



Molly Pomerance. *Kitchen Counter*. Oil on canvas, 24" × 30".

*A better understanding of leukemic stem cells and molecular biology will lead to more effective therapies for leukemic diseases.*

## Considerations for Targeting Malignant Stem Cells in Leukemia

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**Background:** Malignant stem cells have been identified in acute myelogenous leukemia, chronic myeloid leukemia, and some types of acute lymphoblastic leukemia. Like normal stem cells, these leukemic stem cells (LSCs) are able to self-renew, differentiate, and proliferate extensively. Evidence suggests that LSCs are critical for the initiation and perpetuation of leukemic disease.

**Methods:** We reviewed the literature describing the characteristic features of LSCs in various leukemias and the novel molecular approaches being used to specifically ablate the LSC population.

**Results:** Studies have demonstrated the potential importance of ablating LSCs when treating leukemia. The unique characteristics of LSCs that differentiate them from their normal counterparts can be exploited to specifically target the malignant population.

**Conclusions:** Current therapeutic strategies may not effectively ablate the LSC, leaving the potential for disease progression or relapse. A better understanding of LSC cell and molecular biology will allow the design of more effective therapies.

### Introduction

In the past several years, major advances have been made in our understanding of stem cells and their role in human ontogeny and homeostasis. Indeed, a role for stem cells has been defined, or as least implicated, for nearly every major organ system.<sup>1-7</sup> However, despite the marked increase in stem cell studies in many areas, relatively little attention has been focused on the role of stem cells in human malignancy. Given the clear prevalence of stem cells in normal physiology, the genesis of many human cancers may also find their origin in a stem or progenitor cell population.<sup>8,9</sup> While strong evidence for

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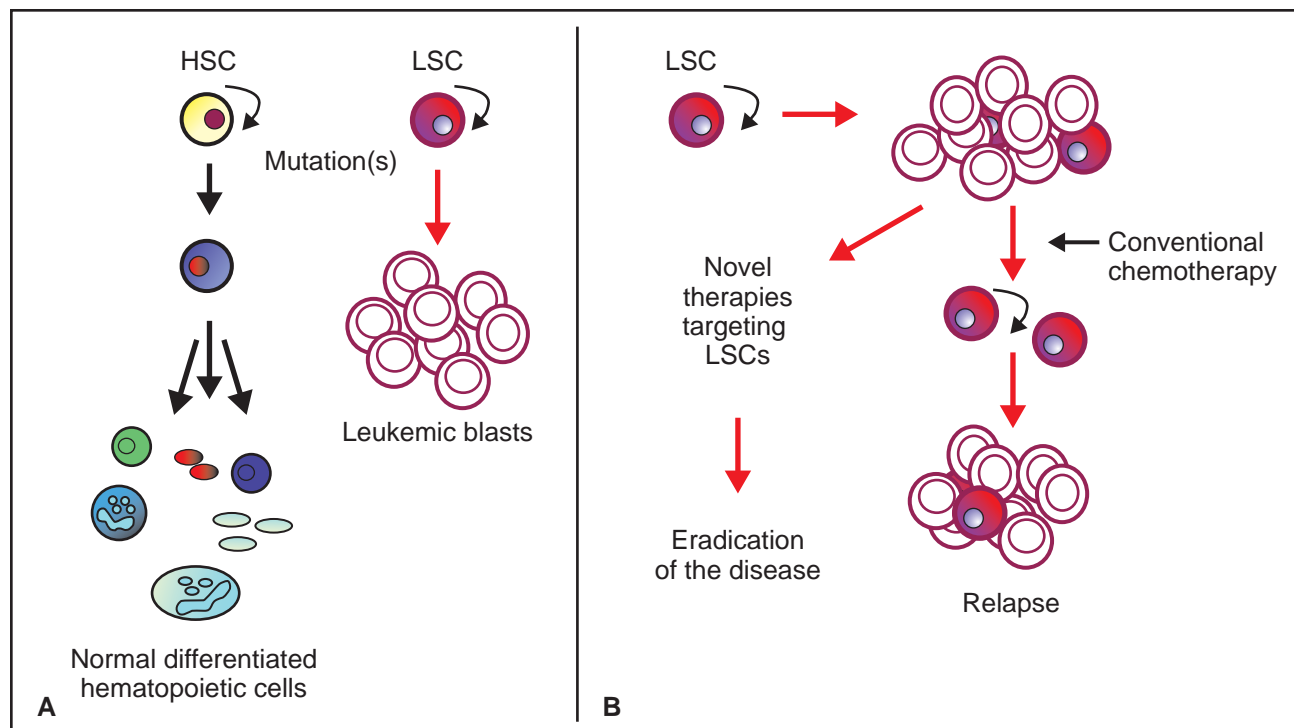
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this postulate remains to be determined for most cancers, recent studies in the hematopoietic system have begun to directly characterize malignant (leukemic) stem cells. This increased understanding of cancerous stem cells in blood-forming tissues will achieve two important goals. First, by defining unique properties of leukemic stem cells (LSCs), it will be possible to design more effective therapeutic strategies. Second, establishing a base of knowledge in the hematopoietic system may provide important paradigms for future studies of malignant stem cells in other types of cancer. Consequently, in this article we review current knowledge of LSCs and discuss how their distinctive features may guide the development of novel therapeutic strategies.

