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Therapeutic targeting of microenvironmental interactions in leukemia: mechanisms and approaches

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Abstract

In hematological malignancies, there are dynamic interactions between leukemic cells and cells of the bone marrow microenvironment. Specific niches within the bone marrow microenvironment provide a sanctuary for subpopulations of leukemic cells to evade chemotherapy-induced death and allow acquisition of a drug-resistant phenotype. This review focuses on molecular and cellular biology of the normal hematopoietic stem cell and the leukemia stem cell niche, and of the molecular pathways critical for microenvironment/leukemia interactions. The key emerging therapeutic targets include chemokine receptors (CXCR4), adhesion molecules (VLA4 and CD44), and hypoxia-related proteins HIF-1α and VEGF. Finally, the genetic and epigenetic abnormalities of leukemia-associated stroma will be discussed. This complex interplay provides a rationale for appropriately tailored molecular therapies targeting not only leukemic cells but also their microenvironment to ensure improved outcomes in leukemia.

Keywords

Bone marrow microenvironment; leukemia; molecular targeted therapy; CXCR4; AMD3100; stem cell niche; drug resistance

1. Key components of the bone marrow microenvironment: home for leukemic stem cells

1.1. Normal hematopoietic stem cells and their physiological niches

Hematopoietic stem cells (HSC) reside within specialized areas of the bone marrow (BM) microenvironment, defined as two distinct microenvironmental niches: “osteoblastic (endosteal)” and “vascular” niches (Perry and Li, 2007). Recent studies demonstrate that these niches work in concert (Figure 1). The osteoblastic niche, localized at the inner surface of the bone cavity and with abundant bone-forming osteoblasts, provides a microenvironment for long-term HSC which are capable of contributing to hematopoiesis as quiescent or slow-cycling cells (Perry and Li, 2007; Arai et al., 2004; Calvi et al., 2003; Zhang et al., 2003). In turn, the vascular niche, which consists of sinusoidal endothelial cells lining blood vessels, promotes proliferation and differentiation of actively cycling, short-

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term HSC (Passegue et al., 2005, Kopp et al., 2005). Coordination between the osteoblastic and vascular niches regulates HSC self-renewal, proliferation, differentiation and mobilization in and out of the BM. HSC leave the osteoblastic niche, mobilize to the vascular niche, and enter the blood vessel. They subsequently may undergo transendothelial migration from the peripheral circulation and return first to the vascular niche and then to the osteoblastic niche (Cancelas and Williams, 2006; Lapidot et al., 2005). In BM, HSC are found in close proximity to endosteum and/or sinusoids (Adams and Scadden, 2006a; Kiel et al., 2005; Kiel et al. 2007; Nilsson et al., 2001). However, several recent reports have questioned the notion of two distinct niches, indicating that the vascular niche may localize within close proximity to the osteoblastic niche. It has been estimated that two-thirds of HSC in the BM are adjacent to sinusoids (Kiel et al., 2005). Xie et al. recently demonstrated that the endosteum forms a well-vascularized special zone that frequently is localized near N-cadherin–positive preosteoblastic cells, and that this special niche promotes expansion of HSC in response to BM damage (Xie et al., 2009).

1.2. Bone marrow architecture

Hematopoietic cell development is tightly regulated by BM stromal cells (BMSC) through production of cytokines, chemokines, and intracellular signals initiated by cellular adhesion. BMSC encompass a variety of cell types, including osteoblasts, osteoclasts, endothelial cells, perivascular reticular cells, and mesenchymal stem/stromal cells (MSC), all of which are critical for the regulation of HSC maintenance and localization (Adams et al., 2006; Arai et al., 2004; Calvi et al., 2003; Kollet et al., 2006; Zhang et al., 2003; Sacchetti et al., 2007; Sugiyama et al., 2006; Morrison and Spradling, 2008). Although the nature of the true “mesenchymal stem cell” remains enigmatic, CXC chemokine ligand 12 (CXCL12)–expressing $CD146^+$ MSC were recently reported to be self-renewing progenitors that reside on the sinusoidal surfaces and contribute to organization of the sinusoidal wall structure (Kiel et al., 2005), produce angiopoietin-1 (Ang-1), and are capable of generating osteoblasts that form the endosteal niche. By generating functionally distinct cell types (osteoblasts and adipocytes) and structures (bone surface and sinusoidal walls), MSC play a crucial role in supporting hematopoiesis as key components of the hematopoietic microenvironment (Sacchetti et al., 2007).

Cytokines and chemokines produced by BMSC concentrate in particular niches secondary to varying local production and through the effects of cytokine-binding glycosaminoglycans. Of these, CXCL12/stromal cell-derived factor-1alpha (SDF-1α) positively regulates HSC homing, while transforming growth factors, FMS-like tyrosine kinase 3 (Flt-3) ligand, and Ang-1 function as quiescence factors. BM engraftment involves subsequent cell-to-cell interactions through the BMSC-produced complex extracellular matrix (ECM) (Zuckerman and Wicha, 1983; Wight et al., 1986). As such, vascular cell–adhesion molecule-1 (VCAM-1) or fibronectin is critical for adhesion to the BMSC (Miyake et al., 1991; Garcia-Gila et al., 2002).

1.3. Leukemic microenvironment: niche retreats for leukemic stem cells

A small fraction of myeloid leukemic cells, leukemia stem cells (LSC), exhibits the capacity for long-term self-renewal (Holyoake et al., 2002; Warner et al., 2004; Liesveld et al., 2004) within the BM microenvironment, which is required for maintenance of the malignant clone (Braun and Shannon, 2008). LSC are able to generate leukemic blasts, and the leukemic clone is organized as a hierarchy (Zhang et al., 2003). BMSC are capable of promoting the growth, survival and drug resistance of leukemic cells by providing the necessary cytokines and cell contact-mediated signals to LSC (Dazzi et al., 2006; Ramasamy et al., 2007). MSC were shown to induce upregulation of anti-apoptotic molecules and promote resistance of acute myelogenous leukemia (AML) cells to the chemotherapeutic agent cytarabine

(Konopleva et al., 2002). The molecular mechanisms for maintaining quiescence of normal stem cells may also facilitate LSC survival. Whereas LSC share certain features of self-renewal and differentiation with HSC, LSC differ in their dysregulated proliferation and ability to invade and spread. The majority of leukemias respond to initial treatment; however, relapse is common, indicating resistance of LSC to current therapies. For LSC survival, proliferation, and differentiation, both the osteoblastic and vascular niches are critical (Arai et al., 2004; Calvi et al., 2003; Zhang et al., 2003; Nilsson et al., 2005; Kiel et al., 2005; Naveiras and Daley, 2006; Kopp et al., 2005).

