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Leukemia (PH Wiernik, Section Editor)

Acute Myeloid Leukemia Mutations and Future Mechanistic Target to Overcome Resistance

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Opinion statement

Cytogenetics and mutation identification in acute myeloid leukemia have allowed for more targeted therapy. Many therapies have been approved by the FDA in the last 3 years including gilteritinib and azacitidine but the overall survival has remained stagnant at 25%. The inability to achieve complete remission was related to the residual leukemic stem cells (LSCs). Thus, the relationship between bone marrow niche and LSCs must be further explored to prevent treatment relapse/resistance. The development of immunotherapy and nanotechnology may play a role in future therapy to achieve the complete remission. Nano-encapsulation of drugs can improve drugs' bioavailability, help drugs evade resistance, and provide combination therapy directly to the cancer cells. Studies indicate targeting surface antigens such as CLL1 and CD123 using chimeric antibody receptor T cells can improve survival outcomes. Finally, new discoveries indicate that inhibiting integrin αvβ3 and acid ceramidase may prove to be efficacious.

Introduction

Acute myeloid leukemia (AML) is an aggressive, heterogeneous malignancy presenting with bone marrow failure due to uncontrolled proliferation of leukemic (blast) cells in bone marrow. Leukemic stem cells (LSCs) give rise to myoblasts that continue to proliferate without differentiating, leading to immature blasts [1].

An estimated 21,450 patients were diagnosed with AML in 2019 and there were 10,920 deaths due to AML [1]. Risk factors associated with diagnosis include blood disorders, radiation exposure, prior chemotherapy treatment, and older age with the median diagnosis at 68 years [1]. AML has a poor prognosis, with overall survival (OS) of 25%, and it can be associated with rapid progression of disease along with resistance to current treatment options [1].

Cytarabine administered over 7 days and daunorubicin given over the first 3 days of treatment (7+3) has been the mainstay of AML treatment for the last half century [2]. Toxicity and resistance ultimately lead to treatment failure and relapse. This treatment is accompanied by severe liver, gastrointestinal, and BM toxicity [2]. Thus, new treatment methods are needed to treat AML through different mechanistic approaches.

Cytogenetics and targeted therapy have given us different angles to attack AML to reduce toxicity and destroy resistant malignant cells. There has been more progress made in AML therapy in the last 3 years than ever before, with a plethora of targeted therapies approved, as depicted in Table 1. However, rapid drug resistance to newer therapy has proven to be a problem, and studies indicate that we need to change the approach to AML treatment.

Historically, complete remission (CR), defined as BM blast count of less than 5%, has been the endpoint for the majority of clinical trials and treatments [3]. This often leaves minimal residual disease (MRD) in the BM, which is highly associated in relapse. A retrospective study analyzing 245 adults showed that 2-year relapse was ~80% in patients who were MRD+ regardless of CR [4]. High relapse rate associated with MRD+ patients is due to the presence of leukemic stem cells (LSCs), responsible for the leukemogenesis. MRD– patients had a relapse rate of ~35%, showing that MRD should be the endpoint in clinical trials to assess for relapse-free survival (RFS) [4].

LSCs are cancer hematopoietic cells that can self-proliferate, differentiate, and survive independently [5]. They express the same receptor markers as normal hematopoietic stem cells, such as CD34 and CD38, which makes them difficult to target [6]. Standard therapy often eliminates the majority of malignant cells but leaves residual LSCs in the BM. They form niches in the BM that give them the tools to evade chemotherapy and receive pro-survival signals. Although patients can reach CR, residual LSCs are mutating and proliferating and will eventually lead to patient relapse [6]. To ensure patients do not relapse, they must reach CR and MRD –. Given AML's heterogeneity and rapid disease progression, patients need to be treated quickly and aggressively with combination therapy to completely eliminate the disease.

This review will briefly go over updated treatment options that are currently in practice. Then we explore the complex mechanism of AML resistance and look at preclinical and clinical trials that indicate ways to overcome it. Finally, we discuss what the future of AML treatment may look like through discovery of new mechanistic targets and development of new technologies.

Current treatment regimen

Newly diagnosed patients with AML are first treated with induction therapy and then consolidation therapy once they are in remission. The backbone of AML treatment, the 7+3 regimen (cytarabine for 7 days along with an anthracycline infusion for the first 3 days), results in remission of 60–80% of younger patients (<65 years) and 40–60% of older patients (≥65 years) [7]. Based on genetic and mutational identification, more specific treatment methods have been developed within the last 3 years. Table 1 summarized the FDA-approved drugs for AML during the past 5 years.

