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REVIEW

Targeting survival cascades induced by activation of Ras/Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways for effective leukemia therapy

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The Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways are frequently activated in leukemia and other hematopoietic disorders by upstream mutations in cytokine receptors, aberrant chromosomal translocations as well as other genetic mechanisms. The Jak2 kinase is frequently mutated in many myeloproliferative disorders. Effective targeting of these pathways may result in suppression of cell growth and death of leukemic cells. Furthermore it may be possible to combine various chemotherapeutic and antibody-based therapies with low molecular weight, cell membrane-permeable inhibitors which target the Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways to ultimately suppress the survival pathways, induce apoptosis and inhibit leukemic growth. In this review, we summarize how suppression of these pathways may inhibit key survival networks important in leukemogenesis and leukemia therapy as well as the treatment of other hematopoietic disorders. Targeting of these and additional cascades may also improve the therapy of chronic myelogenous leukemia, which are resistant to BCR-ABL inhibitors. Furthermore, we discuss how targeting of the leukemia microenvironment and the leukemia stem cell are emerging fields and challenges in targeted therapies.
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Signaling pathways and hematopoietic cancer

Approximately 10% of all cancer deaths result from leukemia and lymphomas in the Western world. While vastly improved therapeutic approaches have been developed for chronic myeloid leukemia (CML), acute promyelocytic leukemia (APL) and childhood acute lymphocytic leukemia (ALL), therapy for adult acute myeloid leukemia (AML) and chronic lymphocytic leukemia has not yet yielded significant advancements. A list of abbreviations used in this review is present in Table 1. While improvements in the outcomes have been observed with young AML patients over the last 40 years, progress in the treatment of older AML patients has not been as significant.[1] A range of 50–75% of adult AML patients achieve complete remission with combination chemotherapy which consists of the deoxycytidine analogue cytarabine and an anthracycline antibiotic (doxorubicin, daunorubicin, idarubicin or the anthracenedione mitoxantrone, which inhibit the enzyme topoisomerase IIa). However, this treatment is not always effective as only approximately 25% of these patients enjoy long-term survival.[1] The incidence of AML increases with age, 1.2 cases per 100 000 at age 30 and >20 cases per 100 000 at age 80.[2] Unfortunately the survival success decreases with the age of the patient. As the average life span increases due to improvements in health care and lifestyles, AML will be an increasing problem in global health care.

While approximately 50% of AML cases have genetic aberrations which can be identified (for example, deletions such as 5q-, translocations such as t(8;21) *AML-ETO*, (the AML1 gene fused to the ETO gene) or duplications such as FMS-like tyrosine kinase 3 (*FLT3)* internal tandem duplication (ITD)), the other 50% do not have currently identifiable genetic mutations.[3] Unlike CML where the *BCR-ABL* translocation is present in virtually all patients and the majority of the patients are sensitive to imatinib which inhibits the BCR-ABL oncoprotein, treatment with a targeted 'upstream' inhibitor (for example, Flt-3 inhibitor) would be ineffective in many AML cases.

Mutations of upstream receptors such as *FLT3* (20–30%), *KIT* (7–17%), and granulocyte colony-stimulating factor receptor (*G-CSFR*) have been documented in AML and *FMS* (12% in myelodysplasic syndrome (MDS) and may cause activation of the Ras/Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways which can result in prevention of apoptosis (Figure 1).[4–9] Furthermore overexpression of vascular endothelial growth factor receptor (VEGF-R) and NRAS mutations have been observed in AML, which could also result in initiation of these pathways.[10,11]

Upregulation of the Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways and phosphorylation of the downstream target Bad are observed frequently in AML patient specimens and associated with a poorer prognosis than patients lacking these changes.[11–16] Aberrant expression of multiple signaling pathways is associated with a worse prognosis.[13] *FLT3* ITD mutations are present in 20–30% of AMLs, and these patients have a poorer prognosis than patients lacking these mutations.[17] *FLT3* and other mutations may result in activation of the Ras/Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways.[18–24] An overview of the effects of these mutations and how they may yield Achilles' heels for leukemia therapy and the treatment of other hematopoietic disorders is presented in Figure 2. It should

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Table 1 List of abbreviations