Ninomiya et al. modeled the homing, proliferation, and survival sites of human leukemia cells and of cord blood $CD34^+$ cells (Ninomiya et al., 2007). The transplanted leukemia cells initially localized on the surface of osteoblasts in the epiphysial region and then expanded to the inner vascular and diaphysial regions. Eight weeks after transplantation, the number of leukemia cells transiently increased by as much as 50%, predominantly in the epiphysial region. After administration of high-dose cytarabine, residual leukemia cells clustered and adhered to the blood vessels as well as to the endosteum, suggesting that leukemia cells receive antia-poptotic signals not only from osteoblasts but also from vascular endothelium (Ninomiya et al., 2007).

1.4. Leukemic stem cell niche, integrins, CD44, ILK

Integrins are required for LSC to lodge in the BM niche. The attachment of AML cells to the BM microenvironment through interaction between very late antigen–4 on leukemic cells and fibronectin on MSC has been shown to be crucial for the persistence of minimal residual disease in AML (Matsunaga et al., 2003). Mudry et al. (2000) showed that the maximum viability of acute lymphoblastic leukemia (ALL) cells during exposure to cytarabine and etoposide required interaction with the MSC adhesion molecule VCAM-1.

The adhesion molecule CD44 has been demonstrated to be a key regulator of AML LSC homing to microenvironmental niches and maintaining a primitive state (Jin et al., 2006). CD44 mediates adhesive cell-cell and cell-ECM interactions through binding to its main ligand, hyaluronan, a glycosaminoglycan highly concentrated in the endosteal region (Avigdor et al., 2004). Other ligands include osteopontin, fibronectin, and selectin, all of which are involved in cell trafficking and lodgment. Beyond its adhesion function, CD44 can also transduce multiple intracellular signal transduction pathways when ligated with hyaluronan or specific function-activating monoclonal antibodies (Turley et al., 2002). Manipulation of CD44 function with the H90 monoclonal antibody resulted in marked reduction of the leukemic burden in NOD-SCID mice transplanted with primary AML cells through alteration of AML LSC fate and abrogation of AML LSC homing.

Integrin ligation triggers activation of prosurvival signaling cascades. As such, integrin-linked kinase (ILK) directly interacts with β integrins and phosphorylates Akt in a phosphoinositol-3-kinase (PI3K)–dependent manner. Direct contact of leukemic cells and BMSC results in activation of ILK/Akt/GSK3β signaling, which modulates Notch signaling and phosphorylation of signal transducer and activator of transcription 3 (STAT3) and promotes survival of leukemic cells (Tabe et al., 2007). Blockade of PI3K or ILK signaling with pharmacological inhibitor LY294002 or QLT0267 specifically inhibited stroma-induced phosphorylation of Akt and GSK3β, suppressed Stat3 and ERK1/2 activation, and decreased Notch1 and Hes1 expression in leukemic cells. In turn, leukemic cells growing in direct contact with BM stromal elements induced activation of Akt, ERK1/2, and Stat3 signaling in MSC, accompanied by significant increases in Hes1 and Bcl2 proteins, which were all suppressed by PI3K/ILK inhibitors. These results indicated reciprocal activation of ILK/AKT in both leukemic and BM stromal cells and suggest that ILK/AKT is a proximal signaling pathway critical for survival of leukemic cells within the BM microenvironment.

Hence, disruption of these interactions by ILK inhibitors represents a potential novel therapeutic strategy to eradicate leukemia in the BM microenvironment by simultaneous targeting of both leukemic cells and activated BM stromal cells (Tabe et al., 2007).

It is well known that ALL cells are sensitive to asparagine depletion, and asparaginase is a major component of ALL therapy. Iwamoto et al. (2007) demonstrated that a high level of asparagine secretion by MSC caused asparaginase resistance of ALL cells that reside in MSC niches, and this protective effect correlated with levels of asparagine synthetase expression in MSC. If the cells residing in these privileged niches have stem cell properties, they might ultimately be capable of initiating leukemia recurrence. These findings provide the basis for understanding asparaginase resistance in ALL, and suggest that depletion of asparagine secretion by MSC might enhance the antileukemic action of asparaginase and other antileukemic agents.

2. CXCR4/CXCL12 interactions and migration of leukemic cells

CXCL12 (SDF-1α) is a chemokine that functions through its receptor CXCR4, a seven-transmembrane G-coupled receptor protein (Loetscher et al., 1994, Jazin et al., 1997). CXCL12-CXCR4 signaling is involved in homing of HSC into BM during ontogeny as well as survival and proliferation of colony-forming progenitor cells (Nagasawa et al., 1996; Lataillade et al., 2000; Broxmeyer et al., 2003). The CXCR4-selective antagonist–induced mobilization of HSC into the peripheral blood further indicates a role for CXCL12 in retaining HSC in hematopoietic organs (Broxmeyer et al., 2005). Cells pass in and out of the circulation through sinusoids, a reticular network of fenestrated vessels (Morrison and Spradling, 2008) that provides the nutrient-rich microenvironment with high concentrations of oxygen and growth factors (Abkowitz et al., 2003). Sugiyama et al. (2006) demonstrated that perivascular reticular cells secrete much higher levels of CXCL12 than other constitutive sources of CXCL12, such as osteoblasts, fibroblasts, and endothelial cells. These reticular cells, defined as CXCL12-abundant reticular (CAR) cells, reside predominantly in sinusoids but occasionally in endosteum, and directly contact HSC. CAR cells may serve as a transit pathway for shuttling HSC between the osteoblastic and vascular niches, where essential but different maintenance signals are provided (Perry and Li, 2007).

Importantly, CXCR4 levels are significantly elevated in leukemic cells from patients with B-cell chronic lymphocytic leukemia (B-CLL) (Mohle et al., 1999) or B-cell but not T-cell ALL (Bradstock et al., 2000; Shen et al., 2001; Dialynas et al., 2001), and to a lesser degree in AML (Mohle et al., 2000; Voermans et al., 2002). Leukemic cells expressing CXCR4 are, in general, highly responsive to CXCL12, demonstrating CXCL12-induced calcium flux, integrin-mediated cell adhesion, chemotaxis, and migration. CXCR4 also mediates the homing to and engraftment of pre-B-ALL cells in the BM of NOD/SCID mice (Shen et al., 2001). In line with these findings, the association between CXCR4 expression and poor outcome in patients with B-CLL (Ishibe et al., 2002), pre-B-ALL (Crazzolara et al., 2001), and AML (Rombouts et al., 2004; Konoplev et al., 2007) has been reported.