Table 1. FDA-approved drugs for AML during the past 5 years

Drug	Approved date	Mechanism of action	Manufacturer	Indication
Rydapt (midostaurin)	Apr 2017	1st-generation FLT3 inhibitor	Novartis Pharmaceuticals	Newly diagnosed AML w/ FLT3+
Idhifa (enasidenib)	Aug 2017	IDH2 enzyme inhibitor	Celgene Corp	R/R AML w/ IDH2 mutation
Vyxeos (daunorubicin/cytarabine) Liposomal combination fixed 1:5 ratio	Aug 2017	Daunorubicin-intercalation between DNA base pairs Cytarabine-pyrimidine analog	Celator Pharmaceuticals	Newly diagnosed therapy-related AML or myelodysplasia-related AML
Mylotarg (gemtuzumab ozogamicin)	Sep 2017	CD-33 monoclonal antibody drug conjugate with igG4 antibody linked to calicheamicin derivative	Pfizer	Newly diagnosed or R/R AML in CD33-positive AML
Tibsovo (ivosidenib)	Jul 2018	IDH1 enzyme inhibitor	Agios Pharmaceuticals	Newly diagnosed AML in adults at least 75 years of age w/IDH1 mutation or R/R AML w/IDH1 mutation
Xospata (Gilteritinib)	Nov 2018	FLT3 inhibitor targeting FLT3-ITD, FLT-TKD, and FLT-D835 mutation	Astellas Pharmaceuticals	R/R AML with FLT3 mutation
Venclexta (venetoclax)	Nov 2018	BCL-2 inhibitor	Genentech Inc.	Newly diagnosed AML in patients 75 years or older
Daurismo (glasdegib)	Nov 2018	Hedgehog inhibitor	Pfizer	Newly diagnosed AML in patients 75 years or older
Onureg (azacitidine)	Sep 2020	Hypomethylating agent	Celgene Corp	Continued treatment of AML patients who achieved first CR or CRi following intensive induction chemotherapy who are not able to complete intensive curative therapy

Abbreviations: *AML* acute myeloid leukemia, *FLT3* fms-like tyrosine kinase 3, *IDH* isocitrate dehydrogenase, *ITD* internal tandem duplication, *TKD* tyrosine kinase domain, *R/R* relapsed/refractory, *CR* complete remission, *CRi* complete remission with incomplete hematologic recovery, *BCL-2* B cell lymphoma

FLT3

FLT3 is a transmembrane receptor ubiquitous in myeloid cells and responsible for cell development. It is activated by the binding of FLT3 ligand and promotes the dimerization of the receptor and triggers autophosphorylation and signal transduction. These signals favor cell proliferation, survival, and differentiation in hematopoietic and progenitor cells [8]. In malignant cells, there are high levels of FLT3 activity along with mutations to these receptors seen in roughly 30% of AML patients, and FLT3-Internal Tandem Duplication (ITD) mutation

in particular is found in 25% of these patients. A FLT3-ITD mutant/wild-type allelic ratio of >0.5 is associated with unfavorable outcomes [9].

Over the last 3 years, a variety of FLT-3 inhibitors were introduced and had modest effects on patient survival. First-generation FLT3 inhibitors such as midostaurin (FDA approved in 2017), sorafenib (phase 3), lestaurtinib (phase 2), and sunitinib (phase 2) are broad spectrum FLT3 inhibitors that target both FLT3-ITD and FLT3-TKD mutations. These are often given with chemotherapy and data have shown efficacy in OS and event-free survival (EFS) [10]. Compared to other first-generation FLT3 inhibitors, midostaurin showed benefit in FLT3 mutations with the lowest amount of toxicity. It had minimal antileukemic activity when used alone, but its tolerance in patients warranted clinical trials to test its efficacy in combination therapy [11]. The Randomized AML Trial in FLT3 in patients less than 60 Years old (RATIFY) studied midostaurin and standard chemotherapy combination and is the largest AML FLT3 mutation study to date with 717 patients. This phase 3 clinical trial showed improved OS in AML patients with both FLT3-ITD and FLT-TKD mutations using midostaurin in combination with standard induction therapy [12]. It is important to note that midostaurin was more effective in patients who received allogeneic hematopoietic stem cell transplant after CR from induction therapy [11].

Gilteritinib and quizartinib are second-generation FLT3 inhibitors with higher potency for FLT-ITD compared to first-generation FLT3 inhibitors [13]. Quizartinib is still under investigation because AML cells were quick to develop resistance through tyrosine kinase AXL activation and FLT3-D835 (a point mutation) [14]. Gilteritinib on the other hand has affinity to FLT-ITD, AXL, and FLT3-D835. In a phase 1/2 study of 252 patients, Perl et al. showed that gilteritinib is effective in treating patients with relapsed and refractory (R/R) AML with an overall response rate (ORR) of 40% [15]. Gilteritinib was more effective in $FLT3^{mut+}$ with an ORR of 52%. It also showed that once daily dosing (20–450 mg) was enough to treat cancer cells without causing toxicity, primarily due to its long elimination half-life (113 h) and high potency (>90% FLT inhibition with doses ≥80 mg) [16]. Gilteritinib treatment showed prolonged survival (median OS, 31 weeks) compared to salvage chemotherapy (median OS, 15–21 weeks) in treatment of R/R AML patients [15]. Gilteritinib's ability to treat resistance $FLT^{mutant+}$, tolerable dosing, and therapeutic efficacy led to FDA approval in 2018 for R/R AML patients with FLT3 mutation [17••].

IDH1/2

Isocitrate dehydrogenase 1 (IDH1) and isocitrate dehydrogenase 2 (IDH2) are enzymes involved in cellular metabolism, epigenetic regulation, and DNA repair [18]. IDH1 and IDH2 mutations are found in 7–14% and 8–19% of AML patients, respectively [19]. Mutations occur in active sites of these enzymes, leading to an increase in d-2-hydroxyglutarate. Through competitive inhibition of α-ketoglutarate (αKG)-dependent enzymes, d-2-hydroxyglutarate altered cellular metabolism, epigenetic regulation, and DNA repair leading to carcinogenesis [18]. Enasidenib was approved in 2017 for IDH2 mutations and ivosidenib was approved in 2018 for IDH1 mutations. Enasidenib was shown to be an effective salvage therapy in R/R AML patients with actionable IDH2 mutations, inducing an approximately 40% response rate and a CR rate of 19%

[19]. It is also non-cytotoxic so it can be used in older patients with comorbidities who are not eligible for intensive induction therapy [19].

