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Small Molecule Inhibitors in Acute Myeloid Leukemia: From the Bench to the Clinic

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Abstract

Many patients with acute myeloid leukemia (AML) will eventually develop refractory or relapsed disease. In the absence of standard therapy for this population, there is currently an urgent unmet need for novel therapeutic agents. Targeted therapy with small molecule inhibitors (SMIs) represents a new therapeutic intervention that has been successful for the treatment of multiple tumors (e.g., gastrointestinal stromal tumors, chronic myelogenous leukemia). Hence, there has been great interest in generating selective small molecule inhibitors targeting critical pathways of proliferation and survival in AML. This review highlights a selective group of intriguing therapeutic agents and their presumed targets in both preclinical models and in early human clinical trials.

Keywords

acute myeloid leukemia; small molecule inhibitors; therapeutic agents

Introduction

Anthracyclin and cytosine arabinoside-based chemotherapy achieves complete remission (CR) in the majority of patients with acute myeloid leukemia (AML).[1] Despite this fact, approximately 50% of patients will relapse within 1 to 2 years. The 5-year survival rate for patients who are less than 60 years old remains less than 50%.[1–3] In a recent analysis by Wingard et al, residual and recurrent disease was the leading causes of death (43%–47%) in the first nine years following allogeneic bone marrow transplantation.[4] The natural course of AML in patients who are greater than 60 years old is dismal with complete remission rates (CRR) of 40% to 65%, relapse rates of 60%–85% within 2–3 years of diagnosis, median overall survival (OS) of less than 6 months, and a 5-year overall survival (OS) of only 3%–8%.[2,3] The poor prognosis for this older population is thought to arise from a higher rate of drug resistance, co-morbidities, poor tolerance to chemotherapy, overexpression of the

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multidrug resistance genes (MRD1 and other ATP Binding Cassette/ABC genes), unfavorable cytogenetics, and a high treatment-related mortality rate (25%).[2,5–9] Accumulating data relating to the biology and initiating events of cancer have resulted in the identification of prognostic markers in AML and the development of novel targeted therapies in the hope of discovering a more efficient and less toxic alternative to conventional chemotherapy. The two small molecular inhibitors (SMIs), imatinib mesylate, a tyrosine kinase inhibitor that represses the function of BCR-ABL kinase (as well as other related tyrosine kinases), which has resulted in an 80% complete cytogenetic response rate in patients with chronic phase CML, and all-trans retinoic acid (ATRA), which can induce compete responses in patients with the APL (acute promyelocytic leukemia; M3 AML), have spawned great interest in the development of SMIs for the treatment of AML.[10–12] However, unlike in CML and APL, the identification of potential targets in AML has been limited by the heterogeneous clonal architecture of non-M3 AML and by the contribution of numerous driver mutations in its onset and progression. In this article, we will review SMIs for a number of biologically relevant targets in AML that are currently in clinical development, with a refrence to the ongoing clinical trials (Table.1), and the possible mechanisms of action and resistance to these reagents in AML.

Nucleophosmin (NPM1)

NPM1, which encodes a nucleolar phosphoprotein, is mapped to the long arm of chromosome 5. Three isoforms of NPM1 are generated by alternative splicing. It has been implicated in genomic stability and cell cycle progression by acting as a histone chaperone and a nucleus-cytoplasmic shuttle. It participates in chromatin remodeling, ribosomal biogenesis, centrosome duplication, ribosomal RNA cleavage, DNA synthesis, RNA transcription, and DNA repair.[1314] Accumulation of NPM1 protein has been observed in cancerous cells, likely reflecting increased DNA replication.[15,16] Approximately, 35% of AML patients harbor NPM1 mutations, most of which are structurally defined by an insertion in exon 12 with the duplication of a TCTG sequence at positions 956–959, leading to changes in the amino acid sequence of the C-terminal domain and loss of trp^{288} and trp^{290}, thereby resulting in unfolding of the C-terminal region in the NPM1 protein and reduced nucleolar binding. A new nuclear export signal motif is also formed that increases NPM1-CRM1 heterodimerization and export to the cytoplasm.[17–19] NPM1 haploinsufficiency predisposes mice to tumor formation.[20] This is thought to be related to the cytoplasmic dislocation of p19ARF (p14ARF ortholog) thus inhibiting its tumor suppressor effect by allowing mouse double minute 2 homolog (Mdm2) binding and inactivation of TP53, or by eliciting the post-translational sumoyl modification of the NPM1 protein in a TP53-independent mechanism.[21–23] Of note, the nuclear factor-κβ (NF-κβ) has an important role in the promotion of metastasis, angiogenesis, and the survival of cancer cells, and it is hyperactivated in the majority of AML patients.[24–26] The favorable outcome of NPM1-mutated AML has been attributed to NF-κβ binding to the mutated form of NPM1 (NPM1c) resulting in cytoplasmic sequestration and inactivation of NF-κβ, leading directly and indirectly to leukemic cell chemosensitization.[17,27,28]

Different strategies of NPM1c targeting have been proposed. Conceptually, transporting NPM1c from the cytoplasm back to the nucleus is an interesting approach, but it remains

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challenging. Leptomycin B is an Exportin-1/CRM1 inhibitor that exhibits in vitro tumoricidal activity by stabilizing TP53 through disrupting its CRM1-mediated nuclear export.[29–31] However, a phase 1 trial with Leptomycin B was discouraging, without objective responses and with significant toxicity manifesting as marked fatigue and anorexia.[32] New, less toxic CRM1 inhibitors, such as CBS9106 and KPT 330, have been developed.[33–37] Additive effects of CRM1 inhibitors with cytarabine, FLT3 inhibitors, and histone deacetylase inhibitors in AML have been reported in recent preclinical studies.[38,39]

Inhibiting the interaction of NPM1 with other proteins has also been investigated. Cytoplasmic relocalization of HEXIM1 can be mediated by NPM1c, resulting in HEXIM1 inactivation and stimulation of the positive transcription elongation factor (P-TEFb), a cyclin-dependent kinase that regulates mRNA synthesis. A P-TEFb inhibitor, seliciclib was evaluated in a phase 1 trial by Bensen et al.[40,41] Seliciclib was given for 7 days on a 3-week cycle, but tumor responses were not observed. Disease stabilization was observed in 8 out of 21 evaluable patients for up to 18 weeks, with dose-limiting toxicities (DLTs) of fatigue, hypokalemia, and urticarial rash. The investigators linked the lack of clinical responses to the significantly lower plasma concentrations in the study participants compared to the levels achieving tumor regression in human xenograft models.[41] Given the heterozygosity of NPM1 mutations, it is conceivable that leukemic cells possess low nuclear levels of wild type NPM1, contributing to the cell growth. Wild type NPM1 levels are also expected to be lower in heterozygous mutant cells compared to normal cells because of dimerization with the NPM1c.[42] Therefore, targeting the wild type NPM1 might also be an effective therapeutic approach via indirectly inhibiting the NPM1c-mutant and/or signaling pathways.[23].[13]

MDM2

TP53 is a tumor suppressor that responds to stress signals and regulates cell cycle arrest, senescence, and apoptosis to maintain genomic stability.[43] TP53 mutations are found in ~50% of tumors, leading to partial or complete loss of the TP53 function and consequently, to oncogenic transformation.[44–46] TP53 levels and activity are also downregulated by the oncoprotein MDM2 in an autoregulatory circuit, which was proposed as an alternative mechanism of TP53 inactivation in AML rather than TP53 mutation.[47,48] Binding of the N-terminal domain of MDM2 with the N-terminus of the TP53 transactivation domain results in TP53 suppression, nuclear export, and ubiquitination followed by proteasomal degradation.[49,50] This interaction explains the correlation of MDM2 overexpression with chemoresistance and poor outcomes in many tumors and provides a basis for the development of SMI against MDM2 in an attempt to enhance TP53 activity.[51–55] Nutlin-3 is an MDM2 antagonist that binds the TP53 pocket of MDM2. It increases TP53 levels and induces TP53-dependent apoptosis of AML blasts in vitro and an additive/synergistic anti-AML effect when combined with chemotherapy such as doxorubicin and cytosine arabinoside.[56] Furthermore, Nutlin-3 induces a TP53-independent apoptotic effect by interfering with MDM2's interaction with other proteins (e.g., p73, E2F-1, HIF-1α) that share the binding site of TP53.[57–59] Anti-angiogenic properties of Nutlin-3 were also observed in preclinical studies and were attributed to TP53 activation in endothelial cells, leading to the induction of anti-angiogenic proteins, such as TSP-1 and BAL1 and to

