF- κ B inhibition to eliminate primitive AML cells.

Inhibition of BET family proteins

Changes in the epigenetic landscape are thought to play a critical role in AML leukemogenesis as supported by the high frequency of mutations in genes (e.g. *TET2*, *DNMT3A*, *MLL*) that affect DNA methylation and histone modifications [5]. Recent evidence suggests that these mutations contribute to development of leukemia by blocking differentiation and increasing self-renewal [56, 57]. Reversal of these epigenetic changes can potentially deplete LSCs by promoting terminal myeloid differentiation. In order to determine specific dependencies on chromatin regulators in a genetically defined mouse model of AML (MLL-AF9/Nras^{G12D}), Zuber *et al.* performed an unbiased RNA interference screen using a custom library of small hairpin RNAs targeting known chromatin regulators [58]. The screen identified bromodomain-containing 4 (BRD4), which is a member of the BET (bromodomain and extra terminal domain) family that recognizes acetylated histones, to be required for disease maintenance [58, 59]. Inhibition of BRD4 with a small-molecule inhibitor known as JQ-1 was highly effective promoting terminal differentiation of AML LSCs *ex vivo* and also showed anti-leukemic activity *in vivo* [58, 59]. JQ-1 was found to be effective against a broad range of AML subtypes and synergizes with FLT3 [60] and HDAC [61] inhibitors. The mechanism of action appears to be at least partially due to suppression of *MYC* expression [58]. Intriguingly, JQ-1 has been shown to inhibit NF- κ B activity, which may contribute to its anti-leukemic activity [62]. Since these initial discoveries, several inhibitors of the BET family members have now been developed and some are in early phase clinical trials.

Inhibition of anti-apoptotic BCL-2 family members

Similar to other malignancies, numerous studies have established that AML cells are dependent on specific anti-apoptotic members of the BCL-2 family for survival. Anti-apoptotic proteins antagonize the activation of pro-apoptotic proteins by binding to their BH3 domains [63]. In particular, MCL-1 and BCL-2 have attracted the most attention as therapeutic targets.

In a series of experiments utilizing lentiviral vectors that inducibly expressed BH3-like ligands to neutralize specific anti-apoptotic BCL-2 proteins, Glaser *et al.* showed that the majority of AML cell lines and some primary AML samples are highly dependent on MCL-1 for survival [64]. Given its importance in promoting AML survival, there has been considerable interest in developing highly specific and potent direct inhibitors of MCL-1. Unfortunately, this approach has proved to be challenging. An alternative approach is to suppress transcription of the *MCL-1* gene which effectively reduces MCL-1 protein levels

due to its very short half-life (about 2–4 hours in most cells) [65]. Several compounds including flavopiridol [66] and PIK-75 [67] have been shown to suppress *MCL-1* transcription through inhibition of the transcriptional kinases CDK9 and CDK7/9, respectively. PIK-75 treatment was shown to reduce the viability of purified LSCs (CD34⁺/CD38⁻/CD123⁺) from primary AML samples *ex vivo* with minimal toxicity to normal bone marrow CD34⁺ progenitor cells. Intriguingly, although PIK-75 was identified in a screen based on the ability to suppress *MCL-1* levels, it also possesses inhibitory activity against phosphatidylinositol 3-OH kinase, which synergizes with CDK7/9 inhibition to kill AML cells [67].

Numerous studies in the past 20 years have demonstrated dependency on BCL-2 for survival in AML cells [68, 69]. These observations prompted initial attempts to use antisense oligonucleotides to suppress BCL-2 expression in AML patients. Although these clinical trials showed activity in some patients [70, 71], issues with enzymatic degradation and short half-lives limited its clinical utility. Recent developments in the ABT series of small molecule inhibitors have revived interest in targeting BCL-2 in AML. ABT-737 was the first molecule discovered in this series and is a highly potent inhibitor of BCL-2, BCL-XL, and BCL-W [72]. ABT-263 was later developed as an orally bioavailable version of ABT-737 [73]. Konopleva *et al.* showed that treatment of primary AML samples with ABT-737 *ex vivo* depleted the LSC population defined as CD34⁺CD38⁻CD123⁺ in most but not all cases [74]. More recently, Lagadinou *et al.* demonstrated that ABT-263 is selectively toxic to a subpopulation of AML cells with low levels of reactive oxygen species (ROS), which they found is enriched for LSC activity [75]. The mechanism of action appears to be due to suppression of oxidative phosphorylation (OXPHOS) that occurs with BCL-2 inhibition. This non-canonical role of BCL-2 has previously been reported [76]. The authors found that LSCs, unlike normal HSPCs, are unable to increase glycolysis to compensate for the decrease in OXPHOS, resulting in ATP depletion and ultimately cell death.