BCL-2

There are other targeted therapies such as venetoclax, a B cell lymphoma-2 (BCL-2) inhibitor, approved by the FDA in 2018 in combination with a hypomethylating agent (HMA) or low-dose cytarabine (LDAC) in patients 75 years or older. BCL-2 is an oncogene that functions to inhibit apoptosis. Once encoded, it plays a role in inhibiting BAX and BAK, which are pro-apoptotic proteins. B cell lymphoma-extra-large (BCL-XL) and myeloid cell leukemia-1 (MCL1) are also anti-apoptotic proteins involved in inhibiting BAX/BAK and preventing release of cytotoxic chemicals from the mitochondria. In normal cells, these pro-apoptotic and anti-apoptotic factors are in balance, but there is upregulation of anti-apoptotic genes in AML [20]. Thus, targeting these anti-apoptotic factors has been successful. Venetoclax in combination with LDAC or HMA has shown CR/CRi (complete remission with incomplete hematologic recovery) rates of 54% and 67%, respectively. The median OS was 10.4 months for combination with LDAC and 17.5 months for combination with HMA [21, 22].

Hedgehog inhibitors

Glasdegib is a hedgehog (Hh) inhibitor that was approved by the FDA in 2018 in combination with LDAC for AML patients who are 75 years of age or older. The hedgehog signaling pathway is normally silenced in adults [23]. Aberrant activation of this pathway is seen in AML patients and allows for leukemic stem cell survival and proliferation [24]. This pathway starts with Hh ligand binding to its receptor, leading to the degradation of pathway repressor patch (PTCH1). PTCH1 normally inhibits smoothened (SMO), but its inactivation allows SMO, a G protein–coupled receptor, to translocate to the cilia. Elevated levels of SMO stabilize glioma-associated oncogene homolog transcriptional factors (GLI TF), which are normally repressed by SUFU and other kinase phosphorylation. GLI TF in its active form translocates to the nucleus and promotes cell survival proteins [25]. This is seen in relapsed patients because activation of this pathway allows for leukemic cells to develop resistance. It was shown that AML cells with overexpression of hedgehog signaling correlate to chemo-resistance compared to AML with less hedgehog signaling [26]. Thus, inhibition of this pathway using glasdegib + LDAC resulted in a 49% reduction in the risk of death compared to LDAC. The ORR with combination therapy was 26.9% compared to 5.3% with LDAC alone in a study involving 132 patients [27].

Even with the rapid treatment advancements made in the last 3 years, the OS in AML patients is still poor. The therapies currently available cannot induce consistent CR^{MRD-} and lead to eventual relapse.

Microenvironment-mediated drug resistance

The poor OS in AML patients even after the plethora of new targeting medications led to questions on mechanisms of resistance [28]. Although this subject is

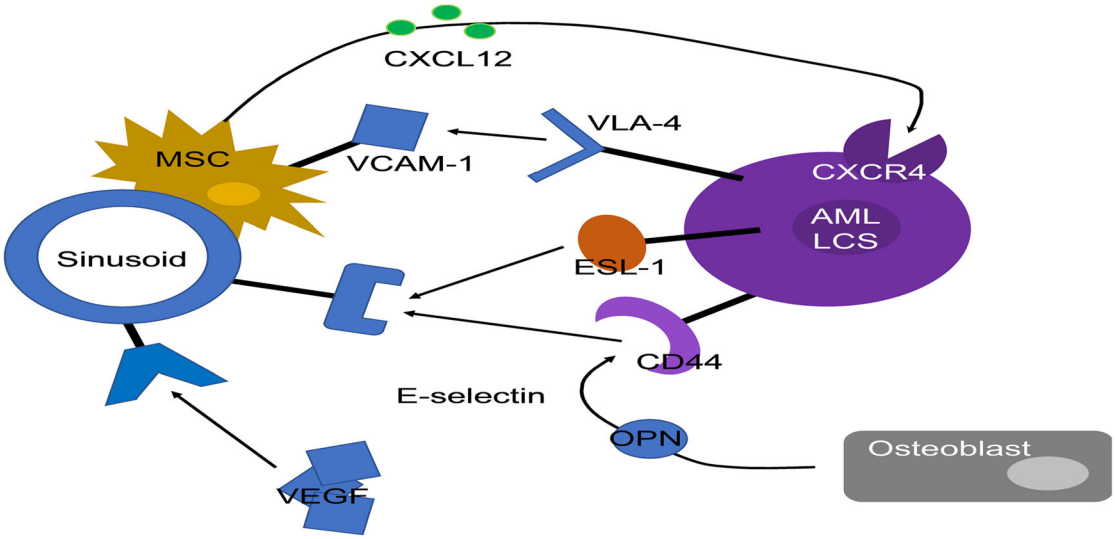
not completely understood, the role of the BM in drug resistance cannot be ignored.