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downregulation of pro-angiogenic proteins such as VEGF, bFGF, FGF-BP, and COX-2.[60,61] The initial results of a phase 1 trial conducted by Andreeff et al in patients with AML using RG7112, a new oral MDM2 antagonist, were promising. Of 31 evaluable patients, CR was reported in 5 after 1 cycle, with signs of hematological improvement in 12 other patients.[62] RG7112 was further studied by Yee and colleagues in combination with cytarabine using 2 schedules. The DLT were diarrhea, rash and thrombocytopenia. The ORR was 52%. Of interest, CR was seen in an elderly patient with disease refractory to previous cytarabine therapy and that all patients with CR had TP53 wild type status. Bridging to transplant was also possible in 3 patients with relapsed/refractory disease.[63]

An analysis of 109 AML specimens after in vitro exposure to the MDM2 inhibitor MI-219 revealed resistance in all samples harboring TP53 mutations and in 30% of those with wild type TP53, with an enhanced response in association with high MDM2 expression.[64] Drug resistance despite the presence of a wild type TP53 phenotype was assumed to be related to a post-translational modification of TP53 that reduces TP53 activity or to impaired TP53 pathways. Of interest, 84% of the FLT3-mutant (ITD) responded well to MI-219 ($IC_{50} < 5$ μM), suggesting a therapeutic potential of MDM2 inhibitors in FLT3-mutated AML. Various compounds that block MDM2–TP53 binding are currently being studied in clinical trials in both hematological and solid tumors. Initial studies, although limited, suggest reasonable safety and efficacy.[65,66]

Aurora kinases

Aurora kinases A, B, and C (AKA, AKB, and AKC) are serine/threonine kinases that regulate mitosis and cytokinesis and have been implicated in both tumor formation and progression.[67–71] The AKA gene maps to chromosome 20q13.2.[72] In RNA interference studies, AKA inhibition disrupts spindle assembly, centrosome maturation and segregation, thereby leading to G2/M arrest and cell death.[73,74] AKA is also a negative regulator of TP53 activity: the phosphorylation of TP53 at Ser315 by AKA induces MDM2-TP53 interaction, thereby causing TP53 inactivation.[75,76] Thus, AKA inhibition increases TP53 levels and activity resulting in G2/M arrest followed by apoptosis. In addition, AKA overexpression in cancer has been linked to chromosomal instability and shortened survival.[77–80] Of note, $CD34^+/CD38^-$ leukemic stem cells exhibit higher expression of AKA as compared with normal CD34+ progenitors, with a selective and potent anti-proliferative and apoptotic response to AKA inhibition.[81]

AKB is a chromosomal passenger protein encoded on chromosome 17p13.1.[69] Inhibition of AKB's function is associated with cytokinesis defects, polyploidy, and apoptosis.[82] Its upregulation in patients with non–small-cell lung cancer, ovarian cancer, oral and laryngeal squamous cell carcinoma, and hepatocellular carcinoma correlates with the increased likelihood of metastasis, chemoresistance and poor survival.[83–87] Recent data suggests a supportive role of AKC to AKB function and that AKC overexpression can promote malignant transformation.[88,89] Preclinical data support the antitumor activity of several of the aurora kinase inhibitors (AKIs). A phase 1 trial of patients with advanced hematological malignancies treated with AT9283, a small molecule inhibitor of AKA, AKB, JAK2/3, and ABL1, resulted in only a transient blast reduction of 30% in patients with AML despite

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reduced levels of the phosphorylated histone H3, a downstream target of AKB. Significant DLTs were reported, including pneumonia, myocardial infarction, and tumor lysis syndrome.[90] Of note, cardiac toxicity manifesting as QTc prolongation was also reported with another AKI (MK-0457) and was attributed to hERG channel suppression.[91]

In contrast to patients with solid tumors, AML patients demonstrated a better response to the selective AKB inhibitor barasertib (AZD1152).[92,93] Barasertib was given as a 7-day infusion on a 21-day cycle, with an overall hematological response of 25%. The majority of the patients enrolled in this study had relapsed AML. Recently, a randomized phase 2 study from MD Anderson Cancer Center reported higher CRR and CRp (CR with incomplete platelet recovery) in older patients with AML in response to a 7-day infusion of barasertib at a dose of 1200 mg/day as compared with those receiving a low dose of cytarabine of 20 mg/day subcutaneously for 10 days every 28 days (35% vs. 12%). The median duration of remission ranged from 28 to 321 days in the barasertib group and from 30 to 85 days in the cytarabine group.[94] Responses were observed mostly after two cycles and included patients from all cytogenetic risk subsets in the barasertib-only arm while being limited to patients with good and intermediate risk cytogenetic profiles in the cytarabine arm. Treatment-related mortality was comparable between the two cohorts 30 days after the start of treatment, but it was lower in the barasertib arm at 90 days. However, this study was hampered by the small number and uneven distribution of patients in terms of age and performance status. Interestingly, some reports with AT9283 suggested TP53 mutation as a predictor of sensitivity.[95] Alternatively, increased pHH3 marks a tumor response to the selective AKA inhibitor MLN8054.[96] Because AKA stimulates the mTOR/Akt pathway and is implicated in taxane and carboplatin resistance, AKIs have been combined with taxane and carboplatin to overcome drug resistance, with encouraging results.[97–99] One interpretation of the limited clinical response to AKIs is the fact that they target the mitotic process, which might limit their activity to the proliferating cells.[70] Several AKIs are in early development with promising results (e.g., TLK60404, CHR-3520 and Aki-100).[70]

Farnesyl transferase

RAS proteins regulate intracellular signal transduction and several critical pathways involved in cell growth, adhesion, migration, invasion, and apoptosis.[100] Approximately 12% to 27% of patients with AML harbor activating mutations in the RAS proto-oncogene family predominantly at codon 12 of N-RAS and K-RAS.[101–108] RAS upregulation without an activating mutation has also been reported with AML.[109] One mechanism of RAS inhibition is via the inhibition of farnesyl transferase, an essential enzyme for the post-translational modification of RAS. Farnesyl transferase adds a farnesyl moiety to the cysteine residue of the C-terminal CAAX motif, thereby allowing RAS to anchor itself to the inner leaflet of the plasma membrane. Data support the ability of farnesyl transferase inhibitors to induce apoptosis in multiple cancer cell lines (including AML lines), most likely by inhibiting the activity of the pro-survival proteins (e.g., AKT, mTOR, MAP kinases) and by activating pro-apoptotic proteins (e.g., Bax, PUMA, BAK).[110–116]

A phase 1 trial of a farnesyl transferase inhibitor, lonafarnib, demonstrated limited clinical activity in patients with myelodysplastic syndrome (MDS) and secondary AML. Excessive

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toxicity was noted in the majority of participants, many of whom required dose reductions after the first treatment cycle or cessation of therapy.[117] Tipifarnib is another farnesyl transferase inhibitor with a more favorable toxicity profile. The initial results of the CEP-20 trial demonstrated that tipifarnib induced CRR/CRp in 14% of poor-risk older AML patients (>70 years old) with a median survival of 18 months in responders irrespective of RAS mutational status. The dose used in this study was 600 mg twice a day for 3 weeks during every 4-week cycle.[118] Of note, a larger trial comparing this regimen with best supportive care failed to replicate these results (CRR was only 8%, with no difference in survival).[119] The AML16 trial that combined tipifarnib with low-dose cytarabine also failed to demonstrate significant differences in CRR and 12-month OS rates, leading to the premature closure of the study.[120] Jabbour and colleagues reported similar negative results in a phase 1/2 trial of the addition of tipifarnib to idarubicin and cytarabine in patients with AML and poor-risk patients with myelodysplastic syndrome (MDS).[121] However, subanalysis revealed a higher CRR among patients with unfavorable cytogenetic abnormalities involving chromosomes 5 and 7 as compared with historical controls receiving chemotherapy alone (58% vs. 19%). This observation was consistent with similar analysis performed for another study by Brenwin and colleagues, which suggested heightened sensitivity to tipifarnib-based therapy in the high-risk population.[122]

Geranylgeranylation of K-RAS and N-RAS is proposed as another way to circumvent farnesyl transferase inhibition and to restore RAS activation.[123,124] In support of this is the fact that the mutation of H-RAS, which does not undergo geranylgeranylation, renders tumors more sensitive to farnesyl transferase inhibitors as compared with those tumors carrying N-RAS and K-RAS mutations.[125,126] Dual targeting of the farnesyl and geranylgeranyl transferases effectively produced an anti-tumor response in vivo, but a high level of toxicity was initially encountered.[127,128] L-778,123 is a dual inhibitor that was investigated in patients after being well tolerated in vivo.[129] Despite successful inhibition of geranylgeranylation, L-778,123 did not suppress K-Ras prenylation in patient samples. Appels et al evaluated AZD3409, another dual inhibitor, in patients with solid tumor malignancies (n=29).[130] The MTD was 750 mg twice a day, with DLTs of diarrhea and nausea. There were no objective responses. Interestingly, the pharmakodynamic data indicated two important points: 1) the maximal inhibition of farnesyl transferase activity was only 49% of pretreatment levels; 2) the maximal farnesyl transferase inhibition occurred at plasma levels considerably lower than those associated with drug-induced toxicity.