Stem cells are typically defined by three distinct properties: self-renewal, differentiation capability (often for 2 or more lineages), and proliferative capacity. (Detailed reviews are available elsewhere.<sup>10,11</sup>) These characteristics allow stem cells to give rise to and maintain a diverse and specialized group of tissues. In normal mammalian hematopoiesis, hematopoietic stem cells (HSCs) have the capacity to replenish the entire blood system. This process is dynamic and strictly regulated by balanced growth/death signals that dictate the fate of each cell in the hematopoietic system. Consequently, mutation of stem cells has the potential to rapidly affect the entire hematopoietic system. If such mutations confer a growth advantage and/or cause developmental arrest of partially differentiated cells, the result is typical-

ly some form of leukemia. Importantly, stem cell-based leukemogenesis is largely analogous to normal hematopoiesis. A model for leukemogenesis (Fig 1A) proposes that the malignant transformation of normal hematopoietic stem/precursor cells would give rise to LSCs.<sup>12-15</sup> Importantly, LSCs retain the key characteristics of self-renewal and proliferative capacity but do not properly differentiate. The resulting failure to produce functional and mature blood cells, as well as the uncontrolled proliferation (and/or the failure to undergo apoptosis) of abnormal cells, is defined as leukemia. Thus, LSCs lie at the heart of leukemic growth and are central to leukemia pathogenesis. Given the critical nature of LSCs for disease progression, it is important to understand the unique characteristics of this rare population that distinguish them from their normal counterparts. Such differences represent potential targets that can be exploited to selectively render malignant LSCs more susceptible to therapeutic intervention.

Most of the currently used therapies for leukemia have been designed based on general biological properties of malignant blast cells, such as rapid cell cycle activity. However, since LSCs can be found in a quiescent state,<sup>16,17</sup> the use of these strategies may not effectively target the LSC population and consequently the disease may not be eradicated (Fig 1B). Therefore, to more effectively ablate malignant clones, it is necessary to specifically target the LSC population. Characteristics of LSCs from several types of leukemia are described below.



Figs 1A-B. — (A) The leukemic stem cell (LSC) model proposes that leukemic blasts originate from a common primitive progenitor that has the capacity to self-renew. (B) Conventional therapy regimens for leukemia have been designed to eliminate leukemic blasts. These regimens may not effectively ablate the LSC population, which eventually recapitulates the disease. The ability to design therapies that can target LSCs should yield more effective eradication of the disease.

## Acute Myelogenous Leukemia

Among the various forms of leukemia, the myeloid diseases have been best characterized with regard to a stem cell origin. Interestingly, acute myelogenous leukemia (AML) is known to display marked clinical heterogeneity (types M0-M7 according to the French-American-British [FAB] classification system).<sup>18</sup> However, at the stem cell level, there appears to be substantial conservation. Indeed, LSCs with common immunophenotypic, functional, and molecular characteristics have been described for AML subtypes M0, M1, M2, M4, and M5.<sup>15,19</sup> In these types of leukemia, LSCs are phenotypically defined as CD34<sup>+</sup>, CD38<sup>-</sup>, CD71<sup>-</sup>, CD90<sup>-</sup>, HLA-DR<sup>-</sup>, CD117<sup>-</sup>, and CD123<sup>+</sup>.<sup>15,19,24</sup> Even though most of these antigenic features are shared with normal HSCs (CD34<sup>+</sup>, CD38<sup>-</sup>, CD71<sup>-</sup>, and HLA-DR<sup>-</sup>), at least three markers have been found to be unique to LSCs (CD90<sup>-</sup>, CD117<sup>-</sup>, and CD123<sup>+</sup>). This leukemia-specific phenotype has allowed researchers to purify stem cell populations of leukemic origin. Notably, the LSC distinct phenotype may also serve as a tool to separate normal HSCs from LSCs for transplantation purposes. In addition, detailed cell cycle analyses and functional studies have demonstrated that LSCs, like normal HSCs, are mostly quiescent.<sup>17,25</sup> (More extensive reviews about HSC properties are available elsewhere.<sup>10,11</sup>) Therefore, LSCs should be less vulnerable to standard chemotherapeutic agents, which generally target actively cycling cells.