As discussed earlier, LSCs' survival post-therapy leads to disease relapse. Studies show that the LSCs may be able to develop a niche in the BM where they can evade therapy and receive pro-survival signals. AML cells can upregulate adhesion receptors such as very late antigen-4 (VLA-4), CD44, and E-selectin ligand-1 (ESL-1), which then interact with molecules such as vascular adhesion molecule-1 (VCAM-1), fibronectin (FN), hyaluronan (HA), and osteopontin (OPN) to engraft in the BM. They interact with mesenchymal stromal cells (MSCs) that provide signals that range from chemokines to pro-inflammatory mediators to survive and replicate. This environment is normally used by hematopoietic stem cells (HSC) but is taken over by LSCs. The cancer stem cells can remodel the niche to their favor through angiogenesis and changes in MSC phenotype. This protection in the BM along with its ability to mutate makes AML difficult to treat. Even if therapy is effective enough to destroy a majority of malignant cells, the residual LSCs can eventually produce resistant leukemic progenitor, resulting in a more aggressive disease. The goal is to completely eradicate LSCs by targeting the BM niche and prevent any MRD. To do that, we must first understand the complex mechanism that encompasses this environment. The microenvironment interaction is highlighted in Fig. 1.

VLA-4 allows AML cells to interact with VCAM-1 located on MSCs, endothelial cells, and extracellular components [29]. The binding of VLA-4 and VCAM-1 between LSCs and MSCs plays two important roles in carcinogenesis. First, this adhesion interaction promotes homing and retention of AML cells to the BM. Second, the VLA-4/VCAM-1 interaction promotes the transfer of pro-survival and proliferation signals such as nuclear factor kappa B (NF-kB), mammalian target of rapamycin (mTOR), and mitogen-activated protein kinase (MAPK) between LSCs and MSCs. VCAM-1 can also bind to FN on stromal cells, which activates phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt)/Bcl-2 pathway. Ultimately, these cell signals provide chemo-resistive properties for malignant cells [30]. Thus, targeting VLA-4 has potential to overcome resistance. Currently, there is a clinical trial ongoing studying chemotherapy combination with FN inhibitor to see if it can overcome resistance (NCT01010373).

Overexpression of CD44 in AML blasts plays an important in engraftment in the BM [31]. Engraftment depends on the CD44 binding to HA and OPN. Ellis et al. showed that homing of transplanted HSC occurred near the trabecular bone-rich region of the femur where there are plenty of blood vessels that supply HA [32]. Gutjahr et al. showed that CD44/HA binding triggered activation of VLA-4 on receptors on the surface of malignant cells and increased the VLA-4/VCAM-1 binding strength with MSCs [33]. This CD44-VLA-inside-out activation is not seen in normal progenitors, indicating this is a form of acquired resistance by tumor cells and making CD44 an interesting target for the future [33].

AML cells can take advantage of osteoprogenitor cells to support their survival. Battula et al. reported that AML cells induce osteogenic differentiation in MSCs while inhibiting adipogenic differentiation, which is something that is not seen in normal MSCs [34]. Overexpression of pre-osteoblast and osteoblast will lead to tumor growth and engraftment mediated by the release of granulocyte colony-stimulating factor, OPN, and chemokines [35]. Using mice



Abbreviations: VLA-4=Very late antigen-4; ESL-1=E-selectin ligand-1; MSC=mesenchymal stromal cells; OPN=osteopontin; CxCL12=CXC motif ligand 2; CXCR4=chemokine receptor 4; VCAM-1=vascular adhesion molecule-1

Fig. 1. LSCs hijacking the normal BM and HSC interactions. ESL-1 and CD44 interacts with E-selectin on the surface of sinusoids, which initiates the initial rolling on the endothelium cells. VLA-4 on the surface of LSCs can bind to VCAM on the surface of MSCs to promote homing in the BM. This interaction allows for transfer of pro-survival and proliferation signals. MSCs produce CXCL12 cell signals, which promotes cell migration and adhesion in the BM, and ultimately cell survival. MSCs can differentiate into osteoblasts and they produce OPN, which promotes tumorigenesis. VEGF production stimulates angiogenesis, and this is essential in the growth and development of leukemia in the BM.

models, Zhang et al. showed that increased osteoblast activity is directly correlated to increased HSC count and niche size [36]. Chemokines such as CXCL12 expression by osteoblast are crucial in mobilization for LSCs.

E-selectin is another adhesion molecule found in the endothelium of the BM. This ligand can bind to ESL-1 or CD44, which are both overexpressed in AML cells, causing engraftment and Wnt signal activation responsible for cell survival [30]. Uproleselan, an E-selectin antagonist, showed clinical efficacy (CR/CRi of 39%) and tolerability in combination with chemotherapy in 91 patients [37]. This is now in phase 3 clinical trial for R/R AML in 380 patients NCT03616470.

AML cells often acquire overexpression of chemokine receptor 4 (CXCR4) that can bind to CXC motif ligand 2 (CXCL12), a chemokine produced in the BM [38]. This interaction plays a role in AML cells developing their niche in the BM through engraftment, migration, and cell signaling via MSCs [39]. High levels of CXCR4 are associated with poor prognosis and treatment resistance [30]. A phase 1–2 study analyzed the combination of plerixafor, a CXCR4 antagonist, with standard chemotherapy in 57 patients. The results were CR/CRi of 50% in primary refractory patients and 47% among early relapse patient [40]. This shows the great promise of CXCR4 inhibition to overcome resistance.

Evidently, the AML-MSC relationship is important to develop a niche in the BM. Borella et al. conducted a preclinical trial comparing AML-MSC to healthy BM donors (h-MSC) [41]. They found that AML-MSC cells proliferate up to 21 days and original AML blasts are still able to keep their clonogenicity properties

and cell surface markers/proteins. AML-MSC proliferated at a much faster rate (40% increase) compared to h-MSC and induced faster osteogenic differentiation, with osteogenic differentiation by AML-MSC starting at day 7 while h-MSC started at day 21.