Histone deacetylase

Histones are alkaline proteins that associate with DNA to form small packaging units called nucleosomes. Histone deacetylases (HDACs) are involved in the post-translational modification of histones via removing ε-N-acetyl lysine residue, resulting in increased coiled DNA winding around the histone core, thereby reducing the accessibility of transcription factors and rendering functional genes inactive. Non-histone substrates to HDACs have also been identified, including TP53, heat shock protein 90 (HSP90), and PTEN.[131–133] The theoretical role of HDAC inhibitors (HDACIs) as anticancer agents is thought to be through the re-expression of epigenetically silenced tumor genes and tumor

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suppressor genes resulting in tumor regression. Trials of HDACIs in cutaneous T-cell lymphoma led to the approval of two HDACIs (vorinostat and romidepsin).[134,135]

The HDAC inhibitory activity of valproic acid (VPA) was tested in a phase 2 study of patients with MDS (n = 43) and AML (n = 32), which resulted in overall response rate (ORR) of 30% and 16%, respectively. The addition of ATRA did not increase VPA responses.[136] Enhanced responses (52%) were noted in the MDS subgroup of patients with normal blast counts. By contrast, further studies with VPA, both as a single agent and with ATRA in high-risk patients with MDS and AML, demonstrated lower response, thereby suggesting that VPA may be more effective for low-risk patients with MDS.[137,138] Treatment of 11 AML patients (70 years or older), unfit for chemotherapy with VPA, ATRA, and theophylline resulted in one CR, 2 CRi, and stable disease for 4–6 months in 3 patients.[139] This combination was also evaluated in 20 AML patients with one CR, 2 PR and eradication of peripheral blood blasts (but not the bone marrow) in one patient.[140] Fredly et al studied the addition of either hydroxyurea, 6-mercaptopurine or both to a combination of ATRA, VPA and theophylline in five patients with advanced AML. This was found to be an effective palliative strategy in reducing blast counts.[141] Another study was conducted by Fredly et al in 36 AML patients unfit for intensive chemotherapy. The patients were treated with continuous VPA, and low-dose cytarabine (10mg/m2 for 10 days), and intermittent ATRA (for 14 days) on every 12-week cycle. Either hydroxyurea or 6-mercaptopurine was used to control non-responding hyperleukocytosis to low-dose cytarabine. Nine patients demonstrated hematological improvement, with a CRR of 6%, which is lower than the CRR of another study (18%) that used a higher dose of cytarabine (20mg/m^2 twice daily for 10 days every 4–6 weeks).[142,143] On the other hand, the CRR of Fredly et al study is similar to another trial (7.4%) that used cytarabine alone at 20 mg/m^2 once a day for 10 days on monthly basis.[144,145]

Depsipeptide is another HDACI that demonstrated preclinical anti-leukemic activity with evidence of c-Myc downregulation. However, its clinical development was halted as a result of limited clinical responses and significant side effects, including profound fatigue and gastrointestinal toxicity.[146] MGCD0103 (mocetinostat) is an HDACI with a prolonged half-life and selectivity to the HDAC isotypes 1, 2, 3, and 11. MGCD0103 was administered three times a week (20 to 80 mg/m^2) to patients with refractory or relapsed AML (n = 22), CML (n = 1), ALL (n = 1), and MDS (n = 5). The MTD was 60 mg/m^2. Three of the 23 evaluable patients achieved a CR after one to three cycles at higher dose levels of 60 to 80mg/m^2. Two of these patients had refractory AML.[147]

Garcia-Manero and colleagues studied vorinostat in patients with hematological malignancies (31 of 41 had AML). The drug was given two to three times daily according to a 2-weeks-on/1-week-off schedule.[137] The ORR was 17% (two patients had CR, two had CRp, and three had a >50% reduction in blasts), which continued for up to 53 weeks. All responders had AML, and bone marrow transplantation was successfully performed after treatment response in three patients. DNA hypermethylation and histone deacetylation could mutually reinforce epigenetic silencing of tumor suppressors in AML.[148,149] The modest single-agent activity of HDACI and the evidence of a synergistic interaction with cytotoxic and DNA hypomethylating agents in preclinical work have prompted several combination

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clinical trials. E1905 study is a phase 2 randomized study that compared the hyopmethylating agent, azacitidine (AZA) alone (50 mg/m2 for 10 days) with the combination of AZA and etinostat (a class I HDACI, with a prolonged half-life of 4.5 days) in patients with AML (n=52) and MDS (n=97). Of interest, the median OS was unexpectedly worse in the combination group (13 months) compared to the AZA alone group (18 months), as well as the rate of infections and thrombocytopenia. Correlative studies showed less global hypomethylation in the combination arm which was attributed to etinostat-mediated inhibition of AZA-induced cell cycle arrest. It has therefore been suggested to administer the two reagents sequentially rather than simultaneously to mimic the successful in vitro model.[150] On the basis of preclinical work indicating enhanced ATRA activity in association with VPA and 5-azacytidine (5-AZA), a total of 53 patients with AML and poor-risk patients with MDS were treated with VPA at escalating doses in combination with 5-AZA (75 mg/m^2) and ATRA (45 mg/m^2, started on day 3) during a 7-day course.[151] Hematological responses of 42% including a CRR of 22% were observed. The CRR in this study is similar to another study using the same combination despite a prolonged exposure to ATRA (21 days).[152] The combination of decitabine (20 mg/m^2 for 10 days), another hypomethylating agent, with escalating doses of VPA (days 5 through 21) was also investigated, but the ORR (44%) of this small study did not differ from that of decitabine alone.[153] In another study, neurotoxicity was encountered at higher doses of VPA (35 to 50 mg/m^2) when it was used with decitabine (15 mg/m^2 for 10 days). An ORR of 22% (50% among untreated elderly patients) was obtained.[154] But, in contrast to the previous study, the response was not linked to plasma VPA levels, despite the significant increase in histone acetylation with higher VPA doses.

Idarubicin (12 mg/m^2) and vorinostat were administered to 41 patients with refractory or relapsed AML. CR was noted in 17% of the evaluable patients. Garcia-Manero and colleagues administered vorinostat at a dose of 500 mg three times daily for 3 days followed by cytarabine and idarubicin infusion followed by consolidation therapy for those AML patients in remission.[155] Vorinostat was continued as a maintenance therapy for up to 1 year in 17% of the participants. Vorinostat dose was reduced in 66% of the patients due to bone marrow suppression or grade 3 or 4 gastrointestinal toxicity. The ORR of 85% (100% in the presence of FLT3-ITD) was considered promising given the patients’ poor risk profile. Gogo and colleagues treated advanced and high-risk AML patients (n = 21) with vorinostat at escalating doses for 7 days followed by etoposide (100 mg/m^2) and cytarabine (1 to 2 g/m^2) on days 11 to 14.[156] They documented an ORR of 33% and a median remission time of 7 months. The CR was achieved in 46% of those patients treated with the MTD of 200 mg twice daily.

Panobinostat (LBH589) is a hydroxamic acid analogue and a pan-HDACI that produced only transient reduction in AML blasts in a phase 1 study by Giles et al.[157] In a phase 1b/2 trial conducted by Tan and colleagues, the administration of Azacytidine (75mg/m^2 for 5 days) with escalating doses of panobinostat (3 times a week for 7 doses) on monthly basis in AML (n=29) and high risk MDS (n=10) patients resulted in ORR of 31% in AML and 50% in MDS patients. Fatigue was reported as the main DLT.[158] The phase 2 trial of Panobinostat in relapsed/refractory acute lymphoblastic leukemia (ALL) and AML was recently completed, but the results have not yet been reported (NCT00723203).