Since the expression of CXCR4 on leukemic progenitor cells contributes to their homing to the BM microenvironment (Nagasawa et al., 1996; Ma et al., 1998), disrupting these interactions with CXCR4 inhibitors may represent a novel strategy for targeting leukemic cells within their BM microenvironment. This has been demonstrated in preclinical models of CLL, ALL, and AML. The active CXCR4-specific antagonists abrogated the antiapoptotic effect of synthetic CXCL12 and alleviated stromal protection of CLL cells from spontaneous or fludarabine-induced apoptosis through inhibition of actin polymerization, chemotaxis, and migration of CLL cells beneath stromal cells (Burger et al., 2005). Further, CXCL12-induced phosphorylation of p44/42 mitogen-activated protein

kinase and STAT3 was abolished by CXCR4 antagonists in CLL cells (Burger et al., 2005). A small-molecule reversible inhibitor of CXCL12/CXCR4, AMD3100, completely blocked CXCL12-induced chemotaxis, attenuated the migration of pre-B-ALL cells into BMSC layers and enhanced the cytotoxic and antiproliferative effects of vincristine and dexamethasone (Juarez et al., 2003).

In a murine model of acute promyelocytic leukemia (APL), administration of AMD3100 in combination with chemotherapy triggered an increase of circulating APL cells with decreased tumor burden and improved overall survival compared to chemotherapy alone (Nervi et al., 2009). In primary CLL and AML cells, a specific peptide targeting CXCR4 inhibited CXCL12-induced chemotaxis, inactivated prosurvival signaling pathways, and partially abrogated the protective effects of BMSC on chemotherapy-induced apoptosis (Zeng et al., 2008).

Significantly increased CXCR4 expression has been reported in Flt3/internal tandem duplication AML compared to FLT3/wild-type AML (Rombouts et al., 2004). This finding and additional pre-clinical data indicate that Flt3 axis participates in the trafficking of transformed hematopoietic cells through CXCR4. Recent report demonstrated that CXCR4 inhibition indeed increased sensitivity of FLT3-mutated leukemic cells to the apoptogenic effects of the FLT3 inhibitor sorafenib under stromal co-cultures. Most importantly, CXCR4 inhibitor AMD3465 alone or in combination with granulocyte colony-stimulating factor induced mobilization of AML cells and progenitor cells into circulation and prolonged survival of the animals treated with sorafenib in vivo, presumably through recruitment of leukemic cells out of their protective microenvironmental niches (Zeng et al., 2008). These results suggest that CXCL12/CXCR4 interactions contribute to the resistance of leukemic cells to signal transduction inhibitor-induced apoptosis in the BM microenvironment.

In chronic myelogenous leukemia (CML), primary CML blasts show attenuated migratory and adhesive responses to CXCL12 and/or decreased CXCR4 expression (Geay et al., 2005; Salgia et al., 1999; Peled et al., 2002). Notably, the BCR-ABL tyrosine kinase inhibitor imatinib restored CXCR4 expression under MSC co-culture conditions, which in turn induced migration of CML cells to the BM microenvironment niches and resulted in acquisition of stroma-mediated chemoresistance of quiescent CML progenitor cells (Jin et al., 2008). The increase in CXCR4 levels on CML progenitor cells was also found in samples from CML patients treated with BCR-ABL tyrosine kinase inhibitor imatinib or interferon alpha. However, the mechanisms involved in $p210^{BCR\text{-}ABL}$-induced disruption of CXCR4 expression and the events downstream of CXCL12-dependent CXCR4 signaling are not fully understood. It has been reported that Lyn-mediated pathological crosstalk exists between BCR/ABL and the CXCR4 pathway in leukemia cells, which disrupts chemokine signaling and chemotaxis and increases the ability of immature cells to escape from the marrow (Ptasznik et al., 2002).

Suppression of normal hematopoiesis is observed frequently in leukemia patients with relatively low tumor burden, which does not necessarily reflect occupancy of the anatomic space by leukemic cells. It has been demonstrated recently that leukemic cell growth disrupts normal hematopoietic progenitor cell (HPC) BM niches and creates a tumor microenvironment (Colmone et al., 2008). Leukemic cells initially migrate toward the CXCL12-positive vascular niches in the BM, which in the murine model overlap with normal HPC niches (Sipkins et al., 2005; Colmone et al., 2008). After 1 month of leukemia growth *in vivo*, CXCL12 production in the tumor vascular niche was markedly downregulated, and the normal human $CD34^+$ cells transplanted in leukemic mice migrated to tumor niches through a CXCL12-independent mechanism, by virtue of stem cell factor (SCF) abundantly secreted by leukemic cells in the tumor niches. These findings indicate

that the alteration of signaling mechanisms of BM niches used by normal HSC homing may be “hijacked” by LSC (Li and Neaves, 2006).

Altogether, disruption of CXCL12/CXCR4 interactions with CXCR4 inhibitors represents the strategy of blocking LSC homing to a BM niche and/or sensitizing leukemic cells to chemotherapy or kinase inhibitors by targeting their protective BM microenvironment. Concerns have been raised over potential toxicity of these inhibitors, in particular when combined with cytotoxic drugs, because mobilized normal HPCs that normally are protected in the BM microenvironment would then be exposed to the toxicity of chemotherapy. A leukemia cell-targeted therapy, such as a monoclonal antibody (anti-CD33, anti-CD20 or anti-CD52) or selective kinase inhibitor (sorafenib, imatinib), in combination with CXCR4 antagonists could avoid these potential side effects. In turn, targeting of SCF may inhibit HPC interaction with tumor niches and conceivably maintain normal progenitor cell function in the setting of malignancy (Colmone et al., 2008).

3. Approaches to modulate the LSC stem cell niche via hypoxia/HIF-1α/ VEGF signaling

Hypoxia is the major stimulus for angiogenesis, and hypoxia-inducible transcription factor-1 alpha (HIF-1α) is its key mediator. Recent reports indicate that the endosteum at the murine bone-BM interface is hypoxic (Parmar et al., 2007). Data in a rat model demonstrate that leukemic cells infiltrating bone marrow were markedly hypoxic compared to cells in bone marrow of healthy rats (Mortensen et al., 1998). Leukemic cells are able to proliferate even under hypoxic conditions, indicating that the cells are able to adapt to hypoxic conditions (Mortensen et al., 1998, Jensen et al., 2000). Further, overexpression of the oxygen-regulated component of HIF-1, HIF-1α, has been observed in clusters of leukemic cells in BM specimens from patients with primary ALL (Wellmann et al., 2004).