LSCs are attracted to hypoxic niches in the BM. These oxygen-deprived areas lack blood flow, therefore making it difficult for drugs to reach them. A study showed that glycolysis process was higher in the femur of LSC transduced mice compared to healthy mice, indicating LSCs can reprogram metabolism [42]. Under normal conditions, hypoxia-inducible factor 1α (HIF-1α) is hydroxylated and is then recognized by von Hippel-Lindau protein, leading to ubiquitination. Under hypoxic conditions, HIF-1α is not hydroxylated and proceeds to form a dimer complex, HIF-2α. This complex can conduct transcription of hypoxic responsive elements (HRE) that upregulate CXCR4, CXCL12, Bcl-2, and VEGF, promoting drug resistance. Preclinical data supported the use of TH-302, a prodrug containing chemotherapy activated under hypoxic conditions, because it showed an increase survival of mice [43]. The therapy went to phase 1 clinical trial but unfortunately failed due to lack of efficacy [44]. Nevertheless, the concept of a hypoxic activated drug gives us another mechanistic aspect to target solely malignant cells.

The idea that LSCs can overtake the BM niche is not one to be ignored. Without addressing this microenvironment-mediated resistance, relapse rates cannot improve. Researchers have done a great job in identifying key targets involved in microenvironment-mediated resistance so far, and now these areas should be furthered explored in clinical trials.

Resistance towards novel therapy

Gilteritinib

When discussing FLT3 inhibitors, gilteritinib proved to be the most effective in R/R AML due to its efficacy against FLT3-D835 mutation, AXL activation, and dose-dependent inhibition of FLT3-F691 substitution. Yet treatment failure still occurs due to BM cytokines providing protection and resistance against therapy [13]. Studies show that acquired resistance to gilteritinib can form through alternative cell signaling in the RAS/MAPK pathway. These cells that develop acquired resistance undergo clonal expansion to give rise to sub-clones that are immune to gilteritinib. One study also identified 2 patients who developed BCR-ABL1 fusion mutation, conferring resistance [45].

Sung et al. conducted an in vivo study in mice to analyze AML cell resistance to FLT3 inhibitors and highlighted a different cell signaling pathway used [13]. This study identifies granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 2 (IL-2) pathway as a redundant survival tool. This activates the downstream pathway of JAK2/STAT5/PIM that allows for cell survival, but not proliferation. The FLT-ITD-mutant mice that received only a FLT3 inhibitor had malignant cell survival without proliferation. The combination of ruxolitinib (JAK2 inhibitor) or INCB05391 (PIM inhibitor) with a FLT3 inhibitor stopped cell survival and proliferation, sensitizing FLT-ITD-mutant cells to FLT3 inhibitors [13]. This shows promise that JAK2/PIM inhibitor can overcome FLT3 inhibitor resistance when given in combination. Currently, there are not any clinical trials with FLT3 inhibitors combined with JAK2/PIM inhibitors; however, it indicates that combination therapy must be used to

destroy malignant cells before they can mutate.

Venetoclax

Treatment failure related to venetoclax in combination with standard therapy can be linked to BM-mediated resistance (BMMR). Its inhibitory properties are towards BCL-2, without any specificity towards other anti-apoptotic proteins such as BCL-XL or MCL-1. In the presence of BCL-2 inhibition, stromal cells can upregulate MCL-1 expression, providing an alternative anti-apoptotic route, leading to treatment failure [46]. O'Reilly et al. in an in vitro and in vivo study supported this by showing that inhibition of BCL-2 and MCL-1 reversed resistance towards 7+3 treatment [46].A study conducted by Han et al. looked into the combination of venetoclax with a mitogen-activated protein kinase (MEK) inhibitor to overcome this resistance [47]. Mechanistically, inhibiting MEK/extracellular signal–regulated kinase (ERK)/MAPK cascade prevents the stabilization of MCL1, therefore preventing the inactivation of pro-apoptotic proteins. In this study, cobimetinib, an FDA-approved medication against metastatic melanoma, was used as an allosteric MEK inhibitor in combination with venetoclax. The study tested cells collected from the BM and peripheral blood of healthy and AML patients. Results supported the hypothesis that the combination of the two therapies had synergistic effects. Alone, the therapies were minimally effective in part due to resistance and lack of efficacy as monotherapy. In combination, the therapy showed significantly more efficacy in malignant cell death in vitro, inducing apoptosis in over 60% of resistant cell lines compared to 16.7% with venetoclax alone [47]. This combination therapy is currently in phase 1 clinical trial and is estimated to be completed in February 2021 (NCT02670044).

Hedgehog inhibitors

Glasdegib is unique because it is the first AML therapy that targets LSCs through the Hh signaling pathway, providing a tool to attack dormant stem cells [48]. Even so, several mechanisms of resistance have already been identified. One mutation is in the ligand-binding pocket of SMO receptor and impairs SMO inhibitors from binding to the active site. Mutations can occur outside of the binding site as well for continuous activation regardless of SMO inhibitor inhibition. Another mutation is downstream activation leading to glioma-associated oncogene homolog 1 (GLi1) immunity against suppressor of fused (SUFU) inhibition and depletion of SUFU. Studies are now showing that GLi1 can be activated independently of the SMO pathway through alternative cell signaling such as MAPK, mTOR, and TGFß. Upregulation of GLI2 can be used as a another resistance tool [48–50]. The resistance mechanisms for novel therapy are summarized in Table 2.