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Most recently, pracinostat gained Orphan Drug status by the FDA in 2014 for the future development in AML. It was shown to induce prolonged CRR of 14% in a phase 1 trial of older AML patients, lasting for 206 and 362 days (unpublished data). An ongoing phase II trial is currently evaluating pracinostat in combination with azacitidine in newly diagnosed AML (NCT01912274). Alternatively, the combination of pracinostat with the JAK2/FLT3 inhibitor pacritinib had significant synergetic anti-proliferative effects in vitro and in vivo, particularly in AML cell lines carrying JAK2^{V617F} and activating FLT3 mutations.[159] This synergistic effect is presumably due to the ability of this combination to counteract the rise in FLT3 and pSTAT5 (observed when using pacritinib alone), or to the suppression of stroma-mediated release of growth factors and cytokines.[159] Of note, McCormack et al has also shown that combining the pan-HDACI, VPA with Nultin-3 (MDM2 antagonist) in vitro increases p53, acetylated p53, and p21 expression, and enhances the apoptotic response of AML cells, predominantly of those with higher CD34 expression.[160]

The exact mechanism by which HDACIs act as anti-neoplastic agents is not yet clear. Increased reactive oxygen species levels (ROS) and antioxidant gene expression following HDACI exposure were observed in vitro.[133] Further, a correlation was found between antioxidant gene expression and vorinostat resistance, suggesting ROS-mediated apoptosis in HDACI-treated cells.[137] Consistent with this hypothesis, the anti-leukemic activity of vorinostat was enhanced by resveratrol likely through inducing ROS production. Two additional pathways were proposed: 1) Sirt1 activation leading to the abrogation of HDACI-mediated p65 acetylation leading to NF-κβ inactivation and 2) S-phase cycle arrest, sensitizing cells to vorinostat-induced apoptosis.[161]

In addition, accumulating evidence suggests that HDACIs induce autophagy, presumably by increasing the expression of LC3B (a modulator of autophagy) or via the hyperacetylation and inhibition of HSP90 and subsequent degradation of AKT (a client protein of HSP90 and a negative regulator of autophagy).[162–166] This is supported by Wei and colleagues' findings of increased autophagy and apoptosis when HDACIs are combined with GX15-070 (a BH3 mimetic) and the reversal of this effect by chloroquine (an autophagy inhibitor).[167] The inhibition of Akt/mTOR pathway by HDACIs was shown in prostate and mantle cell lymphoma cell lines.[168,169] Also, HDACI-mediated apoptosis and differentiation of AML cells was significantly increased by the addition of an mTOR inhibitor.[170] Sandilya et al reported CR in two older AML patients, with high-risk features when treated with VPA (500 mg three times a day) and sirolimus, an mTOR inhibitor (1 mg per day). Of interest is that one of the patients failed 7+3 induction prior to enrollment in the study and that the responses lasted for over 9 months.[171] A detailed review of the activity and toxicity of HDACIs was published by Bruserud and colleagues.[172]

Proteasome inhibitors

Proteasomes are intracellular organelles responsible for polyubiquitinated protein degradation including oxidized, damaged, and misfolded proteins through trypsin-like, chemotrypsin-like, or peptidyl-glutamyl peptide hydrolyzing activities.[173,174] In addition, proteasomes regulate the levels of proteins involved in the cell cycle (e.g., TP53, cyclins, p27), apoptosis, and intracellular signaling pathways.[175–177] Proteasome inhibitors have

successfully induced the in vitro killing of multiple cancers, including hematological malignancies. [178,179178,179178,179178,179178,179178,179178,179178,179178,179178,179]

Bortezomib is a reversible inhibitor of chemotrypsin-like activity in the proteasome that has been approved for the treatment of multiple myeloma and mantle cell lymphoma.[180,181] Guzman and colleagues demonstrated that bortezomib induced an apoptotic response of leukemic stem cells by suppressing the degradation of (and thus increasing the levels of Iκβ), a negative regulator of NF-κβ.[182] Bortezomib also improves the response of myelomonocytic leukemic cells to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) in vitro, which is also related to bortezomib-mediated abrogation of NF-κβ activation, downregulation of the anti-apoptotic proteins C-FLIP and XIAP and expression of the TRAIL receptors DR4 and DR5.[183,184]

Another proposed mechanism of bortezomib is the induction of G2/M cell cycle arrest via the stabilization of cyclin and the cyclin-dependent kinase inhibitors p21 and p27.[182,185] In a study by Cortes et al, bortezomib was administered to 15 patients with relapsed/refractory AML at escalating doses (0.75 to 1.5 mg/m^2) twice weekly for 4 weeks every 6 weeks. Only a transient response was observed in five of the six patients who received the MTD dose of 1.25 mg/m^2, with DLTs at 1.5 mg/m^2 including postural hypotension, fluid retention, nausea, and diarrhea. Four patients experienced a greater than 50% reduction in peripheral or bone marrow blast count, and one demonstrated an increasing neutrophil count.[186]

Sarlo and colleagues treated 14 high-risk older patients with previously untreated and refractory or relapsed AML with bortezomib at a dose of 1.5 mg/m^2 twice weekly for 2 weeks of a 3-week cycle. Treatment response was limited to reduced blast counts in peripheral blood and bone marrow in 61% of the participants. Neurological toxicity developed in 54% of patients, resulting in bortezomib discontinuation in 30% of cases.[187] Alternative more limited bortezomib exposure was well tolerated in 31 patients with newly diagnosed AML and older patients with relapsed AML, with higher doses of up to 1.5 mg/m^2 used in combination with cytarabine and idarubicin as induction therapy. The CR achieved among patients with relapsed AML (67%) exceeded the previously reported CR in literature (20–40%), with median disease-free survival of 15.3 months among responders.[188] A similar response was achieved in a phase 2 CALGB study.[189]

Blum and colleagues treated 19 high-risk and refractory patients with decitabine and escalating doses of bortezomib (up to 1.3 mg/m^2), with an ORR of 37%.[190] This response was similar to a previous study of decitabine alone. Correlative studies suggested that bortezomib could potentiate decitabine cytotoxicity by increasing the expression of miR-293, thereby leading to the inhibition of the SP1/NF-κβ complex and ultimately to FLT3 downregulation. Therefore, an alternative sequential therapy of bortezomib followed by decitabine was proposed to increase the clinical benefit. Extrapolating evidence from multiple myeloma studies, the authors proposed subcutaneous and once-weekly dosing to minimize bortezomib-related neurotoxicity while maintaining its therapeutic efficacy.[191,192] A synergistic anti-leukemic interaction was found between bortezomib and the HDACI belinostat in vitro.[193,194] This combination was investigated in a phase I trial conducted in hematological malignancies, including AML (n=19). Results suggest safety of this regimen,

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without DLT. One CR (despite prior resistance to 7+3 regimen) and 2 PR were observed in AML patients which allowed these patients to proceed to allotransplantation.[195]

SDF1/CXCR4

The chemokine receptor CXCR4 (CD184) is an adhesion molecule widely expressed on many cell types. The interaction of CXCR4 with its ligand SDF1 (CXCL12) is implicated in the migration, homing, and quiescence of hematopoietic and leukemic stem cells in the bone marrow.[196–199] The heterodimerization of SDF1 with CXCR4 also activates the intracellular signaling of several survival pathways (e.g., PI3K/AKT, MAPK, PKC, JAK/STAT).[200] In AML, CXCR4 overexpression has been linked to poor clinical outcomes.[201,202] Plerixafor (AMD3100) is a bicyclam SMI that blocks SDF1 binding to CXCR4 and it was approved as a peripheral stem cell mobilizer for the treatment of multiple myeloma and non-Hodgkin's disease. Plerixafor has been used successfully in preclinical mouse models of AML to sensitize AML to chemotherapy in vivo presumably by "mobilizing" leukemic stem cells and detaching them from the anti-apoptotic effects of the hematopoietic niche.[203]

Uy and colleagues published the results of 46 patients with refractory or relapsed AML who were treated with plerixafor (0.24 mg/kg/day) and a regimen of mitoxantrone, etoposide, and cytarabine.[204] The authors described a twofold increase in circulating leukemic blasts, which peaked at 6 to 12 hours after plerixafor administration. The CRR/CRp was 46% representing modest improvements as compared to chemotherapy alone in previous reports. Additional preclinical data from MD Anderson demonstrated anti-leukemic synergy between CXCR4 blockers and FLT3 inhibitors.[205] A preliminary report on the response to plerixafor, granulocyte colony-stimulating factor (GCSF), and sorafenib in relapsed/refractory FLT3-ITD$^+$ AML demonstrated a CRR/CRp of 77%. This was associated with increased peripheral blood counts of blasts by 41-fold and of leukemic stem cells by 148-fold (NCT00943943).[206] The degree of leukemic stem cell mobilization was noted to be related to baseline blast counts and VLA-4 expression. Of note is that a response was observed in three patients for whom prior treatment with FLT3 inhibitors was not effective.