Notably, HIF-1α was recently found to regulate *CXCL12* (*SDF-1*α) gene expression in endothelial cells, resulting in selective *in vivo* expression of CXCL12 in ischemic tissue in direct proportion to reduced oxygen tension (Ceradini et al., 2004). The HIF-1α–induced CXCL12 expression increased the adhesion, migration and homing of circulating CXCR4-positive progenitor cells into the ischemic tissue. In AML, total and surface CXCR4 expression were upregulated under hypoxic conditions in leukemic cell lines and patient samples (Fiegl et al., 2009). Consistent with the findings that HIF-1α regulates CXCR4 (Staller et al., 2003), these data suggest that a hypoxic BM microenvironment represents a conditional stem and progenitor cell niche in which HIF-1α–induced stabilization and activation of both the trafficking stimulus (CXCL12) and receptor (CXCR4) facilitate recruitment and retention of leukemic progenitor cells. In this context, HIF-1α may represent an important molecular target within the tumor microenvironment (Figure 2). Lee et al. recently reported that the anthracyclines doxorubicin and daunorubicin, commonly used in the therapy of AML, are in fact potent HIF-1α inhibitors (Lee et al., 2009). Other strategies specifically targeting HIF-1α are being explored in solid tumor models. These include a novel antisense oligonucleotide against HIF-1α, which was shown to inhibit tumor angiogenesis, down-regulation of vascular endothelial growth factor (VEGF) expression and enhancement of the antitumor effects of doxorubicin in a hepatocellular carcinoma mouse model (Liu et al., 2008). The direct HIF-1α inhibitor PX-478, currently undergoing Phase I clinical trials, decreased expression of hypoxia-mediated, but not of normoxic, VEGF expression and demonstrated antitumor activity against even large tumor xenografts (Koh et al., 2008).

One of the most advertised functions of hypoxia and HIF-1α is upregulation of VEGF and stimulation of angiogenesis. Formation of new vessels by angiogenesis represents an

adaptive response to hypoxia and involves endothelial cell proliferation, a process stimulated by hypoxia-inducible growth factors, such as VEGF. The microvasculature is an active component of the BM microenvironment and is responsible for supplying appropriate oxygen and nutrients. Increased angiogenesis is observed in myelodysplastic syndrome (MDS) (Pruneri et al., 1999; Korkolopoulou et al., 2001), AML (Hussong et al., 2000; Kini et al., 2001), ALL (Perez-Atayde et al., 1997; Koomagi et al., 2001), and multiple myeloma (MM) (Vacca et al., 1999; Rajkumar et al., 2000). As an essential regulator of physiologic and pathologic angiogenesis, VEGF plays a vital role in the growth and metastases of solid and hematologic malignancies (Ferrara et al., 2003). VEGF secreted by leukemic cells activates receptors on both leukemic and endothelial cells, and stimulates their proliferation. VEGF was found to inhibit apoptosis in leukemic cells after exposure to etoposide and doxorubicin by inducing MCL1, a member of the BCL2 family. Moreover, VEGF was observed to promote the survival of MM cells by inducing BCL2 expression and inhibiting apoptosis via VEGF receptor 2 (Dias et al., 2002). In CML, the CML-associated oncogene *BCR-ABL* induces expression of the *VEGF* and *HIF1*α genes via a PI3K/mTOR-dependent pathway (Mayerhofer et al., 2002). The tyrosine kinase inhibitor imatinib has been shown to inhibit c-Kit-induced HIF-1α activity and VEGF expression in small cell lung cancer cells, indicating that activation of c-Kit by SCF results in HIF-1α–mediated enhancement of VEGF expression and that inhibition of c-Kit signaling by imatinib could result in inhibition of tumor angiogenesis (Litz and Krystal, 2006).

The proteasome is a multicatalytic enzyme complex that is responsible for degradation of intracellular proteins and controls cell proliferation, differentiation and apoptosis (Clarke, 2002; Jesenberger and Jentsch, 2002). The proteasome inhibitor bortezomib (PS-341) is used for the treatment of several hematological malignancies, most commonly MM (Richardson et al., 2006) because of its ability to induce apoptosis of MM cells by affecting their interaction with the microenvironment (Mitsiades et al., 2006; Blade et al., 2005), and to inhibit MM-associated angiogenesis (Roccaro et al., 2006). Bortezomib exerts an anti-angiogenic effect by inhibiting VEGF and interleukin 6 (IL-6) secretion by MM endothelial cells (Roccaro et al., 2006; Strieter, 2005). When human umbilical vein endothelial cells (HUVECs) were induced to express HIF-1α prior to bortezomib treatment, bortezomib was observed to have a greatly enhanced pro-apoptotic effect (Veschini et al., 2007). This indicates that HIF-1α upregulation under hypoxic conditions may sensitize endothelial cells to the anti-angiogenic and pro-apoptotic effects of bortezomib and might be exploited to target tumor-associated vessels. Another example of the complexity of HIF-1α regulation is exemplified by the anti-apoptotic protein Bcl-2. Curiously, while Bcl-2 increases VEGF via HIF-1α–dependent transcription by stimulating phosphorylation of AKT and ERK1/2 under hypoxic conditions, Bcl-2 had no effect on these proteins under non-hypoxic conditions. Bcl-2 mRNA interference decreased ERK1/2 phosphorylation and VEGF secretion in *hypoxic* Bcl2-overexpressing cells but not in control cells (Trisciuoglio et al., 2005). Thus, Bcl-2 overexpression and hypoxia have synergistic effects on HIF-1α–dependent gene expression in tumor cells.

Targeting angiogenesis is arguably the most advanced approach of influencing the tumor/leukemia microenvironment. The anti-angiogenic agents have demonstrated clinical activity in myeloma, myelodysplastic syndrome, and leukaemias. The anti-VEGF monoclonal antibody that neutralizes VEGF-A, Bevacizumab, is the first antiangiogenic agent which has been validated as a cancer therapy. Other types of antiangiogenic agents, such as tyrosine kinase inhibitors (sunitinib; sorafenib) and anti-cytokine drugs (thalidomide; lenalidomide) have now entered clinical practice (William et al., 2008). Bevacizumab administered as monotherapy in patients with heavily-treated refractory AML resulted in a time-dependent reduction in VEGF expression without clinical response (Zahiragic et al, 2007). However,

bevacizumab combined with ara-C and mitoxantrone has been demonstrated to improve overall response rate of 48% in the Phase 2 study (Karp et al, 2004).