Future target mechanisms

Acid ceramidase

Acid ceramidase (AC) is an enzyme that metabolizes ceramide into sphingosine. Increased levels of AC lead to reduced ceramide and higher levels of sphingosine, which is then phosphorylated by sphingosine kinase to form sphingosine 1-phosphate (S1P). S1P promotes cell migration, cell cycle progression, and cell survival. S1P activates the NF-kB pathway, which leads to an

Table 2. Mechanisms of drug resistance to current therapies

Therapy	Mechanisms of Resistance
Cytarabine/daunorubicin (7+3)	1) Downregulation of ENT1 responsible for drug uptake in cell 2) Activation of AKT and mTOR pathways 3) Secretion of soluble factors such as interleukins and G-CSF 4) Increase P-gp efflux expression 5) Activation BCL-2 genes
Midostaurin	1) TP53 mutation 2) Activation MAPK cascade 3) FLT3-F691L mutation
Idhifa (enasidenib)	- $IDH2^{R140Q}$ mutation changing the catalytic site
Mylotarg (gemtuzumab ozogamicin)	- Overexpression of $ABCA_3$ transporter and HFE gene preventing GO internalization into AML cells to induce cytotoxicity
Xospata (gilteritinib)	1) Activation of MAPK cascade 2) Secretion of soluble factors such as interleukins and G-CSF 3) CYP3A4 metabolism
Venclexta (venetoclax)	1) Activation of BCL-XL and MCL-1 anti-apoptotic pathways 2) Deletion of BAX gene
Daurismo (glasdegib)	1) Mutation on or outside of ligand-binding pocket of SMO receptor 2) Decrease SUFU or mutation in GL1 leading to immunity against SUFU 3) MAPK and mTOR activation of GLi1 4) Upregulation of GLI2
Onureg (azacitidine)	- Mutations in PTPN11, FLT3, BCOR, and EZH2 in AML cells providing aberrant survival signals.

Abbreviations: *ENT1* equilibrative nucleoside transporter1, *mTor* mammalian target of rapamycin; *G-CSF* granulocyte colony-stimulating factor, *P-gp* permeability glycoprotein, *BCL-2* B cell lymphoma-2, *MAPK* mitogen-activated protein kinase, *FLT3* fms-like tyrosine kinase 3, *IDH* isocitrate dehydrogenase, *$ABCA_3$* ATP-binding cassette sub-family A member 3, *HFE* human homeostatic iron regulator protein, *BCL-XL* B cell lymphoma-extra-large, *MCL-1* myeloid cell leukemia, *SMO* smoothened, *SUFU* suppressor of fused, *GLi1* glioma-associated oncogene homolog 1, *PTPN11* tyrosine-protein phosphatase non-receptor type 11

increase in ATP-binding cassette transporter that is responsible for drug efflux, P-glycoprotein (PGP). Recent studies show that glucosylceramide synthase (GCS), the sphingolipid enzyme, can also increase PGP expression [51]. Its overexpression allows malignant cells to evade chemotherapy by preventing it from entering the cells. Thus, reducing PGP protein expression through targeted therapy can improve the outcome for AML patients.

Targeting PGP has not been successful because of drug toxicity, so targeting AC provides a different mechanism [52]. Overexpression of AC levels has been linked to higher NF-kB and PGP activity. A study published by the American Society for Biochemistry and Molecular Biology goes deeper into these linkages and is discussed below [53].

The cell lines used in this study were parenteral HL-60 cells, HL-60 cells with acquired resistance to vincristine (HL-60/VCR), and HL-60 cells with acquired resistance to the BCL-2 inhibitor ABT-737 (HL-60/ABTR). The resistant cell lines were compared to the parenteral cell lines and the resistant cells had elevated PGP expression with increased levels of AC. These cells were then given a variety

of treatment regimens and compared for efficacy. The study compared standard chemotherapy (cytarabine, daunorubicin, and mitoxantrone) in each of these cell lines with and without an AC inhibitor (LCL204). Using LCL204 overcame chemotherapy resistance for all 3 standard chemotherapy drugs used in the resistant HL-60 cells, and the cells that only received chemotherapy showed resistance to the treatments. In addition, lower levels of AC and PGP were seen in cells that received LCL204. This highlights the potential of AC inhibitors to overcome standard chemotherapy resistance by inhibiting AC and decreasing PGP expression. Furthermore, AC knockdown ranged from 71 to 92%, resulting in PGP reduction of 45–87%. Finally, this study investigated the link between NF-kB and AC using S1P inhibitors and NF-kB inhibitors. There was a positive correlation between AC overexpression and NF-kB activity [53].

Overexpression in AC can lead to resistance through PGP and NF-kB activity. More research needs to be done to determine the specific links.

Integrin αvβ3

Integrin αvβ3 is a heterodimer integrin that plays a role in cell adhesion, cell cycle, and cell proliferation. It is abundantly expressed in tumor cells and provides a mechanism of metastasis and angiogenesis [54]. αvβ3 has a receptor for L-thyroxine (T4) that will increase the expression of programmed death ligand 1 (PDL-1), which can regulate immune checkpoints and allow malignant cells to proliferate and survive [55]. T4 interaction with αvβ3 can induce angiogenesis and cell proliferation [56]. This highlights the important role of overexpression of thyroid hormones in cancer patients and can be associated with poor prognosis.