HSP90

HSP90 belongs to the heat shock protein family and is comprised of two main isoforms: the inducible HSP90α and the constitutively expressed HSP90β.[207] It is a pleotropic chaperone that is upregulated in response to cellular stress and is required for folding, transport, stabilization, and proteasomal degradation of proteins involved in the cell cycle, apoptosis, steroid receptor function, and signal transduction.[208–212] The engagement of a client protein with the HSP70/HSP40/HSP90 complex and p60^{HOP} forms the HSP90 multichaperone complex, in which functional conformation and activation of the client protein require the binding of ATP to the N-terminal domain of HSP90.[208,212–214] Several oncogenic proteins are regulated by HSP90, including c-kit, HER-2, FLT3, BCR-ABL, ZAP-70, Akt, NPM-ALK, Raf-1, and mutated TP53.[209,215–222] High HSP90 levels were identified in many solid tumors.[223] HSP90 is also highly expressed in patients with AML, and it confers chemoresistance and poor survival.[224–226] Furthermore, HSP90 exists predominantly in its active multiprotein complex state in cancerous cells as compared with normal tissues, which

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suggests that there is malignant cell dependence on this protein.[227] Reikvam et al reported increased HSP protein content (including HSP90) in FLT3-ITD$^+$ AML, and that the apoptotic response to HSP90 inhibition is more prominent in FLT3-ITD$^+$ AML samples than in wild-type AML.[228]

HSP90 inhibitors act mostly by blocking HSP90 ATP binding, presumably leading to the trapping of the client oncogenic protein in a misfolded form, which is then followed by proteasomal degradation. The in vitro exposure of AML cells to the HSP90 inhibitor 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG) produced the downregulation of Akt, Raf-1, N-RAS, K-RAS, and mutant p53; increased p21 levels; the activation of apoptotic protein Bax; and G2/M arrest.[229,230] 17-AAG is a potent and tumor-specific agent as a result of its high affinity for the HSP90 heterocomplex.[227] The preclinical activity of 17-AAG positively correlated with the percentage of leukemic cells expressing HSP90, which may define a subset of potentially 17-AAG sensitive AML patients.[226] Eleven patients with refractory or relapsed AML were treated with 17-AAG followed by bortezomib. Hepatotoxicity related to the benzoquinone moiety of 17-AAG was noted as the primary DLT, without hematological response apart from a transient reduction in blast count from 79% to 9% in one patient.[231] QTc prolongation was also reported, and it was attributed to previous anthracyclin therapy and the 17-AAG formulation since it was not encountered in two other studies of multiple myeloma despite higher doses of 17-AAG.[232,233]

Alvespimycin (17-DMAG) is a 17-AAG analog with more potent effects and a longer-half life.[234] It was given intravenously to 24 patients with refractory AML at escalating doses (8 to 32 mg/m^2) twice a week for 2 out of 3 weeks, with a CRR of 17% and blast reduction of more than 50% in one patient. However, there were no further plans for clinical development as a result of the significant toxicity associated with this drug, including neurotopenia, diarrhea, fatigue, and myocardial ischemia.[235] Patients who were co-treated with cytarabine exhibited synergetic anti-myeloid effects.[236,237] HDACIs were also able to synergistically enhance 17-AAG activity against leukemic cells, including those expressing FLT3 mutation.[238,239] The mechanisms of resistance to HSP90 inhibition were extensively reviewed by Piper and colleagues.[240] One of the proposed mecahinsms is upregulation of another molecular chaperone HSP70. Combining 17-DMAG with the HSP70 inhibitor VER-155008 has shown a synergestic activity against primary AML cells in vitro.[241] In addition to CXC4, studies are ongoing to explore the therapeuti potential of other prtective factors in the tumor niche.(table.2).

Kinase Inhibitors

FLT3

The FMS-like tyrosine kinase-3 (FLT3) is a class III tyrosine kinase receptor that is predominantly expressed in hematopoietic progenitors. FLT3 homodimerizes with itself upon binding to its ligand (FLT3L), thereby leading to the activation of the cytoplasmic kinase domain and the trans-phosphorylation of the intracytoplasmic tyrosine residues, which trigger the phosphorylation of critical signal transduction proteins that drive cell differentiation, proliferation, and survival (e.g., STAT5, ERK1/2).[242,243] FLT3 mutations that result in constitutively activated signaling have been described in AML, the most

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notable one being the internal tandem duplication (ITD) that results from a frameshift mutation involving insertions in the juxtamembrane domain of FLT3. FLT3-ITD is recognized as the most frequent mutation among individuals with AML. It is found in 24% of adult patients, and it represents a poor prognostic feature of AML with normal cytogenetics.[244] The adverse effect of FLT3-ITD also overrides the favorable prognosis of NPM1 mutation, especially with high allelic burden.[245] The size of FLT3-ITD (>70bp) was investigated as a prognostic indicator of survival, with conflicting reports.[246,247] Point mutations in the activation loop of the tyrosine kinase domain (FLT3-TKD) were also identified in 7% of patients with AML; these were most commonly a missense mutation of (D835Y) that changes tyrosine to aspartate.[248–250]

Semaxinibis (SU5416) is a SMI against FLT3, c-kit, and VEGFR (1–2). It was tested in a phase II trial of 43 patients with c-kit positive AML (>30%) who had refractory disease or who were deemed unfit for induction chemotherapy.[251] The twice-weekly administration of 145 mg/m^2 was associated with a partial response of more than 50% blast reduction in 7 patients, but this effect lasted only 1 to 5 months, whereas CRp was achieved in only 1 patient, who relapsed after 2 months. The main adverse events were nausea, headache, and severe bone pain. Both high VEGF expression and low c-kit expression were favorable predictors of response. FLT3 phosphorylation was minimally inhibited by SU5416 among the patients who were examined; however, a reduction in bone marrow vessel density was noted in all responders (100%) as compared with 72% of the non-responders. Thus, it was postulated that the clinical benefit of SU5416 is mainly the result of its anti-angiogenic properties and that an additional anti-leukemic effect could be established by targeting c-kit–mediated and FLT3-mediated proliferation.

Sorafenib is an oral multikinase inhibitor approved for the use in patients with renal cell carcinoma, with activity against serine/ threonine kinase Raf-1, c-Kit, FLT-3, PDGFR and VEGFR. A recent German randomized, placebo controlled trial in older AML patients (n=201) revealed that the use of sorafenib (400 mg twice a day) in between induction (cytarabine and daunorubicin) and consolidation chemotherapy followed by maintenance duration after CR for 1 year from the beginning of therapy was associated with higher toxicity and mortality (17 vs. 7%), lower CRR (46 vs. 60%) and ORR (57 vs.64%) after induction, and lack of improvement in event free-survival (EFS) (7 vs. 5 months) and OS (15 vs. 13 months), including those patients with FLT3-ITD.[252] In the SORAML trial, younger AML patients (n=276) received either sorafeninb (800mg a day) or placebo concomitantly with induction and consolidation therapy and for 1 year as maintenance therapy following consolidation. Preliminary results were suggestive of improvement in EFS, but not in the 2-year OS in the sorafenib arm, particularly in patients carrying FLT3-ITD. There was no significant difference in CRR between the placebo and sorafenib arms (56% and 60%, respectively). In a retrospective analysis of refractory and relapsed FLT3-ITD AML patients (n=69), Metzelder et al reported CRR on sorafenib monotherapy in 23% of the cases, with more frequent and durable response (median of 197 days) in patients who relapsed post- allogeneic stem cell transplant compared to those who relapsed after chemotherapy (median of 136 days).[253] This raised the possibility of an enhanced sorafenib activity by graft-versus-leukemia (GVL) effect. Elevated FLT3 ligand levels following chemotherapy resulting in FLT3 receptor activation was postulated as a mechanism of

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resistance to FLT-3 inhibitors.[254] Ravandi et al studied the combination of azacytidine and sorfenib in 43 patients with refractory or relapsed FLT-3 ITD AML. The CRR was 43% (ORR 46%) but with a median duration of only 2.3 months.[255]

Sunitinib (SU11248) is another oral inhibitor of FLT3, c-kit, PDGFR, and VEGFR (1–2). It was evaluated in 15 patients with AML, who were given two doses of 50 mg/day and 75 mg/day for a 4-week cycle followed by 1 or 2 weeks of rest. The responses were limited to partial remissions for short durations (4 to 16 weeks) and morphological remission in 1 patient. These effects were mainly observed in patients with FLT3 mutations. The higher dose of 75 mg was associated with DLTs of cardiac failure, hypertension, and profound fatigue.[256]

Midostaurin (PKC412) is another FLT3 inhibitor that was examined in patients with relapsed or refractory AML and high-risk MDS (n = 95). They were given doses of 50 mg and 100 mg twice daily. The drug was well tolerated and induced hematological improvement in patients with wild-type and FLT3 mutations (42% and 71%, respectively).[257] A subsequent phase 3 CALGB study was launched in 2008, in which patients aged 60 years or less with newly diagnosed AML with FLT3 mutations were randomized to either midostaurin with standard induction and consolidation therapy or standard chemotherapy and consolidation without midostaurin. The results are pending (NCT00651261). Finally, patients with activating mutations of FLT3 who are in first remission will be randomized (phase II) to either observation or mitostaurin maintenance after allogeneic stem cell transplantation (NCT01883362). This trial is just beginning and the results will be expected in the next 3–5 years.