Thalidomide is an inhibitor of angiogenesis that down-regulates VEGF secretion from bone marrow endothelial cells (D'Amato et al, 1994). Thalidomide has been approved by Federal Drug Administration (FDA) in 2006 for the treatment of newly diagnosed multiple myeloma, in combination with dexamethasone (William et al., 2008). Thalidomide was also found to be effective in myeloproliferative disorders (Di Raimondo et al. 2001) and AML (Richardson et al. 2002). Lenalidomide is a less toxic potent thalidomide analog, and the Phase 3 clinical trials enrolled by the relapsed/refractory multiple myeloma patients demonstrated an improved response rate for patients treated by lenalidomide and dexamethasone, compared with dexamethasone alone ($P < 0.001$) (Wang et al, 2006; Dimopoulos et al, 2007; Weber et al, 2007).

Sunitinib (SU011248, Sutent®), an oral small molecular tyrosine kinase inhibitor of VEGF receptor and platelet-derived growth factor (PDGF) receptor that exhibits potent anti-angiogenic and anti-tumor activity in preclinical models, also inhibits other tyrosine kinases, including the SCF receptor c-KIT and the FLT3 and RET kinases (Arora and Scholar, 2005; Mendel et al., 2003; Pawson, 2002), which are important factors, mediating proliferation of lymphoma and leukemia cells (Arora and Scholar, 2005; Heinrich et al., 2002; Schmidt-Arras et al., 2004). In phase I studies of sunitinib in AML, limited clinical activity has been observed. Although cases with FLT3 activating mutations responded better than those with wild-type receptors, responses are of short duration. Combination of such strategies with conventional AML chemotherapy is likely needed to improve treatment outcomes (Fiedler et al., 2005; Chow and Eckhardt, 2007).

The molecularly-targeted antiangiogenic reagents in different stages of clinical development are shown in Table 1:

4. Leukemogenesis: role of the bone marrow microenvironment

In the adult homeostatic organism, the BM niche maintains stem cells primarily in a quiescent state by providing signals that inhibit cell proliferation, and only upon receipt of a stimulating signal does the stem cell become activated to divide and proliferate. In turn, LSC are believed to arise through transforming events targeting HSC, which allow growth-independent survival and proliferation. As an example, Zhang et al. demonstrated that HSC are the primary target of the oncogenic K-ras mutations, which cooperate with subsequent genetic events for development of juvenile myelomonocytic leukemia or T-cell lymphoblastic leukemia/lymphoma (Zhang et al., 2009). Recent data indicate that, in parallel with leukemogenic events in the hematopoietic system, the niche is converted into an environment with dominant signals favoring cell proliferation and growth. In some cases, a combination of these scenarios may be required (Li and Neaves, 2006). Therefore, LSC may receive the support of a BM niche for their survival and may in turn influence deregulation of the BM niche by their dominant proliferation-promoting signals.

Dysfunction of a BM niche may contribute to leukemogenesis by supplying abundant growth factors that promote proliferation and/or inhibit apoptosis (Jones and Wagers, 2008). LSC may differ from normal HSC, furthermore, in their degree of dependence on the niche. For example, loss of the osteoblastic niche, which is believed to maintain HSC quiescence by inhibiting cell proliferation, may lead to HSC expansion. Myeloma cells inhibit Wnt activation in the microenvironment through release of soluble dickkopf homolog 1 (DKK1), which causes an increase in the concentration of RANKL and a decrease in osteoprotegerin production. This results in increased activation of osteoclasts and bone destruction (Qiang et

al., 2008). These findings lead to the identification of new potential targets for therapy in MM, such as RANKL.

Recent discoveries utilizing mouse models have provided the first experimental evidence for genetic changes in the BM microenvironment contributing to or required for leukemogenesis. Walkley et al. demonstrated that dysfunction of retinoblastoma protein (RB) or retinoic acid receptor γ (RARγ) in the BM microenvironment can contribute to development of pre-leukemic myeloproliferative disease. Widespread inactivation of RB, a central regulator of the cell cycle and a tumor suppressor, resulted in extramedullary hematopoiesis and myeloproliferative disease in the murine hematopoietic system. However, myeloid-specific loss of RB did not induce myeloproliferative disease or HSC abnormalities. Therefore, the myeloproliferative-like disorder in the *Rb* mutants is the result of perturbed interactions between hematopoietic cells and the BM microenvironment (Walkley et al., 2007a).

The microenvironment-induced myeloproliferative-like disorder also has been observed in $RAR\gamma^{-/-}$ mice (Walkley et al., 2007b) and developed solely because of the RARγ-deficient microenvironment. The RARγ deficiency led to depletion of the osteoblastic niche and excessive myelopoiesis in BM and spleen, resulting in myeloproliferative disease, whereby HSC are maintained in the vascular niche (Walkley et al., 2007b). Both RB and RARγ deficiency–induced expansion of HSC and progenitor cells may result from loss of inhibitory signals normally provided by the osteoblastic niche. These findings critically underscore the importance of interactions between hematopoietic cells and the BM niche/microenvironment and indicate that additional genetic mutations within the BM microenvironment may be necessary for leukemic transformation.

The significance of the hematopoietic microenvironment to disease initiation has been suggested by studies with mice deficient in phosphatase and tensin homolog (PTEN) (Yilmaz et al., 2006). PTEN deficiency in both in hematopoietic cells and the microenvironment resulted in myeloproliferation that progressed to overt leukemia/lymphoma. However, inducible PTEN deletion in hematopoietic cells in the presence of a wild-type BM microenvironment promoted HSC depletion without evidence of myeloproliferation or leukemic development (Yilmaz et al., 2006). These results suggest that PTEN deficiency in hematopoietic cells alone is not sufficient for malignant transformation. Likewise, activation of NF-κB in myelopoietic cells and the absence of its inhibitor IκBα are not sufficient for induction of hypergranulopoiesis, but these changes in the nonhematopoietic compartment, such as fetal liver, resulted in increased numbers of dysplastic hematopoietic cells with progression into secondary AML (Rupec et al., 2005). This progression was mediated by upregulated perinatal expression of Jagged1 in $I\kappa B\alpha^{\Delta/\Delta}$ hepatocytes and activation of Notch1 in neutrophils. Co-culture of IκBα-deficient hepatocytes with wild-type BM cells induced Jagged1-dependent hypergranulopoiesis, which was abolished by inhibition of Jagged1 (Rupec et al., 2005). In mice with a conditional deletion of IκBα only in the myeloid lineage and not in fetal liver cells, cell-autonomous induction of myeloproliferative disease was not observed. These results indicate that cell fate decisions leading to a premalignant hematopoietic disorder can be initiated by non-hematopoietic cells with inactive IκBα, conceivably via activation of the Notch pathway. Additional studies indicate the role of Notch signaling in the interactions of HSC and the microenvironment (Matsuoka et al., 2008) demonstrated that the tumor suppressor Fbxw7, which negatively regulates cyclin E, Notch, and c-Myc protein levels, plays a role in maintaining HSC quiescence and repressing potential oncogenic activity of HSC. Notably, Notch ligand Jagged is expressed by the HSC niche, and Jagged/Notch activation results in increased HSC number and niche expansion (Calvi et al., 2003).