Functional analysis of malignant stem cells in AML has been achieved using long-term culture (LTC) assays and the nonobese diabetic/severe combined immunodeficient (NOD/SCID) xenotransplant model as tools to identify and characterize LSCs. Several studies have demonstrated that cells bearing the LSC phenotype described above are highly enriched for leukemic stem and progenitor cells, as defined by LTC and NOD/SCID assays.<sup>15,19,21,23</sup> This type of rigorous functional analysis is at the crux of stem cell characterization studies and provides the most compelling evidence to date that AML is a disease of stem cell origin.

Unique molecular differences between normal and LSCs have been also described. For example, two tumor suppressor genes, interferon regulatory factor-1 (*irf-1*) and death-associated protein kinase-1 (*dapk-1*) were found to be constitutively expressed in purified CD34<sup>+</sup>CD38<sup>-</sup> cells from seven different AML patients.<sup>26</sup> *Irf-1* and *dapk-1* protein expression has been associated with proapoptotic functions.<sup>27-29</sup> However, their role in AML has not yet been determined. These results are paradoxical in that the genes encoding these proapoptotic factors are found frequently deleted or hypermethylated in malignant cells.<sup>30-33</sup> It is possible that expression of *irf-1* and/or *dapk-1* indicates partial activity of cellular mechanisms attempting to restrict malignant growth. If so, then it may be possible to exploit the presence of these factors in strategies designed to specifically target the LSC population.

Since cellular life/death decisions are based on a delicate balance between proapoptotic and antiapoptotic factors, one might also expect antiapoptotic factors to play a role in the leukemogenesis process as well. Indeed, antiapoptotic factors have also been found aberrantly expressed in AML cells. Bcl-2, Bcl-x<sub>L</sub> and Mcl-1 were highly expressed in AML patients,<sup>34,35</sup> although only Mcl-1 has been detected in primitive AML populations (M.L.G. and C.T.J., unpublished data, 2004). Interestingly, the CD34<sup>+</sup> fractions display higher expression of these antiapoptotic factors when compared to the CD34<sup>-</sup> fractions.<sup>36</sup> In addition, electrophoretic mobility shift assays performed on highly enriched CD34<sup>+</sup>, CD38<sup>-</sup>, and CD123<sup>+</sup> populations (~97% quiescent) from different primary AML specimens showed the survival factor NF-κB (nuclear factor κB) to be constitutively active.<sup>37</sup> In contrast, normal CD34<sup>+</sup> cells do not activate NF-κB unless they are stimulated with a mitogen.<sup>38</sup> This finding strongly suggests that NF-κB may play a role in the leukemogenic process. The mechanisms of constitutive NF-κB activation in AML cells remain to be determined; however, several of the known mutations found in AML patients could potentially be involved. Mutations resulting in the activation of STAT5, RAS/MAPK, and PI3K/AKT pathways are common in AML, and all have the potential to trigger downstream activation of NF-κB.

## Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is a clonal malignancy resulting from reciprocal translocation between chromosomes 9 and 22 (known as the Philadelphia [Ph] chromosome).<sup>39</sup> This translocation results in expression of the chimeric fusion gene BCR/ABL and has been used to diagnose the disease.<sup>40,41</sup> In addition, this specific cytogenetic characteristic has made the study of stem cell populations in this malignancy more amenable. Notably, although CML has long been considered a stem cell disease,<sup>42</sup> detailed characterization of CML stem cells has been relatively recent.<sup>43</sup> In vitro studies using long-term culture-initiating cell (LTC-IC) assays<sup>44,45</sup> demonstrated the presence of pluripotent stem cells of malignant origin in patients with CML. Subsequent studies using SCID<sup>46</sup> and NOD/SCID<sup>47</sup> mouse transplant models have been used to determine the engraftment potential of CML stem cells, which share phenotypic markers with their normal counterparts. The majority of CML progenitors were found to have a higher proliferative capacity when compared to normal progenitors,<sup>48</sup> which suggested that most CML progenitors were actively cycling. However, more detailed cell cycle analyses of primary CML samples identified a quiescent subpopulation within the CD34<sup>+</sup> progenitors.<sup>16</sup> This population of cells was also found to be capable of initiating factor-independent growth that correlated with an increased expression of interleukin-3 (IL-3).<sup>49</sup> In addition, IL-3 autocrine stimulation in these primitive cell pop-