Quizartinib (AC220) is a new selective FLT3 inhibitor with improved efficacy and a better pharmacokinetic profile. A phase 1 study of 76 patients with relapsed or refractory AML and who were treated with quizartinib (12 to 450 mg/day) revealed an ORR of 53% in the FLT3-ITD^{+} group (CR noted in 24%) and 14% in the FLT3-ITD^{-} group.[258] Quizartinib was further studied in a phase 2 trial of AML, in which an ORR of 68% was achieved in patients with refractory or relapsed AML, including those who relapsed after allogeneic bone marrow transplantation.[259] Allogeneic transplantation after quizartinib response was performed in 35% of both FLT3-ITD^{+} and FLT3-ITD^{-} cases. The OS at 1 year for FLT3-ITD^{+} patients post-allogeneic stem cell transplant was 39% among those achieving complete and partial remission. Alternatively, the OS at 1 year without allogeneic stem cells transplantation after complete and partial remission were 25% and 5%, respectively.[260] A phase 1 clinical trial is currently examining the role of Quizartinib as maintenance in AML post-allogeneic stem cell transplantation (NCT01468467).

On the basis of in vitro analyses of primary AML samples, it was concluded that increased mutant allelic burden (>50%) and disease relapse confer increased sensitivity to FLT3 inhibitors.[261] The type and location of FLT3 mutations have also influenced the therapeutic response.[262–264] Parmar et al demonstrated resistance of CD34^{+}FLT3-ITD^{+} leukemic progenitors in vitro to FLT3 inhibitors when cultured with stromal niche cells.[265] Alternatively, recent publications by Weisberg and colleagues demonstrated the benefits of

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using multikinase targeting agents (e.g., AKT, JAK2, Abl) to overcome stromal-cell–derived protective effects and to sensitize AML blasts to FLT3 inhibitors.[266,267]

C-KIT

The stem cell factor receptor (c-kit) is a type III tyrosine kinase receptor encoded by the proto-oncogene kit. The binding of c-kit to its ligand, the stem cell factor (SCF), results in dimerization and autophosphorylation of the receptor and subsequent activation of multiple pro-survival signaling pathways, including PI3 kinase, RAS-RAF-MAP kinase, and JAK/STAT.[268,269] C-kit is expressed in normal hematopoietic cells and in more than 70% of patients with AML, and it plays important roles in cell differentiation, expansion, and survival.[268,270,271] Constitutive activation of c-kit is most frequently due to point mutations in the receptor tyrosine kinase or to ITDs, and these were associated with an increased risk of relapse, drug resistance, and shorter survival (particularly with t(8;21) AML).[272,273–276] Imatinib is a SMI that blocks the ATP binding site of the tyrosine kinases c-kit, PDGFRα and PDGFRβ, and BCR-ABL fusion protein.[277] The published data regarding the efficacy of imatinib for the treatment of refractory AML are inconsistent. This might be explained by variations in patient selection, c-kit mutational status and level of expression and/or dependency of leukemic cell survival on other genetic alterations.[278–282] The addition of imatinib to the reinduction regimen of mitoxantrone, etoposide and cytarabine in a phase I/II trial (n=33) have shown a good response, particularly in patients with relapsed disease, and responses were correlated with AKT inhibition.[283] Imatinib resistance due to the D816 c-kit and to other secondary mutations in c-kit that develop after exposure to imatinib has been described in patients with GIST and CML.[282] In preclinical experiments, Santos et al showed an apoptotic/anti-proliferative response of AML progenitor cells to dasatinib, a new c-kit and src inhibitor, with an additive effect when administered in combination with chemotherapy.[284] A recent CALGB/Alliance and other clinical trials are testing the role of dasatinib in patients with t(8;21) and inv16 AML, both AMLs that can be negatively impacted by activating mutations of c-kit (NCT00850382, NCT02013648, NCT01876953).

JAK2

Janus kinases (JAKs) are non-receptor tyrosine kinases that include JAK1, JAK2, and tyrosine kinase 2 (TYK2).[285] JAKs are associated with the intracellular domain of the class 1 and 2 cytokine receptors for the transmission of cytokine-induced growth and inflammatory signaling to the nucleus through the phosphorylation of the transcription factors STATs.[286,287] Hyperactivated JAK/STAT signaling is frequently observed in patients with AML, and a negative impact of elevated phosphorylated JAK2 levels on treatment outcome was recently demonstrated.[288–291] The activating mutation $JAK2^{V617F}$ was reported in 0.5% to 8% of de novo AML, with a higher frequency seen in patients with t(8;21) leukemia, in whom it is considered a cooperating mutation.[290,292–295] Alterations in the negative regulators of JAK/STAT signaling have also been identified, including the downregulation of CD45 in patients with t(8;21) AML, and hypermethylation of the suppressor of cytokine signaling-1, and the protein inhibitor of activated stats-2 in 70% of AML population.[296–298]

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The treatment of refractory leukemia with ruxolitinib (Jakafi, a JAK1/2 inhibitor FDA approved for the treatment of advanced myelofibrosis) in a phase 2 trial resulted in CR in only 3 of the 38 participants.[299] All responders had post-myeloproliferative AML, and the JAK2^{V617F} mutation did not influence the treatment response. The toxicity was limited, and it manifested mainly as thrombocytopenia and increased transaminases. Hyperactivated JAK/STAT signaling was postulated as a mechanism of resistance to selective FLT3 inhibitors.[300] The simultaneous targeting of FLT3 and JAK/STAT pathways has shown synergistic effects against AML cell lines.[300,301] Of note, lestaurtinib, a JAK2/FLT3 inhibitor, failed to improve patient responses or survival in a phase 2 study when given 2 days after salvage chemotherapy in patients with FLT3-mutant AML (n = 224). These results were attributed to unfavorable pharmacokinetics resulting in inadequate suppression of FLT3 or to post-chemotherapy elevation in FLT3 ligand levels (thus minimizing FLT3 inhibition) and α_1-acid glycoprotein (thus decreasing the free serum levels of the drug).[302] Another FLT3/JAK2 inhibitor, pacritinib (SB1518), demonstrated impressive preclinical activity in FLT3-TKI resistant AML, which has led to a phase 1/2 study in patients with advanced AML (NCT00719836).[301]

Polo-Like Kinases (Plk)

The Polo-like Kinases (Plks) from the family of serine/threonine kinases (Plk1-4) have emerged as important regulators of cytokinesis and cell cycle progression. But Plk1 (STPK13) is the only isoform that has been shown to have a critical role in activation of Cdc2, centrosomal maturation and normal spindle formation.[303] These family members, especially Plk1, are overexpressed in many tumors and have been thought to be logical therapeutic targets, especially in light of in vitro data suggesting that genetic deletion of Plk1 or knockdown of Plks in cell lines results in either loss of viability or decrease in proliferation. Of note is that this occurred predominantly in those cell lines harboring K-RAS mutationa.[304] Although multiple Plk inhibitors are now in early phase clinical trials for non-hematologic malignancies, no studies have thus far been opened for the treatment on AML.

Hedgehog Pathway

Hedgehog signaling is critical for hematopoietic stem cell differentiation and survival. It is positively regulated by the transmembrane molecule, Smoothened (SMO), and the Glioma family of transcription factors (GLI1, GLI2 and GLI3). The transmembrane proteins, Patched (PTCH1 and PTCH2), have inhibitory effects through the suppression of SMO. When GLI2 and GLI3 are phosphorylated on serine and threonine by glycogen synthase kinase (GSK3β), protein kinase A, and casein kinase, they undergo C-terminal ubiquitination and proteasomal proteolysis, yielding truncated proteins that inhibit the transcription of downstream targets. Ligand binding results in PTCH and SMO dissociation followed by SMO accumulation and stabilization of GLI2 and GLI3, which translocate to the nucleus and induce transcription of GLI1, PTCH1, and PTCH2, which then mediate cell cycle progression, apoptosis, and stem cell renewal.[305] Aberrant Hedgehog signaling can contribute to tumor initiation, angiogenesis, and cancer cell survival via gain-of-function SMO mutations, loss-of-function PTCH mutations, aberrant GLI2 and GLI3 expression,

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upregulation of drug resistance genes (e.g., p-glycoprotein, MDR1, BCRP, and ABCG2) or by influencing the interaction of cancer stem cells with the stroma. [306–312] The expression of SHH, GLI1, and GLI2 has been demonstrated in AML blasts, and an anti-leukemic effect was observed in vitro in response to cyclopamine, an SMI of Hedgehog/SMO signaling.[309]