Structural chromosomal aberrations have been demonstrated in BMSC from 44% of patients with MDS and 54% of those with AML. Although few breakpoints of chromosomes which are typical for leukemia aberrations were observed in BMSC, the majority of cytogenetic aberrations in BMSC were different from those in leukemic cells. These findings suggest enhanced genetic instability of BMSC in MDS/AML, and indicate the potential involvement of BMSC in the pathophysiology of these conditions (Blau et al., 2007). Recently, Lopez-Villar et al. reported the presence of cytogenetic aberrations on MSC from MDS patients by array-based comparative genomic hybridization and fluorescence *in situ* hybridization, some of them specially linked to a particular MDS subtype, the 5q- syndrome (Lopez-Villar et al., 2009).

Additional evidence of MSC dysfunction comes from recent findings in MDS. Marrow stroma from patients with MDS, in contrast to that from more advanced stages of MDS such as chronic myelomonocytic leukemia, expressed 14- to 17-fold higher levels of *IL-32* mRNA than healthy controls, and this constitutive IL-32 expression promoted apoptosis in MDS cells, reproducing the inefficient hematopiesis and extensive apoptosis in MDS marrows (Marcondes et al., 2008). These findings indicate that stroma-produced IL-32 could contribute to the pathophysiology of MDS, and serve as a therapeutic target. Furthermore, this modified microenvironment phenotype was reproduced when the stromal cells exposed to tumor necrosis factor alpha, known to be produced at high levels by MDS cells. Thus, these findings indicate two independent mechanisms of stromal abnormalities in tumors: 1) genetic changes in stromal cells, and 2) secondary, epigenetic changes arising in MSC in response to tumor cells.

5. Conclusion and future directions

By elucidating the role of the BM microenvironment in the pathogenesis of hematologic tumors, recent studies have provided the framework for identifying and validating novel therapies that target both leukemic cells and cells in their surrounding microenvironment (Figure 3). We hypothesize, however, that such drugs will need to be combined with conventional cytotoxic agents to completely eradicate leukemic cells.

The underlying molecular mechanisms involved in stem cell activation and homing to the niche will provide important insight into the precise mechanisms involved in tumor-host interactions that contribute to drug resistance. This understanding will provide a framework for the rational combination of agents in clinical trials to overcome drug resistance and improve patient outcomes. In particular, further understanding of the contribution of the BM niche to the process of leukemogenesis may provide new targets aimed at destroying LSC without adversely affecting normal stem cell self-renewal.

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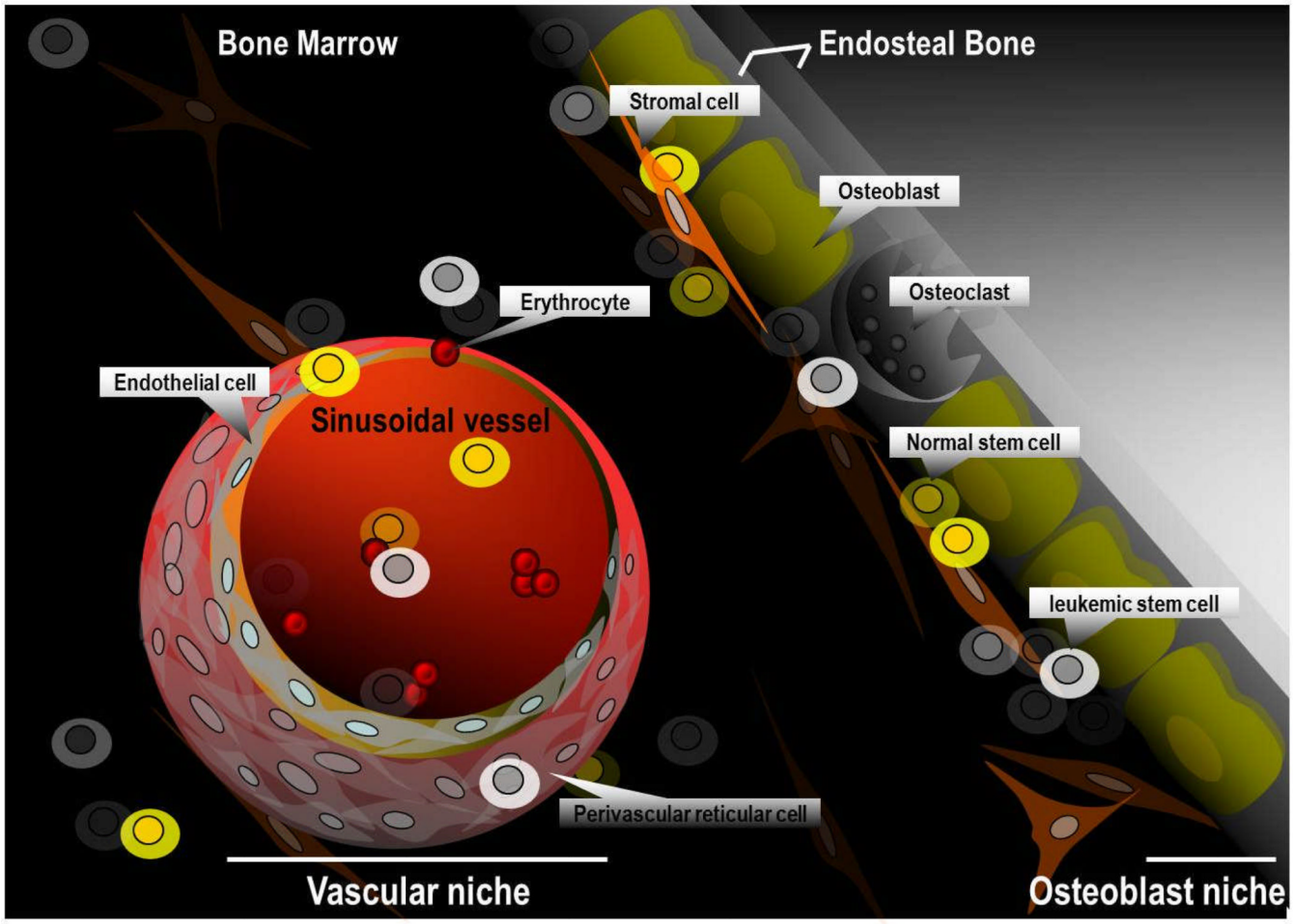

Figure 1. Major components of osteoblastic and vascular niches

The BM microenvironment consists of a complex network of cells. The normal and leukemic stem cells reside in either the osteoblastic or vascular niche. In the osteoblastic niche at or near the endosteum, osteoblasts, octeoclasts and stromal cells may provide a quiescent microenvironment for normal and leukemic stem cells. In the vascular niche around sunusoids, perivascular reticular cells, sinusoidal endothelial cells, and mesenchymal progenitors may facilitate transendothelial migration, homing, proliferation, and differentiation of normal and leukemic stem cells.