Studies show αvβ3 expression is associated with AML leukemogenesis and disease progression through increased levels of syk kinase and HoxA genes, which allow for downstream signaling through NF-kB and PI3k pathways. These cell signaling pathways provide a mechanism of evasion of drug therapy for malignant cells [57]. Azzariti et al. concluded that sorafenib resistance was linked to increased α3β1 (an integrin in the same family) activation in patients with hepatocellular carcinoma [58].

These studies indicate the need for novel mechanistic approaches such as αvβ3 inhibition to inhibit all downstream signaling and prevent disease progression. As mentioned earlier, hyperthyroidism can subsequently activate the αvβ3 because of the T4 ligand, making this a promising target. Tetraiodothyroacetic acid (tetrac) is a T4 analog with αvβ3 antagonistic effects. The use of tetrac has shown promise in not only AML, but prostate, renal, and various other cancer types as well [59, 60]. The preclinical trial seems promising and this calls for further exploration of tetrac in cancer therapy.

Immunotherapy

CAR T cell therapy is a method of engineering T cells with synthetic receptors that recognize specific targets on the surface of malignant cells. The engineered T cells are cultivated and given back to the patient intravenously. Then the patient's immune system is able to identify and eliminate cancer cells based on human leukocyte antigen recognition [61].

Treatment with CAR T cells has shown success in relapsed and refractory acute lymphoblastic leukemia (ALL) and diffuse large B cell lymphoma (DLBL). In 2017, the FDA approved two CD19 CAR T cell therapy for B cell malignancies, KYMRIAH and YESCARTA, giving scientists hope that this technique can be applied to AML treatment [62].

C-type-lectin-like-molecule-1 (CLL-1) and CD33 are potential targets due to their high level of expression in AML cells [63••]. Fang Liu et al. conducted a study using the combination of CD33 and CLL-1 (cCAR) in treating AML in human cell lines, mouse models, and children and adults with R/R AML [64]. The phase 1 trial showed the ability to eradicate LSCs, indicating the potential for cCAR treatment in patients with R/R AML. Specifically, a 6-year-old patient diagnosed with AML with FLT3-ITD having 81% of leukemia blast in the BM was treated with cCAR. Previously, 5 cycles of treatment had failed in this patient, including combination therapy with FLT3 inhibitor, due to AML resistance. The patient achieved complete remission after treatment with cCAR, which highlights its potential in overcoming resistance. cCAR showed the ability to induce total myeloid ablation but at a safer level than standard chemotherapy or radiation therapy [64]. An update on this trial posted in June 2020 reported that 7 out of 9 patients achieved MRD− within 4 weeks of cCAR infusion. Out of the 2 patients that did not achieve MRD−, one was determined to be CD33+/CLL1−, which highlights the importance of targeting CLL1− [65]. Phase 1 clinical trials are still ongoing to support its efficacy and tolerability.

Another antigen that CAR T cell targeting has shown efficacy towards is CD123. Budde et al. conducted a study analyzing the safety and efficacy of CD123 CAR T cell therapy in 6 R/R AML patients following allogeneic hematopoietic stem cell transplantation [66]. Two of these patients were treated with low-dose therapy and 1 patient achieved a morphologic leukemia-free state that lasted 2 months. This patient received a second round of therapy after 3 months and results showed a blast reduction from 77.9 to 0.9%. Of the 4 patients receiving normal dosing, 2 patients had complete remission with 1 patient becoming transfusion independent. The other 2 patients had a reduction in blast count but not complete remission. This trial highlighted the safety of CD123 CAR T cell therapy because there were no dose-limiting toxicities and no treatment-related cytopenias. This study points to a different targeting moiety of CAR T cells that is both efficacious and tolerable.

A recent study published in June 2020 analyzed the use of allogeneic FLT3 CAR T cells with rituximab as an off switch to target malignant cells in the BM while minimizing toxicity. These cells were engineered to target FLT3 along with two short mimotopes that rituximab can target. The study was conducted on human HSCs and mice. FLT3 CAR T cells successfully eradicated leukemic cells. In vitro and in vivo study supported that rituximab successfully turned off the FLT3 CAR T cells, allowing BM recovery after therapy. The efficacy of this in human trials is still questionable due to the difference in expression of FLT3 between human and mouse [67].

Furthermore, the dual targeting therapy towards the antigens expressed on AML cells may be a safer treatment option to eradicate LSCs [68]. He et al. developed a bispecific CAR T cell with CD13 and TIM3 specificity that was highly active in the leukemic microenvironment. The preclinical data showed eradication of AML in the BM, blood, and spleen. CD13 is expressed in normal HSCs but the addition of TIM3 improved selectivity for AML since these cells predominantly express this

marker [69]. The bispecific approach allows for higher malignant cell selectivity and improves eradication. There are a many CAR T cells in trial with unique targeting mechanisms and some are highlighted in Table 3.

Checkpoint inhibitors (CIs) represent a different form of immunotherapy that are currently under investigation. In AML patients, effector T cells are in the "exhausted" state due to overexpression of inhibitory receptors. CI can block the inhibitory molecules for effector T cells to gain back function. Success of CI can be seen solid tumors but its use in AML is still under investigation [70]. Inhibition of PD-1, PDL-1, and CTLA-4 are widely being studied for efficacy. The use of PD-1 inhibitors as monotherapy has shown modest benefits in AML. One out of 8 patients showed minimal response to pidilizumab treatment and 0 out of eight patients showed a response to pembrolizumab [71,72]. However, the combination of PD-1 inhibitor with HMA shows clinical efficacy. A phase 2 study by Daver et al. included 70 AML patients and explored the use of nivolumab (PD-1 inhibitor) + azacitidine. The ORR was 33% with a CR/CRi rate of 24% [73]. This emphasizes the potential of CI in combination therapy, and there are many ongoing trials in place that are highlighted in Table 3. The road to the use of CI in AML therapy seems to be a long one, but the rationale behind its use in combination therapy is warranted.