Minami and colleagues demonstrated that the SMO inhibitor PF-04449913 could inhibit leukemia-initiating cells as assessed by the serial xenotransplantation of primary AML blasts.[313] The initial results from a phase 1 trial evaluating PF-04449913 in multiple hematological malignancies are suggestive of good safety profile and treatment response.[314] PF-04449913 could also reduce the resistance to Ara-C mediated by co-culturing of AML cell lines with bone marrow stromal cells in vitro.[315] A clinical trial examining the effect of PF-04449913 with chemotherapy in patients with AML or MDS is currently underway (NCT01546038). In addition, a GLI2 inhibitor (GANT61) has recently shown promising anti-leukemic activity in vitro.[316]

WNT/β-Catenin Pathway

WNT is a glycoprotein that plays an important role in hematopoietic stem cell growth and renewal and in organogenesis during embryonic development.[317] WNT is normally in an inactive state in adults, but it is overexpressed and activated in patients with multiple cancers.[318] It is also implicated in epithelial-mesenchymal transition (EMT) that increases the metastatic potential of tumors.[319] Growing evidence supports the role of the WNT/β-catenin (canonical) pathway in AML. The activation of this pathway is induced by the engagement of WNT with the Frizzled receptor and its co-receptors, the lipoprotein-receptor–related proteins (LRP5 and LRP6). This leads to the relocation of the multiprotein degradation complex (MDC), which consists of glycogen synthase kinase (GSK3β), adnomatous polyposis coli (APC) and axin proteins to the cell membrane resulting in suppression of MDC function. MDC is normally responsible for the phosphorylation of β-catenin which is then targeted by the E3–ubiquitin–ligase complex for proteasomal degradation. Thus, the inactivation of MDC leads to the intracellular accumulation of β-catenin, which translocates to the nucleus to be associated with the T-cell receptor factor and the lymphoid enhancer-binding factor 1 (LEF1) leading to upregulation of survivin, cyclin-D1, C-MYC, and CD44 among others.[320–323]

β-catenin expression in primary AML specimens has been associated with WNT activation and reduced patient survival.[324,325] Multiple strategies are being investigated to abrogate WNT/β-Catenin signaling; 1)disrupting ligand interaction with WNT and its co-receptors (a soluble ligand binding domain of Fzd8, Fzd8-CRD-FC); 2)blocking β-catenin accumulation via the inactivation of Dishevelled, a protein that interacts with Frizzled and that is involved in MDC inactivation (e.g., niclosamide); or by the upregulation of axin (e.g., XAV939, IWR-1); 3) blocking β-catenin nuclear localization by NSAID; 4)the inhibition of HGF-mediated degradation of E-cadherin, a protein that normally complexes with β-catenin leading to β-catenin intracellular sequestration (e.g., PHA665752); 5)the inactivation of p38 MAPK, a kinase that normally phosphorylates and inactivates GSK3 resulting in upregulation of Snail and the downregulation of E-cadherin (e.g., PH-797804); and 6)blocking the formation and transactivation of β-catenin/LEF/TCF complex (e,g., ICG-001,

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CGP049090, ZTM000990, PFK115-584).[326–337] Several drugs targeting WNT/β-catenin signaling are undergoing clinical evaluation in AML (Table 1).

IDH1 and IDH2

Mutations in isocitate dehydrogenase (IDH1 and IDH2) are detected in around 6–16% and 8–19% of AML patients, respectively.[338] The incidence is higher in patients with normal cytogenetics and NPM1 mutations.[338–340] But the data are conflicting regarding the prognostic significance of such mutations.[338,341] However, recent studies suggested that an elevated serum level of 2-hydroxyglutarate protein (which is produced by IDH1 and IDH-2 mutant cells, and is thought to induce DNA hypermethylation and accelerate leukemogenesis) is a marker of IDH mutation, and disease activity and a predictor of poor outcome.[340,342–345] Cell line studies by Chaturvedi et al demonstrated suppression of growth and MAPK signaling in IDH1- mutant leukemic cells when treated with HMS-101 (a SMI of IDH1 mutation).[346] AG-6780 is a SMI of IDH2/RI40Q mutation that has been shown to stimulate the expression of maturation markers in leukemic cells in vitro, reflecting induction of differentiation.[347] AG-221, is another IDH2 inhibitor that is being evaluated as a single agent in patients with MDS, relapsed/refractory AML, and newly diagnosed, older AML patients carrying IDH2 mutation. The initial report of the phase I trial of AG-221 supports safety of the drug with early signs of efficacy. Responses were observed in 6 of 10 evaluable patients; with 2 CR (one of whom proceeded to allogeneic stem cell transplantation).[348]

Immunotherapy: Monoclonal and Bispecific Antibodies

Gemtuzumab ozogamicin (GO) is a humanized monoclonal antibody- drug conjugate directed against CD33, a transmembrane receptor expressed by the majority of AML blasts.[349] An ORR of 26–30% in several Phase II trials of GO as monotherapy in relapsed AML led to the initial FDA approval in 2000. GO was withdrawn in 2010 due to increased death rate and lack of an additional benefit in a SWOG study. However, emerging data from subsequent phase III trials studying the incorporation of GO to standard chemotherapy indicated an improved disease-free and overall survival, especially in the low risk groups.[350–352] It was presumed that such controversy is due to using lower doses of daunorubicin in the GO arm of the SWOG trial.[353] Further, GO was associated with high response and prolonged remission in APL when used as monotherapy (substituting chemotherapy in older patients or in molecular relapse) and as an alternative to anthracyclines in combination with ATRA and arsenic trioxide (for induction therapy of high-risk APL).[354–356]

New technologies in protein engineering have led to the generation of promising immune-based therapeutic agents that could recruit immune effector cells to tumor targets. Such molecules are called bispecific antibodies. They combine two antigen specificities for the simultaneous binding to specific markers expressed by effector cells and tumor cells, thereby leading to immune response activation for the selective eradication of target cells in an antigen-specific and MHC-unrestricted manner.[357–359] Thus, bispecific antibodies could bypass some of the protective barriers of AML blasts against immune responses such as

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MHC-downregulation, impaired immunological synapse formation between T cells and blasts, and expression of negative co-stimulatory ligands (such as PD-1L) on target AML and tumor cells. Currently, the most developed platforms of bispecific antibodies are the bispecific T-cell engagers (BiTEs) and the dual affinity re-targeting molecules (DARTs). A CD3 × CD33 BiTE has shown promising tumoricidal effects in vitro and in vivo preclinical models.[360] Our group has investigated a CD3 × CD123 DART in AML and shown near-complete eradication of the $CD123^{+}$ human AML in the peripheral blood in a primary human AML xenotransplantation model.[361] However, non-specific T cell activation and cytokine storm are potential risks with the use of such bi-specific agents. Blinatumomab (CD3 × C19 BiTE) has been shown to induce high rates of remissions in patients with B-precursor acute lymphoblastic leukemia (B-ALL), but with prolonged B lymphocyte depletion and hypogammaglobulinemia.[181–183] Cytokine release syndrome was observed, manifested with fever, rigors, and hypoxia, which could be ameliorated by steroid prophylaxis.[184] Approximately 15% of patients experienced CNS complications, including convulsions and encephalopathy, which were reversible after drug withdrawal.[362–364] Wong and colleagues demonstrated recently the activation of T cell cytotoxicity against B-precursor CLL (B-CLL) following exposure to Blinatumomab in vitro.[365] Blinatumomab is currently being evaluated in patients with ALL, B-NHL and B-CLL (NCT01466179).

Expert Commentary

Aberrant signal transduction has been considered the driving force of oncogenesis, with a high degree of complexity and cross-talk among the different pathways. The use of SMIs allows for the specific targeting of intracellular kinases and protein–protein interactions, which has been proven effective for improving the survival of GIST, APL and CML and which has prompted substantial interest in AML. However, in contrast with the preclinical results, there has been only a modest clinical benefit of SMIs in patients with AML, especially when used in the absence of chemotherapy. These limited clinical responses may involve the upregulation of the targeted gene, the selective expansion of a resistant clone (as a result of epigenetic alterations or primary or acquired mutations), a protective tumor microenvironment, the presence of other molecular aberrations that maintain leukemic cell proliferation and survival, or the properties of the cell lines or primary AML samples examined in the preclinical work.[185] This argues for the importance of the combination with other targeting and cytotoxic agents while considering the potential toxicity that may result from the off-target effects on normal cells.