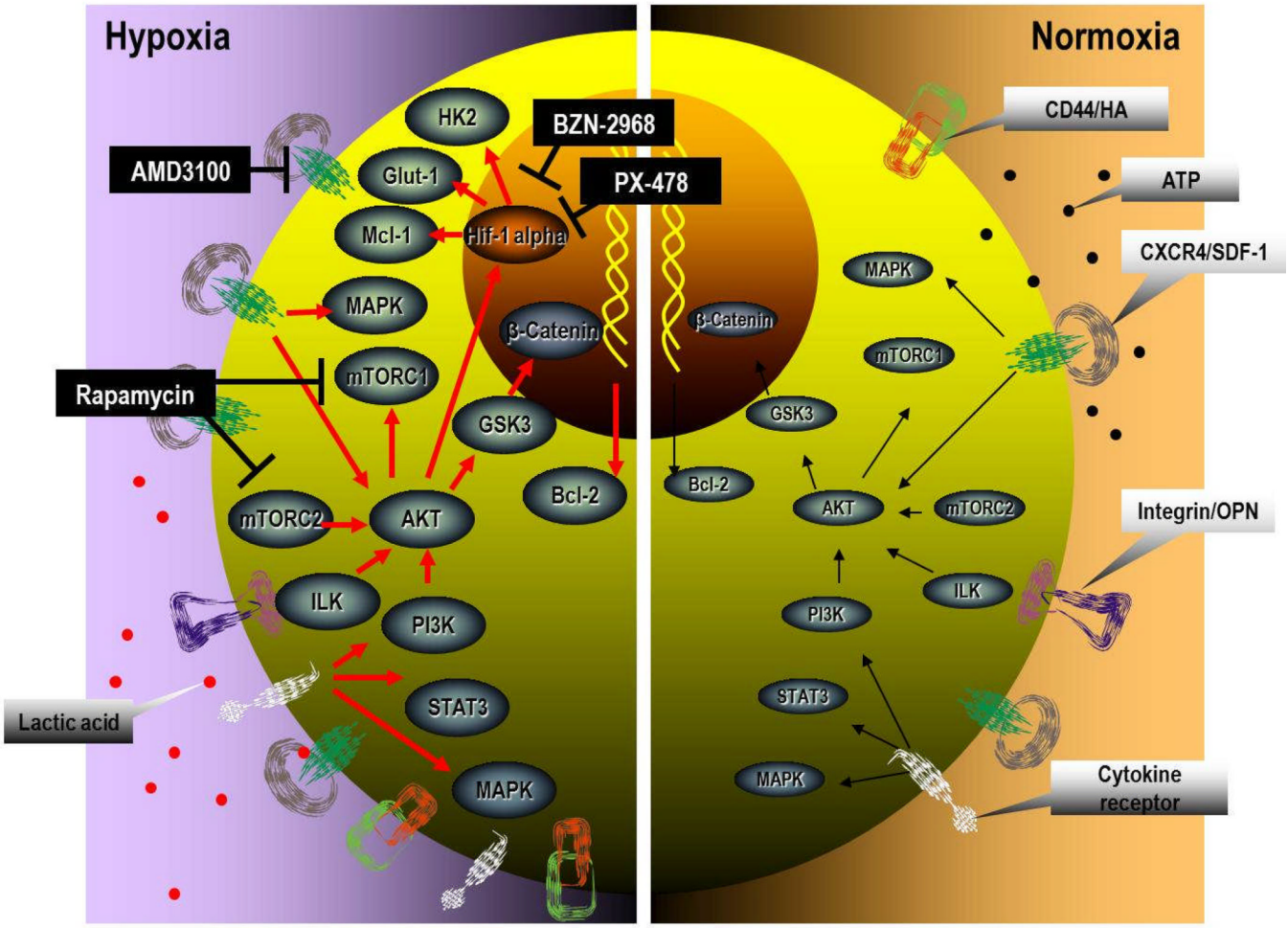

Figure 2. Hypoxia vs. Normoxia: therapeutic targets

The process of recruiting the normal and leukemic stem cells to the BM microenvironment may depend on endothelium-derived SDF-1α. Oxygen tension gradually declines from the vascular niche to the osteoblastic niche. Under hypoxic conditions of the osteoblastic niche, HIF1-α translocates into the cell nucleus, bind to the DNA target genes carrying a hypoxia-response element (HRE), and initiates the transcription of genes involved in angiogenesis, anaerobic glycolysis, and vasodilation. Activation of growth factors and cytokines, stimulation of SDF-1α/CXCR4 signaling and integrin ligation lead to increased HIF1-α by modulating its stability and increased translation through the PI3K/AKT/mTOR pathway.