Although ABT-263 showed promising pre-clinical activity, its clinical use has been hampered by thrombocytopenia which is the major dose-limiting toxicity [77]. This effect is due to the inhibition of BCL-XL which is required for platelet survival and function [78, 79]. In response to this limitation, ABT-199 was developed as a highly specific and potent inhibitor of BCL-2 that does not cause thrombocytopenia [80]. ABT-199 has been recently shown to induce apoptosis in primary human AML blasts and LSCs (defined as CD34⁺CD38⁻CD123⁺) in *ex vivo* assays [81]. ABT-199 is now in early phase clinical trials for a number of hematologic malignancies including AML.

We recently found that BCL-2 inhibition selectively targets AML cells with isocitrate dehydrogenase (*IDH*) mutations through an unbiased large-scale lentiviral RNA interference screen. Importantly, we showed that *in vivo* treatment of *IDH*-mutant primary AML with ABT-199 resulted in not only a decrease in bulk disease burden but also a loss of functional LSC activity with minimal hematologic toxicity. This sensitization effect appears to be due to inhibition of cytochrome C oxidase activity in the mitochondrial electron transport chain (ETC) by high intracellular levels of 2-hydroxyglutarate, an oncometabolite that is generated by the mutant enzymes [82]. We further showed that tigecycline, which disrupts ETC function through inhibition of mitochondrial translation [83], sensitized resistant AML cells

to the pro-apoptotic effects of ABT-199. The anti-leukemic activity of other combinations of ETC inhibitors with ABT-199 on the LSC population is under active investigation.

Inhibition of telomerase activity

The maintenance of telomeres at the ends of chromosomes through telomerase activity is one of the requirements for unlimited cell divisions in cancer cells [84]. Small molecule inhibitors of telomerase activity have been shown to restrain the growth of a variety of tumor types [85]. Recently, Bruedigam *et al.* demonstrated that AML LSCs from a retroviral mouse model (MLL-AF9) and primary AML samples are similarly dependent on telomerase activity for survival and proliferation [86]. This effect appears to be dependent on p53 activation as p53 knockdown partially rescued telomerase-deficient LSCs [86]. The authors also identified a telomerase-regulated gene signature that correlated with patient prognosis. Importantly, treatment of xenograft models of human AML with imetelstat, an antisense oligonucleotide that specifically targets the RNA component of the telomerase complex, impaired leukemia progression and delayed relapse following chemotherapy. Important issues to address in future studies include distinguishing between telomere length-dependent and independent mechanisms, investigating potential off-target effects, and identifying synergistic therapeutic combinations.

The Hematopoietic Niche and its Involvement in Leukemogenesis

The microenvironment for blood forming cells is generally referred to as the hematopoietic niche [87], and is a complex anatomical site composed of various cell types and structural components regulating stem cell function. Among the supportive cells in the niche are perivascular cells, osteoblasts, osteoclasts, endothelial cells, sympathetic nervous cells, immune cells, adipocytes and megakaryocytes (for comprehensive review see Frenette, PS *et al.*, 2014 [88]), some of which are direct descendants of mesenchymal stromal cells (MSCs), an adherent population that can recreate a functional hematopoietic microenvironment upon transplantation to ectopic sites [89–92]. Whereas, it is well known that LSCs prefer to localize within special microenvironments of the bone marrow cavity [93, 94] similar to normal HSCs, it is still unclear whether LSCs require similar microenvironmental support for long-term maintenance of the disease. However there is growing evidence suggesting that cell-extrinsic signals coming from components of the normal HSC niche have important roles in leukemia pathogenesis. Those key signals mainly influence their survival after chemotherapy [14] and homing to the hematopoietic microenvironments [95]. A permissive microenvironment might also play a role in disease initiation by transforming normal HSCs [96].