It is important to note that most of the data available are from early-phase studies. This preludes accurate interpretation in the light of the heterogenousity and small number of the patients, the short duration of follow up, selection bias, and the presence of multiple confounding factors that may influence treatment outcomes (e.g., drug exposure, clinical status, cytogenetics, interval of CR, and number of treatment cycles administered). In another aspect, the high remission rate of FLT3 inhibitors, although it is mostly short-lasting, can be considered as a bridging strategy for transplantation. Combining SMIs with conventional chemotherapy is an interesting investigational approach in AML to reduce disease burden and thereby improve transplantation outcome. Studies have shown the immunomodulatory properties of some SMIs.[366] For example, sunitinib down regulates the

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expression of PDL-1 (by myeloid-derived suppressor cells {MDSCs}), and CTLA-4 and PD-1 (by tumor cells), reduces T-regulatory cells and MDSCs, and increases cytotoxic T cell expansion.[367] It may therefore be reasonable to study such SMIs 1) as maintenance therapy post-transplantation to enhance GVL effect, prolong disease remission and eliminate minimal residual disease, and 2) to enhance the GVL effect of donor lymphocyte infusion for relapsed AML after transplantation. We also agree with previous reports that durable partial response and disease-stabilization of well tolerated SMIs are potential markers of clinical benefit, particularly in older patients, unfit for chemotherapy and transplantation, and in relapsed AML patients with limited therapeutic options.[145]

Five-year view

Compelling evidence supports the heterogenousity of AML, the importance of cytogenetics, and the use of gene expression profiling to stratify the risk and design a treatment plan that is based on the biological characteristics of the disease. It is intuitively appealing to incorporate molecular markers of prognosis and biochemical response on the basis of correlative scientific analyses of the relevant patient specimens for more personalized treatment decisions and clinical trial design. Understanding the redundancy of pro-survival pathways, tumor evolution, and cancerous cell interaction with protective niches is an ongoing challenge to the discovery and validation of new drug targets and combination therapies. Additional effort is needed to exploit the pharmacokinetics/dynamics and mechanisms of action and resistance of the current SMIs for enhanced therapeutic strategies in the future.

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Key issues

- Small molecule inhibitors have been developed to modulate the activity of proteins encoded by mutated or overexpressed genes in patients with acute myeloid leukemia.
- Clonal evolution, dependency on alternative signaling pathways, and pharmacodynamics are potential obstacles to the clinical development of small molecule inhibitors for the treatment of acute myeloid leukemia.
- Multi-targeting and the combination of small molecule inhibitor treatment with chemotherapy may improve treatment efficacy.
- The analysis of patient samples is an important tool to investigate resistance mechanisms and to discover and validate biological markers that could be used for the prediction and assessment of treatment response.

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Table.1

Selected Ongoing Clinical Trials of Small Molecular Inhibitors in Patients with Acute Myeloid Leukemia

Molecular Target	Small Molecule Inhibitor	Phase of the study	Study Design	ClinicalTrials.gov Identifier
Aurora kinase	Alisertib (Aurora A Kinase inhibitor)	Phase 1	alisertib in combination with 7+3 induction chemotherapy	NCT01779843
	AMG 900	Phase 1	Single agent	NCT01380756
Farnesyl transferase	Lestaurtinib	Phase 1/ 2	lestaurtinib + cytarabine + idarubicin relapsed and refractory AML	NCT00469859
Histone deacetylase	Valproic acid	Phase 1/ 2	ATRA+ Valproic acid+ low dose cytarabine	NCT00995332
	Entinostat (MS275)	Phase 2	Entinostat + 5AZC in elderly	NCT01305499
	Vorinostat	Phase 2	Temozolomide + vorinostat in patients more than 60 years old with newly diagnosed or relapsed or refractory AML	NCT01550224
	Vorinostat	Phase 3	Cytarabine + daunorubicin hydrochloride or idarubicin+ cytarabine with or without vorinostat	NCT01802333
Proteasome inhibitors	Bortezomib	Phase 2	Comparing decitabine with decitabine + subcutaneously injected bortezomib in untreated AML	NCT01420926
	Ixazomib	Phase 2	Single agent for NPM1c relapsed/ refractory AML	NCT02030405
CXCR4	Plerixafor	Phase 1	Plerixafor+ decitabine for induction and post-remission therapy in elderly	NCT01352650
	Plerixafor	Phase 1/2	Plerixafor+ GCSF + chemotherapy in refractory or relapsed AML	NCT00906945
	BL-8040	Phase 2A, multicenter, open-label	Single agent therapy for relapsed and refractory AML	NCT01838395
	BMS-936564 (Anti-CXCR4)	Phase 1, multicenter, open-label	Single agent in relapsed AML, and B cell malignancies	NCT01120457
HSP90	Ganetespib	Phase 1/2	To combine either the tyrosine kinase inhibitor AC220, plerixafor, or ganetespib with chemotherapy in older patients with AML and high-risk myelodysplastic syndrome (AML18 Pilot)	NCT01236144
FLT3	Crenolanib	Phase 2	Single agent therapy in relapsed and refractory AML with FLT3- D835 activating mutation	NCT01522469
	Sorafenib	Phase 1/2	Sorafenib + vorinostat, + bortezomib for AML with complex or poor-risk (monosomy 5/7) cytogenetics or FLT3-ITD–positive mutation	NCT01534260
	Sorafenib	Phase 1	Single agent maintenance therapy after allogeneic stem cell transplantation.	NCT01398501
	Sorafenib	Phase 3	Bortezomib+ sorafenib for de novo AML with high allelic ratio FLT3-ITD disease	NCT01371981
	Midostaurin (PKC412)	Phase 2	Single agent therapy for c-kit or FLT3-ITD mutated t(8;21) AML	NCT01830361

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Molecular Target	Small Molecule Inhibitor	Phase of the study	Study Design	ClinicalTrials.gov Identifier
c-kit	Nilotinib	Phase 1/2	Nilotinib+ mitoxantrone, etoposide +high-dose cytarabine (NOVE-HiDAC) induction chemotherapy followed by consolidation therapy for poor-risk patients up to 65 years old with c-kit positive AML	NCT01222143
Hedgehog pathway	PF-04449913	Phase 2	Single agent therapy for high risk AML post-allogeneic stem cell transplantation relapse	NCT01841333
WNT pathway	PRI-724	Phase 1/2	Single agent tharpy for advanced malignancies.	NCT01606579
	CWP232291	Phase 1	Single agent therapy for relapsed and refractory AML, chronic myelomonocytic leukemia, myelodysplastic syndrome or high-risk myelofibrosis	NCT01398462
IDH1/2	AG-120	Phase 1	Monotherapy in advanced hematological malignancies with IDH1 mutation	NCT02074839
	AG-221	Phase 1	Monotherapy in advanced hematological malignancies with IDH2 mutation	NCT01915498

AML, Acute myeloid leukemia; *5AZC,* 5-Azacytidine.

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Table.2

Potential therapeutic targets in the tumor microenvirment

Molecular target	Biological Significance and related Studies	Refrences
VLA-4/VCAM-1	• BIO5192, is a VLA-4 antagonist that causes disruption of the intearction of VLA-4 adhesion molecule on progenotors with its ligand VCAM-1 on mesenchymal stroma cells, and results in significant progenitor cell mobilization. • VCAM-1/VLA-4 intraction stimulates NFκB signaling and is linked to chemoresistance in AML.	[368, 369]
Angiogenesis	• The addition of Bevcizumab to chemotherapy had no additive effect in a phase II tria of older AML patients with newly diagnosed AML. • Inhibition of the PI3K/Akt/mTOR signaling reduced angioregulatory cytokine release from the tumor and the stroma cells (fibroblasts, osteoblasrts and endothelial cells), with an anti-proliferative effect in vitro. • The interaction between AML cells and endothelial cells enhances angiogenesis through upreglation of VEGF and Notch/Dll4 pathway.	[370–372]
HIF-1α	• Upreglates VEGF and induces angiogenesis. • Upregulates CXCR4 and CXCL12 leading to increased CXCR4-CXCL12-mediated chemotaxis, and migration of leukemic stem cell to the protective bone marrow niche. • The cross-talk between HIF-1α and Notch pathway promotes leukemic stem cell survival. • Echinomycin, an HIF inhibitor showed an anti-leukemic activity in vitro and in vivo.	[373–376]
Arginase II	• Released by AML blasts. • Associated with suppressed proliferation of T cells and $CD34^+$ heamatopoietic progenitors, polarization of monocytes to immunosuppressive M2-like phenotype, and increased AML engrafment. • The immunosuppresive effects could be revrersed with the use of the Arginase inhibitor NOHA, and the NO synthase inhibitor L-NMMA. • Clinical development was delayed due to concens of drug toxicity.	[377, 378]
CD44	• Mediates cell-cell and cell-extracelluar matrix adhesion. • Binding to hyaluronan ligand induces intracellur signaling. • CD44 antibody eliminated leukemic stem cells in vivo, as demonstrated by serial transplantation experiment.	[373, 379]

VLA-4 , veruy late antigen-4; *VCAM-1*, vascular cell adhesion molecule; *VEGF*, vascular endothelial growth factor; *HIF-α*, hypoxia induced factor-1 alpha; *AML*, acute myeloid leukemia.