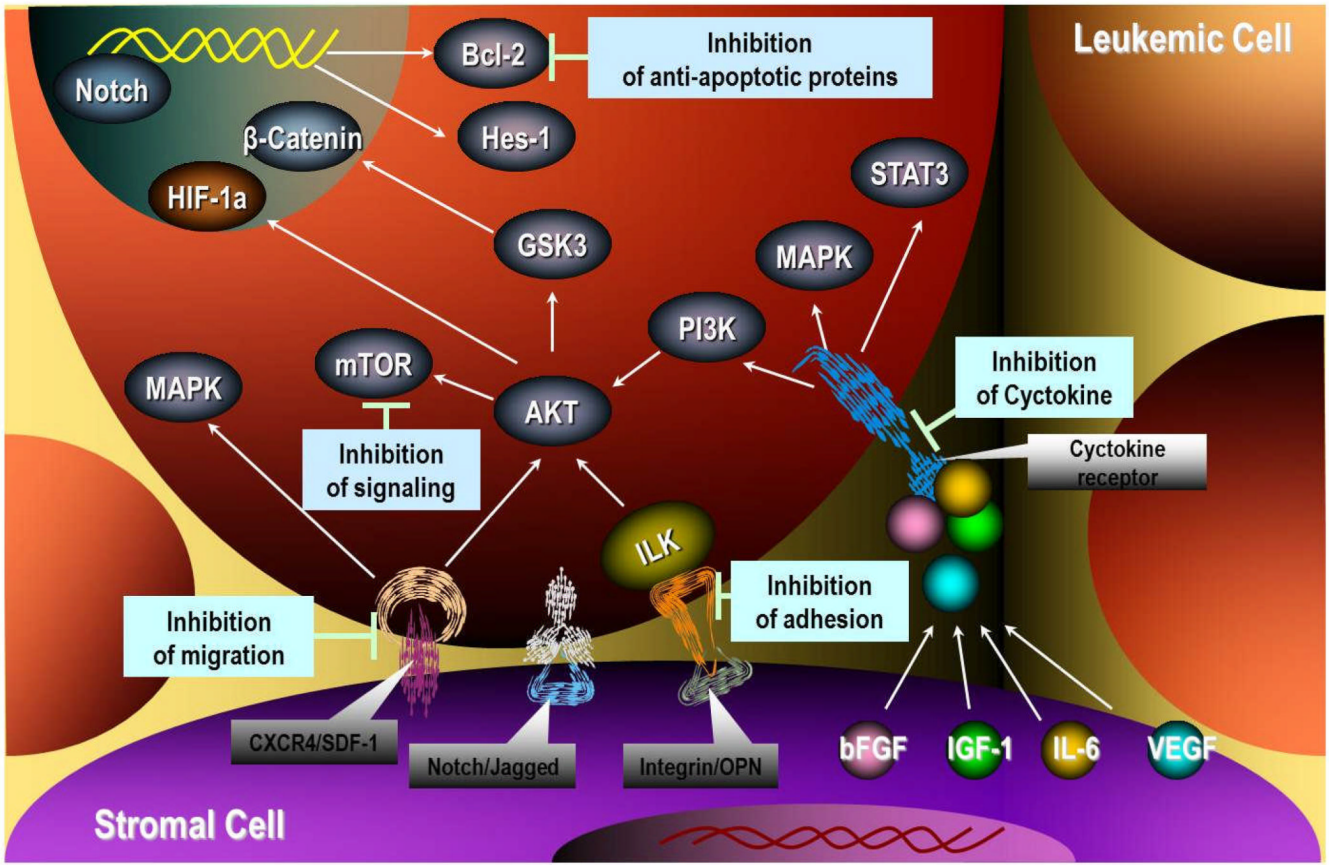

Figure 3. Therapeutic targets in the bone marrow niche

In the BM microenvironment, a complex network of cells, ECM, and secreted molecules work in concert to regulate the normal and leukemic stem cells. Cytokine and chemokine signaling and integrin ligation can activate the PI3K/Akt pathway which regulates several downstream components that are involved in regulation of the normal and leukemic stem cells survival and proliferation. The novel therapeutic agents indicated in the Figure, which target the leukemic cells in their surrounding microenvironment, or interactions between leukemic cells and BM stromal cells, have been shown to destroy LSC without adversely affecting normal stem cell self-renewal.

Table 1

Angiogenesis inhibitors in clinical trials for treatment of hematological malignancies.

Drug	Sponsor	Trial	Mechanism
A. Drugs that block activators of angiogenesis			
Bevacizumab (Avastin™)	NCI Genentech	Phase III, diffuse large B-cell lymphoma (combined with rituximab and CHOP chemotherapy)	Anti-VEGF antibody (binding and inactivation of VEGF)
		Phase III, newly diagnosed mantle cell lymphoma (combined with rituximab, cyclophosphamide, doxorubicin, bortezomib, and prednisone or rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone)	
Vatalanib (PTK787/ZK 222584)	NCI, Novartis Pharmaceuticals Corporation	Phase II, leukemia, myelodysplastic syndromes, myelodysplastic/ myeloproliferative diseases	VEGF receptor (VEGFR) inhibitor
VEGF-trap	NCI, Pharmaceutical/Industry	Phase I, relapsed or refractory non-Hodgkin's lymphoma	Specific VEGF inhibitor
Sorafenib tosylate (Nexavar™)	NCI, Bayer/Onyx	Phase I/II, relapsed or refractory lymphoma or multiple myeloma (combined with everolimus)	VEGFR, PDGFR, Raf-1, Flt-3, c-KIT inhibitor
		Phase I/II, older patients with acute myeloid leukemia or high-risk myelodysplastic syndromes (combined with low-dose cytarabine)	
Cediranib meleateate	NCI, Pharmaceutical/Industry	Phase I, pediatric patients with refractory or recurrent acute myeloid leukemia	VEGFR2, VEGFR1–3 inhibitor
		Phase I, lymphoma (combined with AZD2171)	
B. Drugs that inhibit endothelial cells directly			
Thalidomide	Commercially available, approved for leprosy: Celgene and Lafal; France, NCI	Phase III, multiple myeloma, maintenance therapy after autologous stem cell transplantation (combined with prednisone)	VEGF, basic fibroblast growth factor (bFGF) inhibitor, TNF-alpha inhibitor, interleukins and interferons activation inhibitor
		Phase I/II, newly diagnosed B-cell chronic lymphocytic leukemia (combined with fludarabine)	
		Phase II, relapsed or refractory low-grade non-Hodgkin's lymphoma	
Lenalidomide (Revlimid)	NCI	Phase IV, previously treated multiple myeloma (combined with dexamethasone)	Thalidomide analog VEGF, basic fibroblast growth factor (bFGF) inhibitor, TNF-alpha inhibitor
		Phase III, maintenance therapy for B-cell CLL following second line therapy	
		Phase III, low- or intermediate-1-risk	

Drug	Sponsor	Trial	Mechanism
		myelodysplastic syndromes and symptomatic anemia (combined with epoetin alfa)	
		Phase I/II, relapsed mantle cell lymphoma (MCL) and diffuse large B-cell lymphoma (combined with rituximab)	
C. Drugs that inhibit the proteasome			
Bortezomib (Velcade)	NCI Millennium	Phase III, multiple myeloma, consolidation therapy for elderly patients	Nuclear factor (NF)-kappaB inhibitor (interfering with NF-κB-mediated) angiogenesis
		Phase III, newly diagnosed mantle cell lymphoma (combined with rituximab, cyclophosphamide, doxorubicin)	
		Phase I/II, relapsed or refractory indolent B-cell lymphoproliferative disorders or mantle cell lymphoma (combined with rituximab, cyclophosphamide, and prednisone)	
		Phase I/II, relapsed or refractory aggressive B- or T-cell non-Hodgkin's lymphoma (combined with gemcitabine and hydrochloride)	