Several reports underline the potential contribution of an aberrant hematopoietic microenvironment on leukemogenesis. All of these reports share the common approach of selectively targeting genes in niche-specific cells. Deletion of retinoic acid receptor gamma (RAR γ) from BM-microenvironment initiated the formation of a myeloproliferative syndrome [97], whereas targeted deletion of miRNA-processing endonuclease Dicer1 in Osterix-expressing osteoprogenitors recapitulated key features of myelodysplasia and/or acute myeloid leukemia [96]. Similarly, a mutation of beta-catenin in osteoblasts resulted in

upregulation of Jagged-1, thereby activating the Notch-pathway in hematopoietic cells and leading ultimately to AML development in mice [98]. Furthermore, signals that emanate from neoplastic hematopoietic cells can alter the biology of the niche. Schepers *et al.* showed that neoplastic cells are capable of remodeling the niche into a self-reinforcing leukemic niche [99]. In addition, rather than directly influencing hematopoietic cells and leading to malignant transformation, aberrant niche cells could also simply increase proliferation of HSCs, thereby increasing the potential pool of cells capable of acquiring genetic abnormalities [100]

The growing evidence that i) signals from the BM-microenvironment can drive or select for subsequent transforming events in leukemia stem cells, and ii) signals from leukemia can remodel existing niches to support its growth, implies that such signals may represent candidate targets in novel therapeutic strategies.

Targeting LSC-Niche Interactions

If LSCs depend on specific niche interactions, targeting these interactions might be an effective approach to eradicate the disease. This treatment could either directly influence LSC survival or help sensitize them to conventional cytotoxic agents that are able to get rid of bulk leukemia but usually fail to eliminate LSCs. Most LSCs reside within the chemotherapy-protective endosteal and vascular niches [94]. Dislodging LSCs from their protective microenvironment through systemic administration of granulocyte-colony stimulating factor (G-CSF) could sensitize the cells to chemotherapy and increase long-term therapy success [14]. Other potential targets include blocking the interaction of CXCL12 (SDF-1) on niche cells with CXCR4 expressed on LSC using CXCR4-inhibitors. This has been shown to increase response rates and decrease leukemia burden in murine leukemia models and led to the initiation of several clinical trials [101, 102]. Combining CXCR4-inhibition with neutralizing antibodies to TGF-beta has been shown to further increase efficacy [103]. Ligation of the adhesion molecule CD44 on LSCs with monoclonal antibodies and thereby blocking their lodging and trafficking to supportive niches is another promising way proven to be effective in selectively killing AML LSCs [95].

Conclusions

Although treatment strategies and corresponding long term survival for AML patients have not changed drastically over the past three decades, we now are at the frontier of a promising new era of novel targeted therapies that have the potential to specifically attack LSCs without adversely affecting normal hematopoietic stem and progenitor cell populations. One of the biggest future challenges will lie in early and efficient identification of patient and/or leukemia subtype specific targets within leukemic stem cells that can be tackled in a combinatorial manner using established therapeutics and novel approaches, both biologic and small molecules. Additionally implementing new modalities that attack interactions with the leukemic stem cell microenvironment are promising strategies that might further improve future leukemia therapy and patient outcomes.

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Table 1

Biological therapies engineered to specifically target LSCs or CD33+ leukemic progenitors that are currently under investigation

Target	Description	Antigen-specific Bio-therapeutic	Stage of Development	References
CD123	Interleukin-3 receptor alpha chain, up-regulated on blasts and LSCs in majority of patients	Humanized blocking monoclonal antibody (CSL360) with improved Fc CD16 binding (CSL362)	Phase I/II	PMID 19570512, PMID 24705479 Clinical Trial (CSL360): NCT01632852 Clinical Trial (CSL362): NCT00401739
		Bispecific CD3 and CD123 antibody fusion	Pre-clinical	PMID 22740616
		Trispecific Fv derivative against CD123 and CD33	Pre-clinical	PMID 20636437
		Chimeric antigen receptor (CAR)	Pre-clinical	PMID 24030378 PMID 24596416
		Interleukin-3 diphtheria toxin fusion protein	Phase I	PMID 18297533
CD47	Inhibitory ligand for SIRPα, overexpressed on LSCs, inhibits phagocytosis by macrophages ("don't eat me signal")	Humanized monoclonal antibody	Phase I	PMID 19632179 PMID 19632178
		SIRPα high affinity engineered variant	Pre-clinical	PMID 23722425
		SIRP-Fc fusion protein	Pre-clinical	PMID 22945919
CD33	Myeloid specific antigen expressed on committed myeloid precursors and majority of AML blasts but low expression on CD34+CD38-LSCs	Antibody-drug conjugate (Gemtuzumab ozogamicin)	Phase III	PMID 11432892
		Chimeric antigen receptor (CAR) directed T cells	Phase I/II	PMID 22851554
		Antibody-drug conjugate (SGN-CD33A)	Phase I/II	PMID 25174587
		Monoclonal antibody (SGN-CD33, lintuzumab)	Phase II/III	PMID 23770776 PMID 19557623
CD44	Hyaluronan and osteopontin receptor	Monoclonal antibody H90 (mouse IgG1)	Pre-clinical	PMID 16998484
CLL-1	C-type Lectin-like molecule expressed on 87% of CD34+CD38-LSCs but not normal cells	Monoclonal antibody	Pre-clinical	PMID 15548716 PMID 19648166
CD96	Type I membrane protein in immunoglobulin superfamily; present on majority of CD34+CD38-LSCs	Humanized monoclonal antibody	Potential role in purging cell products prior to autologous transplantation	PMID 17576927 PMID 22879978
TIM3	Mucin-domain containing molecule useful in separating LSCs from HSCs	Monoclonal antibody	Pre-clinical	PMID 21383193 PMID 21112565