ulations resulted on constitutive STAT5 activation.<sup>50</sup> These studies have begun to define unique molecular properties of CML stem cells and provide a possible explanation for the difficulty in targeting this rare quiescent population with standard chemotherapeutic regimens.

## Acute Lymphoblastic Leukemia

Although LSCs have most often been associated with myeloid leukemias, recent studies suggest that at least some lymphoid diseases may also arise from malignant stem/progenitor cells. Acute lymphoblastic leukemia (ALL) is characterized by the malignant expansion of immature cells from lymphoid lineages. Approximately 85% of diagnosed ALL cases are a result of the expansion of B-cell precursors, and 15% correspond to T-cell precursor aberrancies. As in the malignancies described above, the blast population has a limited proliferative capacity. This finding suggests the existence of LSCs that can perpetuate lymphoid disease. Further, for Ph<sup>+</sup> ALL, functional studies have defined an LSC population. The BCR/ABL translocation is found in 5% to 25% of ALL, the majority of which are B-cell lineage. NOD/SCID xenotransplantation studies were used to assess the functional characteristics of CD34<sup>+</sup>, CD38<sup>-</sup> cells from ALL patients carrying the BCR/ABL translocation.<sup>51</sup> The phenotypically primitive cells were able to successfully engraft the marrow of experimental animals, but more mature cells could not. This finding demonstrates that malignant cells were generated exclusively by the stem cell population (CD34<sup>+</sup>/CD38<sup>-</sup>). Another study in B-cell ALL using patients with known T-cell receptor rearrangements, showed that primitive cell populations (CD34<sup>+</sup>, CD38<sup>-</sup>) isolated from remission patients maintained the leukemic T-cell receptor configuration. This was observed in nearly 50% of the samples analyzed.<sup>52</sup> Even though it was not demonstrated that these CD34<sup>+</sup>, CD38<sup>-</sup> cells were capable of causing relapse, the study further supports the concept that ALL may arise from a population of stem/progenitor cells and that such cells may be resistant to standard chemotherapy.

## Experimental Therapeutic Strategies

As the properties of malignant stem cells become better defined, it is possible to more thoroughly evaluate current therapeutic strategies. Notably, while several new therapies seek to attack specific molecular characteristics of leukemic cells, none of these approaches has been validated for LSCs. Perhaps most prevalent among the new "rationale" drugs is the c-Abl inhibitor imatinib mesylate (Gleevec). This agent is a potent inhibitor of Abl protein kinases and is also an effective inhibitor of c-Kit and the platelet-derived growth factor receptor.<sup>53,54</sup> While the

remarkable clinical efficacy of the drug has revolutionized CML treatment, a concerning observation is that imatinib mesylate may not be directly cytotoxic to CML stem cells.<sup>55</sup> Laboratory studies indicate that the drug readily induces death of most CML cells but is only cytostatic for the most primitive CML progenitors.<sup>56</sup> Further, CD34<sup>+</sup> cells from CML patients with complete cytogenetic responses were found to be of malignant origin with the capacity to give rise to CML blasts.<sup>57</sup> These findings indicate that a reservoir of CML-initiating cells could be maintained in patients who otherwise demonstrate no evidence of disease.

The success of imatinib mesylate has encouraged identification and targeting of analogous pathways in other forms of leukemia. In AML, activating mutations have been identified in tyrosine kinase receptors that are strongly associated with cell growth and transformation. Flt-3 activation, now described as the single most common aberrancy in AML, is found in 15% to 40% of AML patients,<sup>58-62</sup> and c-kit mutations are found in approximately 5% of AML specimens.<sup>61,63,64</sup> Small molecular inhibitors of these kinases show substantial antileukemic activity *in vitro* and are currently in clinical trials.<sup>65</sup> These agents are designed to inhibit autophosphorylation and subsequent activation of downstream targets of the tyrosine kinase receptors.<sup>66</sup> Whether inhibition of such kinases is cytotoxic to quiescent LSCs remains to be determined.