Table 3. Investigational therapies for AML resistance treatment

Drug	Investigational phase	Mechanistic target	Research group
SEL24/MEN1703 NCT03008187	I/II	PIM/FLT inhibitor oral combination	Menarini Group
Camrelizumab + Decitabine NCT04353479	II	Humanized monoclonal immunoglobulin targeting PD1 combined with DNA methyltransferase inhibitor	Shanghai Jiao Tong University School of Medicine
Lintuzumab-Ac225 in combination with CLAG-M Chemotherapy NCT03441048	I	Monoclonal antibody radio conjugate (Lintuzumab-Ac225) targeting CD33 along with standard CLAG-M chemotherapy	Medical College of Wisconsin
Meresitinib + LY287445 NCT03125239	I	Meresitinib is a MET kinase inhibitor combined with LY2874455 a FGFR inhibitor	Dana-Farber Cancer Institute
CD123/CLL1 CAR T cells NCT03631576	II/III	Biological CAR T cell therapy targeting CD123/CLL 1	Fujian Medical University
CLL-1, CD33 and/or CD123-specific CAR gene-engineered T cells NCT04010877	I/II	Biological CAR T cell therapy targeting CLL1, CD33 +/– CD123	Shenzhen Geno-Immune Medical Institute
Tislelizumab+ DNA hypomethylating agent +/– chemotherapy NCT04541277	II	Inhibiting PDL-1 in combination with DNA methylation +/– standard therapy for R/R AML	Chinese PLA General Hospital
Atezolizumab+ Guadecitabine NCT02935361	II	PDL-1 Inhibitor + DNA hypomethylating agent	University of Southern California

Abbreviations: *FLT3* SEL24/MEN1703; *PD1* programmed cell death protein 1; *CLAG-M* cladribine, cytarabine, G-CSF, and mitoxantrone; *CAR T cell* chimeric antigen receptor T cell; *PDL-1* programmed death-ligand 1

Nanotechnology

Nanoparticles (NPs) as a drug delivery system allows encapsulation of medications to enhance permeability, reduce adverse effects, improve bioavailability, overcome multi-drug resistance, and deliver combination medications. NPs can be formulated to target malignant cells based on surface markers in order to spare normal cells [74]. Vyxeos is a nano-formulated combination of cytarabine + daunorubicin (5:1 ratio) approved in 2017 for treatment-related AML (t-AML) and AML with myelodysplasia-related changed (AML-MRC) [75]. Jazz Pharmaceuticals sponsored a study of 309 patients with t-AML or AML-MRC and the results showed Vyxeos improved OS by 9.6 months compared to 5.9 months with 7+3 treatment (NCT01696084). Nano-encapsulation allowed Vyxeos to overcome multi-drug resistance by evading PGP efflux pumps and protecting cytarabine and daunorubicin from metabolism. The NPs increased drug exposure to the BM and allowed for a synergistic chemotherapeutic effect [75].

An alternative study used NPs to incorporate parthenolide (PTL), an NF-kB inhibitor, with an antiCD44 conjugate. PTL is not soluble, but the encapsulation with poly lactide co-glycide (PLGA) NPs improved its bioavailability while the antiCD44 increased drug targeting to AML cells in the BM. The study showed increased tumor uptake of PTL-PGLA-antiCD44 bioavailability compared to PTL-PGLA due to target specificity and prolonged half-life [76••].

Furthermore, Sudha et al. incorporated nanotechnology to combine tetrac with chemotherapy and improve drug delivery [77]. Tetrac was conjugated with polyethylene glycol (PEG) to improve duration of tetrac binding to its receptor and encapsulated with paclitaxel and doxorubicin. The study showed improved drug uptake by malignant cells and increase anticancer effects of therapy [77].

The ability to provide therapy in combination while evading biological pharmacokinetics make NP-drug delivery systems an interesting topic. Scientists can encapsulate our current therapies and add a targeting moiety to destroy LSCs in the BM before they can mutate. Further research is being conducted to determine the safety and to determine which combinations can improve outcomes.

Conclusion

We have discussed mechanisms of AML resistance that cause patients to relapse. AML therapy was stagnant for 4 decades until 2017 when midostaurin was approved. Since then we have seen many new targeted drug therapies such as glasdegib, ivosidenib, and venetoclax approved for treatment. Still, the OS for AML patients remains poor due to the inability to achieve CR^{MRD-}. Studies show that residual LSCs often lay dormant in the BM where they are protected by the microenvironment. This makes BM targeting of CXCR4, CD44, VLA-4, and LSC-MSC integration very promising.

Other promising leads such as integrin $\alpha v\beta 3$ and acid ceramidase have been discovered. $\alpha v\beta 3$ inhibition can decrease expression of downstream pathways such as NF-kB and PDL-1. CAR T cell therapy could be more beneficial. Clinical trials needed to provide more data regarding the safety and efficacy. These new target mechanisms and potential treatment options indicate that the future of AML treatment is bright.

Declarations

Conflict of Interest

Rehan Uddin declares that he has no conflict of interest. Noureldian H. E. Darwish declares that he has no conflict of interest. Shaker A. Mousa declares that he has no conflict of interest.

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