Table 2

Small molecule therapeutics targeting AML LSCs that are currently under investigation.

Target(s)	Agent(s)	Proposed mechanism(s) of action	References
Regulators of Apoptosis			
BCL-2, BCL-XL, BCL-W	<ul style="list-style-type: none"> • ABT-737 • ABT-263 	<ul style="list-style-type: none"> • Inhibits the anti-apoptotic proteins BCL-2, BCL-XL, and BCL-W • Suppresses oxidative phosphorylation 	PMID: 25379408 PMID: 23333149
BCL-2	<ul style="list-style-type: none"> • ABT-199 	<ul style="list-style-type: none"> • Inhibits the anti-apoptotic protein BCL-2 	PMID: 24346116
MCL-1 and PI3K	<ul style="list-style-type: none"> • PIK-75 	<ul style="list-style-type: none"> • Suppresses MCL-1 through inhibition of CDK7/9 • Inhibits PI3K signaling 	PMID: 23775716
XIAP	<ul style="list-style-type: none"> • Dequalinium chloride • AEG35156 	<ul style="list-style-type: none"> • Induces apoptosis and differentiation of AML LSCs • Induces apoptosis by suppressing XIAP mRNA levels 	PMID: 24952669 PMID: 20938744
Cellular inhibitor of apoptosis protein-1	<ul style="list-style-type: none"> • Birinapant 	<ul style="list-style-type: none"> • Promotes death receptors/ caspase-8-mediated extrinsic apoptosis 	PMID: 24526787
NF-κB pathway			
Multiple targets	<ul style="list-style-type: none"> • Parthenolide • DMAPT (Parthenolide analog) 	<ul style="list-style-type: none"> • Inhibits NF-κB activity • Activates p53 pathway • Induces ROS levels and depletes glutathione through inhibition of GCLC and GPX1 • Synergizes with inhibition of PI3K/mTOR pathways 	PMID: 15687234 PMID: 12451177 PMID: 24089526 PMID: 20889920
NF-κB, Hsp90	<ul style="list-style-type: none"> • AR-42 	<ul style="list-style-type: none"> • Inhibits NF-κB activity and increases degradation of Hsp90 client proteins 	PMID: 24934933
Proteasome complex	<ul style="list-style-type: none"> • Bortezomib • MG-132 	<ul style="list-style-type: none"> • Inhibits NF-κB signaling through prevention of IκBα degradation 	PMID: 24382349
IκB kinase (IKK)	<ul style="list-style-type: none"> • BMS-345541 	<ul style="list-style-type: none"> • Synergizes with bortezomib to suppress NF-κB signaling 	PMID: 23416210
Multiple targets	<ul style="list-style-type: none"> • Triptolide • MRx102 (triptolide analog) 	<ul style="list-style-type: none"> • Inhibits NF-κB activity • Induces ROS and inhibits Nrf2/HIF-1α pathways • Decreases expression of anti-apoptotic proteins XIAP and Mcl-1 • Inhibits RNA synthesis 	PMID: 24309935 PMID: 21904380 PMID: 17804695