Aside from receptor tyrosine kinases, several other molecular pathways are currently under investigation in leukemia. For example, constitutive activation of JAK/STAT pathways has been described in nearly 70% of the AML patients. Interestingly, the activation of these pathways could potentially be mediated by either *flt3* mutations<sup>67,68</sup> or autocrine cytokine stimulation loops, thus providing a link between apparently disparate mutations. Novel therapies are being considered to target the JAK/STAT pathway in an effort to selectively kill leukemic cells.<sup>69</sup>

Molecular targeting strategies have also been directed towards the Ras pathway. Mutations that activate Ras signaling have been described for 15% to 25% of AML cases.<sup>70-72</sup> In addition, in the absence of ras mutations, this signaling pathway can be activated by autocrine/paracrine cytokine signaling. Inhibition of Ras activation can be achieved using farnesyltransferase inhibitors (FTIs), which prevent the adequate processing of Ras through prevention of membrane attachment and further signal transduction.<sup>73</sup> Since a downstream effector of Ras is the MEK signaling cascade, MEK inhibitors might be used as an alternative to inhibition of Ras. MEK inhibitors have been examined in considerable detail and are able to kill AML cells.<sup>74,75</sup>

Another strategy recently employed for cancer therapy has been the use of monoclonal antibodies that specifically recognize tumor antigens. Agents such as rituximab (anti-CD20, Rituxan) for lymphoma therapy have demonstrated that this approach can be effective for at least

some cancers.<sup>76</sup> For myeloid leukemia an analogous strategy has been established by targeting the CD33 antigen, which is commonly expressed on primary AML cells. The drug developed for this approach, gemtuzumab ozogamicin (Mylotarg), consists of an anti-CD33 antibody conjugated to the toxic antibiotic calicheamicin. While this strategy is appropriate for AML cells bearing CD33, the expression of this antigen in the AML stem cell compartment has not been clearly described. Clinical evidence showing drug efficacy in a minority of patients<sup>77,78</sup> suggests that CD33 expression may be variable or heterogeneous among more primitive AML cell types.

The use of all-trans retinoic acid (ATRA) for treatment of acute promyelocytic leukemia (APL) represents a potential therapeutic targeting of primitive leukemic cells. However, because a specific LSC has not been formally described in APL, it remains unclear whether ATRA treatment specifically targets the stem cell population.

While all of these strategies are derived from sophisticated studies of leukemia cell and molecular biology, to our knowledge none have been carefully analyzed in the context of LSCs. Given the potential importance of targeting LSCs, we suggest preclinical studies that directly assess drug efficacy in primitive cell types should be a high priority for new leukemia therapies.

## Future Directions: Targeting Leukemic Stem Cells

Given the quiescent status of LSCs and their relatively low frequency, ablation of this population is likely to be a significant challenge. Furthermore, whereas some malignant cells, such as CML blasts, succumb to blockade of a single pathway, evidence from recent studies suggests that manipulation of multiple signals may be necessary to eliminate LSCs. The finding that NF- $\kappa$ B is activated in the LSC population suggests one possible strategy to impair the growth of malignant stem cells. NF- $\kappa$ B is a survival factor found constitutively active in most hematologic malignancies<sup>37,79-83</sup> as well as many other cancers.<sup>84</sup> Its inhibition is considered an important strategy to induce apoptosis in malignant cells.<sup>84</sup> Under normal circumstances, NF- $\kappa$ B is kept out of the nucleus by interaction with its own inhibitor, I $\kappa$ B. Upon stimulation, a series of events result in the phosphorylation, ubiquitination and subsequent degradation of I $\kappa$ B. Consequently, NF- $\kappa$ B translocates to the nucleus where it activates transcription of a wide variety of genes.<sup>85</sup> Hence,