Target(s)	Agent(s)	Proposed mechanism(s) of action	References
Multiple targets	<ul style="list-style-type: none"> Fenretinide 	<ul style="list-style-type: none"> Suppression of NF-κB and Wnt signaling Induces ROS generation 	PMID: 23513221
TAK1	<ul style="list-style-type: none"> 5z-7-oxozeaenol AZ-TAK1 	<ul style="list-style-type: none"> Blocks NF-κB pathway 	PMID: 25287709
Multiple targets	<ul style="list-style-type: none"> Niclosamide 	<ul style="list-style-type: none"> Inhibits NF-κB pathway Induces ROS levels 	PMID: 20215516
PI3K/AKT/mTOR pathway			
PI3K/AKT mTORC1	<ul style="list-style-type: none"> PI-103 	<ul style="list-style-type: none"> Suppresses signaling downstream of PI3K/AKT/mTOR Synergizes with arsenic to eradicate non-APL LSCs 	PMID: 23335068 PMID: 21803735 PMID: 18548104
mTOR complex 1 (mTORC1)	<ul style="list-style-type: none"> Temsirolimus Rapamycin 	<ul style="list-style-type: none"> Inhibits mTOR signaling Synergizes with clofarabine to induce cell cycle arrest, apoptosis, and autophagy Synergizes with etoposide to eradicate AML LSCs 	PMID: 23271044 PMID: 16150937
Kinases			
Janus kinase (JAK) 2	<ul style="list-style-type: none"> AZD1480 Ruxolitinib TG101209 AZ960 	<ul style="list-style-type: none"> Inhibits the signaling pathways (e.g. STAT3 and STAT5) downstream of JAK2 	PMID: 24668492 PMID: 23812420 PMID: 21128225
Casein kinase 1 α (Csnk1a1)	<ul style="list-style-type: none"> D4476 	<ul style="list-style-type: none"> Decreases ribosomal protein S6 phosphorylation and activates p53 through inhibition of Csnk1a1 	PMID: 24616378
Casein kinase 2	<ul style="list-style-type: none"> Apigenin 	<ul style="list-style-type: none"> Synergizes with PI3K/AKT inhibition to induce apoptosis 	PMID: 21115916
c-Jun N-terminal kinases (JNKs)	<ul style="list-style-type: none"> SP600125 	<ul style="list-style-type: none"> Inhibits the JNK-AP1 pathway which sensitizes LSCs to NF-αB inhibition 	PMID: 24842373
Phosphoinositide-dependent kinase 1 (PDK1)	<ul style="list-style-type: none"> BX-795 	<ul style="list-style-type: none"> Inhibits signaling pathways (AKT/PKC/S6K) downstream of PDK1 	PMID: 24334295
SRC family kinases c-KIT	<ul style="list-style-type: none"> Dasatinib 	<ul style="list-style-type: none"> Activates p53 pathway by inhibiting AKT- mediated phosphorylation of MDM2 	PMID: 23896410
Aurora kinase A	<ul style="list-style-type: none"> MLN8237 C1368 	<ul style="list-style-type: none"> Modulates levels of Bcl-2 family member proteins 	PMID: 23686525 PMID: 23071472

Target(s)	Agent(s)	Proposed mechanism(s) of action	References
Hematopoietic cell kinase (HCK)	<ul style="list-style-type: none"> RK-20449 	<ul style="list-style-type: none"> Suppresses signaling pathways downstream of HCK 	PMID: 23596204
Integrin linked kinase (ILK) & FLT3	<ul style="list-style-type: none"> QLT0267 	<ul style="list-style-type: none"> Inhibits pathways downstream of ILK and FLT3 	PMID: 20193963
PIM Kinase	<ul style="list-style-type: none"> AZD1208 	<ul style="list-style-type: none"> Attenuates STAT5 and destabilizes MYC 	PMID: 25006129
FLT3-ITD	<ul style="list-style-type: none"> Lestaurtinib (CEP-701) Tandutinib (MLN-518) Sunitinib (SU11248) Quizartinib (AC220) 	<ul style="list-style-type: none"> Inhibition of signaling pathways downstream of FLT3 	PMID: 15797998 PMID: 15242881 PMID: 23180436 PMID: 23012328
WNT-β-catenin pathway			
WNT-β-catenin pathway	<ul style="list-style-type: none"> BC2059 Sulindac Indomethacin 	<ul style="list-style-type: none"> Increases proteasome-mediated degradation of β-catenin Reduces β-catenin levels 	PMID: 25482131 PMID: 21811629 PMID: 20339075
Cyclooxygenase (COX)-2	<ul style="list-style-type: none"> Nimesulide 	<ul style="list-style-type: none"> Suppresses β-catenin activation 	PMID: 23645839
Epigenetic regulators			
Histone deacetylase (HDAC) inhibitor	<ul style="list-style-type: none"> Entinostat 	<ul style="list-style-type: none"> Induces expression of Nur77, Nor1, c-Jun, JunB, TRAIL, Bim, and Noxa 	PMID: 23592435 PMID: 23247046
Bromodomain-containing 4 (BRD4)	<ul style="list-style-type: none"> JQ-1 	<ul style="list-style-type: none"> Displaces BRD4 proteins from acetylated histones Suppresses MYC expression 	PMID: 21814200 PMID: 23249862
Histone demethylase KDM1A/LSD1	<ul style="list-style-type: none"> Analogues of tranylcypromine 	<ul style="list-style-type: none"> Suppresses the oncogenic program in MLL leukemia Increases H3K4(me2) and expression of myeloid-differentiation-associated genes. Sensitizes non-APL AML to the pro-differentiation effects of ATRA 	PMID: 22464800 PMID: 22406747
G9a histone methyltransferase	<ul style="list-style-type: none"> UNC0638 	<ul style="list-style-type: none"> Suppresses HoxA9-dependent gene expression 	PMID: 24532712
Polycomb-repressive complex 2 (PRC2)	<ul style="list-style-type: none"> DZNep 	<ul style="list-style-type: none"> Disrupts PRC2 activity, reactivates TXNIP, inhibits thioredoxin activity, and increases reactive oxygen species (ROS) 	PMID: 21734239
Metabolic regulators			
Mitochondrial translation	<ul style="list-style-type: none"> Tigecycline 	<ul style="list-style-type: none"> Disrupts electron transport chain function through inhibition of mitochondrial translation 	PMID: 22094260

Target(s)	Agent(s)	Proposed mechanism(s) of action	References
SIRT1 deacetylase	• Tenovin-6	• Activates p53 signaling and suppresses c-MYC levels	PMID: 25280219
Cell cycle regulators and DNA damage response			
Wee1 kinase	• AZD-1775	• Disrupts cell cycle checkpoint regulation	PMID: 25283841
Checkpoint kinase 1 (Chk1)	• MK-8776	• Potentiates HDAC inhibitor lethality through disruption of intra-S checkpoint, DNA replication, and DNA repair	PMID: 23536721
Multiple targets	• Zalypsis	• Induces dysregulation of DNA damage response	PMID: 21330323
Other mechanisms			
Telomerase	• Imetelstat	• Antisense oligonucleotide targeting the RNA component of the telomerase complex	PMID: 25479751
Multidrug resistance protein 4 (MRP4)	• Probenecid	• Increases intracellular cAMP levels through inhibition of MRP4 activity	PMID: 25301721
Hypoxia-inducible factor (HIF)1 α	• Echinomycin	• Inhibits HIF-1 α DNA binding and transcriptional activity	PMID: 24994068
SUMO-conjugating enzymes	• Anacardic acid	• Restores expression of the pro-apoptotic gene DDIT3 through inhibition of the SUMO pathway	PMID: 24910433
miR-21 and miR-196	• Antagomirs	• Inhibits cellular transformation mediated by HOX-signaling oncoproteins	PMID: 24334453
Lysosomes	• Mefloquine	• Disrupts lysosomes, permeabilizes the lysosome membrane, and releases cathepsins into the cytosol	PMID: 23202731
Unknown	• ErPC3	• Induces cell cycle arrest and apoptosis through JNK- and PP2A-dependent mechanisms	PMID: 20200557
Unknown	• Kinetin riboside	• Exact mechanism unknown but requires adenosine kinase activity	PMID: 22160482
Ribonucleotide reductase	• Ciclopirox	• Chelates intracellular iron and inhibit the iron-dependent enzyme ribonucleotide reductase	PMID: 19589922
AML1-ETO	• Oridonin	• Induces caspase-3-mediated enzymatic cleavage of AML-ETO to a truncated form	PMID: 22461642

Target(s)	Agent(s)	Proposed mechanism(s) of action	References
Multiple targets	<ul style="list-style-type: none">• TDZD-8	<ul style="list-style-type: none">• Induces rapid loss of membrane integrity and depletion of free thiols• Inhibits PKC and FLT3 signaling pathways	PMID: 17785584
Heme oxygenase 1	<ul style="list-style-type: none">• PEG-ZnPP• SMAZnpp	<ul style="list-style-type: none">• Inhibits cytokine-dependent proliferation of LSCs	PMID: 22165967