drugs suitable for inhibition of NF- $\kappa$ B activation are those that can prevent NF- $\kappa$ B localization to the nucleus. One such class of drugs is proteasome inhibitors, which have been demonstrated to induce apoptosis in different malignancies, including AML, through inhibition of I $\kappa$ B degradation.<sup>86</sup> Initial studies demonstrated that proteasome inhibitors can induce apoptosis in 80% to 90% of phenotypically primitive AML cells in only 12 hours while sparing normal hematopoietic progenitors.<sup>37</sup> Subsequent studies used an adenovirus vector encoding a dominant negative allele of I $\kappa$ B to specifically block NF- $\kappa$ B in primary AML cells. Importantly, these experiments resulted in a death rate of only 50% of AML cells over 36 hours of culture, suggesting that inhibition of NF- $\kappa$ B is important but not sufficient to induce the robust cell death observed with proteasome inhibitors.<sup>87</sup> This discrepancy indicates that proteasome inhibitors cause accumulation of cellular factors in addition to I $\kappa$ B that are important in promoting cell death. This is not a surprising result, since proteasomes are known to degrade several proteins involved in cell cycle progression and apoptosis.<sup>88</sup> Interestingly, when low concentrations of proteasome inhibitors were combined with the anthracycline idarubicin, the apoptotic effect observed was more dramatic. It was also found that this drug combination activates p53, presumably as a result of a DNA damage response elicited by the anthracycline.<sup>89,90</sup> Since proteasome inhibitors prevent the degradation of proteins such as p53, the two drugs may cooperate by increasing and accumulating the levels of proapoptotic p53 while simultaneously disabling the NF- $\kappa$ B survival signal. From a more general perspective, it could be hypothesized that two types of events are necessary to preferentially induce apoptosis in the LSC population: (1) activation of stress responses, such as p53, p38, or c-Jun N-terminal kinase (JNK), and (2) inhibition of survival signals unique to LSCs, such as NF- $\kappa$ B (Fig 2). Interestingly, the activation of stress responses can lead to activation of survival pathways in both normal and LSCs.

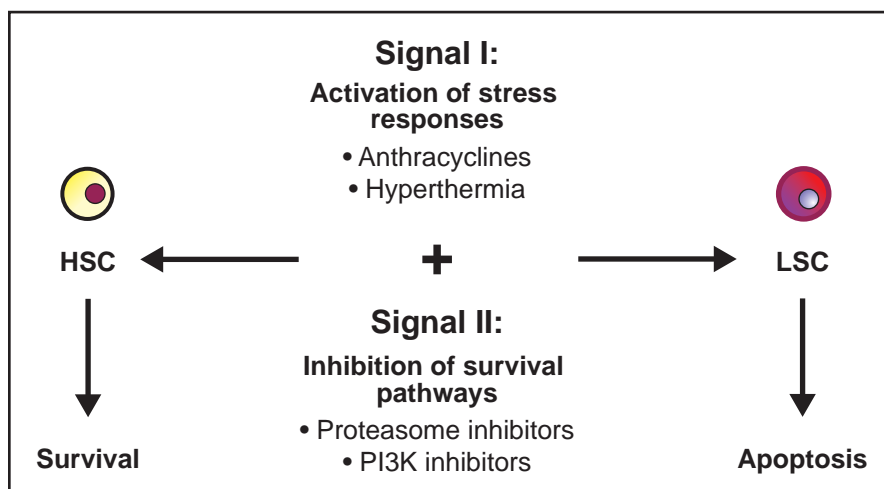


Fig 2. — Model for leukemic stem cell (LSC) apoptosis. Two general types of stimuli are simultaneously required to induce LSC-specific cell death while sparing normal hematopoietic stem cells (HSCs).

However, the inhibition of NF- $\kappa$ B may distinctively affect the malignant population, which may have become dependent on the survival signal. To achieve LSC apoptosis, the appropriate stress response could potentially be achieved using stimuli such as ionizing radiation, chemotherapy drugs, hyperthermia, or reactive oxygen species; while survival signals can be blocked using proteasome inhibitors (PS-341) or inhibitors of the I $\kappa$ B kinase (IKK; eg, parthenolide, sodium salicylate).

A similar paradigm was recently suggested by the findings of Wierenga et al.<sup>91</sup> These investigators demonstrated that LSCs could be killed by exposure to high temperatures (43° C) in combination with a synthetic alkyllysophospholipid (ET-18-OCH<sub>3</sub>). In this case, hyperthermia could be initiating stress responses, and ET-18-OCH<sub>3</sub>, a known inhibitor of the PI3K pathway,<sup>92</sup> may be blocking an important survival signal.

By establishing general parameters for induction of LSC apoptosis, it should be possible to develop more effective clinical therapies. Given the heterogeneity of mutations that give rise to these malignancies, the ability to target the malignant population is not likely to be achieved by a single specific inhibitor. To this end, it is crucial to fully understand the signaling pathways that regulate survival and death in LSC populations. Recent studies have begun to characterize molecular mechanisms that may be relevant to LSC survival. However, more detailed analyses using multiparameter and/or network-based approaches should be a priority for future studies.

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