Abbreviations	*Definitions*
17-AAG	17-Allyamino-17-demethoxygeldanamycin
ABC	ATP-binding cassette
ABCB1	ATP-binding cassette subfamily b, member 1
ABCG2	ATP-binding cassette subfamily b, member 2
ALL	Childhood acute lymphocytic leukemia
AML	Adult acute myeloid leukemia
AML-ETO	AML1 gene fused to the ETO gene
APL	Acute promyelocytic leukemia
ATO	Arsenic tri-oxide
BH3	Bcl-2 homology region-3
BTK	Burton's tyrosine kinase
CASP9	Caspase 9
Chk1	Check point kinase
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
COX-1	Cyclooxygenase-1
DAPT	*N*-(N-(3,5-difluorophenacetyl)-L-alanyl)-S-phenylglycine *t*-butyl ester
DDB2	Damage-specific DNA binding protein 2
FGF-R1	Fibroblast growth factor receptor-1
FLT3	FMS-like tyrosine kinase 3
GCSFR	Granulocyte colony-stimulating factor receptor
GIST	Gastrointestinal stromal tumors
GSS	Glutathione synthetase
HGF	Hepatocyte growth factor
HSP70	Heat shock proteins 70 kDa
IGF-1	Insulin-like growth factor-1
ILK	Integrin-linked kinase
ITD	Internal tandem duplication
KD	Kinase domain
LIF	Leukemia inhibitory factor
MDM-2	Murine double minute-2
MDR/TAP	ATP-binding cassette subfamily b,
MDS	Myelodysplasic syndrome
MRP1	Mutlidrug resistant protein 1
MSH3	Muts homolog 3
NPM	Nucleophosmin gene
p53AIP1	p53-Regulated apoptosis-inducing protein 1
PAK	p21-Activated protein kinase
P-Crk-L	Phosphoprotein c-abl-regulated kinase-ligand
PDGF-R	Platelet-derived growth factor receptor
PKC	Protein kinase C
PML-RARα)	Promyelocytic leukemia-retinoic acid receptor-α
PON2	Paraoxonase 2
PP2A	Protein phosphatase-2A
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
PTPN22	Protein tyrosine phosphatase nonreceptor type 22
RAB-11	Rab-protein-11
SDF-1	Stromal-derived factor-1
SPHK1	Sphingosine kinase-1
TRAP1	Tumor necrosis factor receptor-associated protein-1
VEGF-II	Vascular endothelial growth factor II
VNN1	Vanin 1

be pointed out here, however, that the most commonly found mutation in AMLs occurs at the nucleophosmin (*NPM*) gene. NPM mutant proteins localize aberrantly in the leukemic-cell cytoplasm, hence the term NPM-cytoplasmic positive. *NPM1* mutations in absence of FLT3-ITD identify a prognostically favorable subgroup in the heterogeneous normal karyotype AML category.[25]

Targeted therapy in AML

While treatment of some subsets of AML, such as APL have shown great success with retinoids and arsenic tri-oxide (ATO),

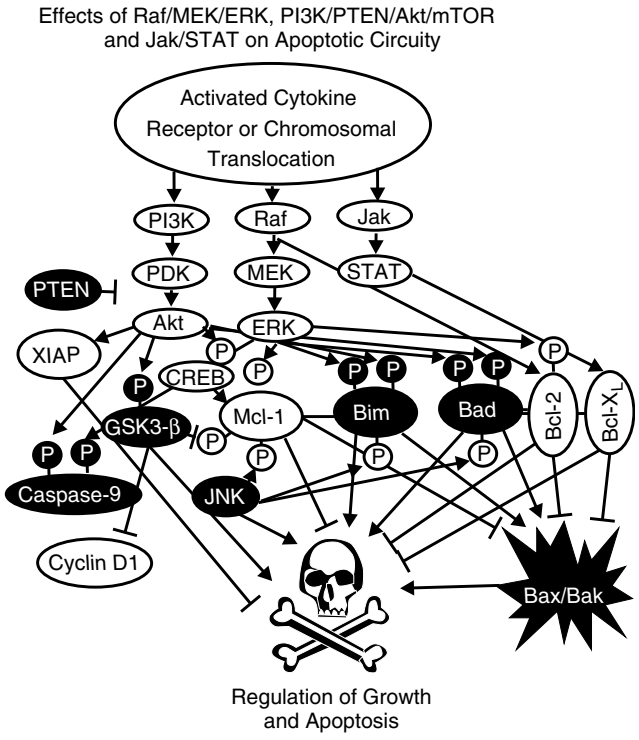

Figure 1 Effects of Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR and Jak/STAT on apoptotic circuitry. These three pathways are often activated by mutations in upstream signaling molecules or chromosomal translocations. These pathways often regulate apoptotic pathways which when deregulated can result in leukemia.

a significant problem in the remainder of AML patients is that most chemotherapy does not ultimately work and eventually the patients relapse and succumb to the disease.[1] Another continuing problem in leukemia therapy is the emergence of drug resistance.[22–24] Unlike the success observed with imatinib and dasatinib in treatment of CML,[26] similar successes have not been observed in AML. Flt-3 inhibitors have been developed, but only approximately 20% of AMLs have mutations at *FLT3*, which render them somewhat sensitive to Flt-3 inhibitor monotherapy.[27] Farnesylation inhibitors (for example, zarnestra) which target RAS, mutated in approximately 10% of AML, have had only moderate activity in phase II studies.[28,29] These inhibitors will probably not be useful in monotherapy settings.

In some AML patients, murine double minute-2 (MDM-2) is overexpressed which enhances the tumorigenicity and the loss of apoptotic processes resulting in a poor prognosis.[29] When MDM-2 ubiquitinates wild type (WT) p53, it becomes targeted for degradation in the proteosome.[30] p53 controls the transcription of many genes and is critical for the fate of the cells. Many of these targets are involved in p53-dependent apoptotic processes (for example, Puma and Noxa). p53 and apoptotic pathways represent emerging therapeutic targets in leukemia treatment.[31–33] Recently, Andreeff and his group have observed that the small molecular weight, membrane-permeable, nutlins bind and inhibit MDM-2 in AMLs with WT p53. This results in increased levels of WT p53 and the induction of apoptosis and cell cycle arrest. Therefore, by using this therapeutic approach, the activities of p53 are enhanced and tumor growth is suppressed. More studies need to be performed, but according to this group, nutlins are a novel strategy in the treatment of AML and other chemorefractory conditions.[31] Thus targeting MDM-2 with nutlins may be a therapeutic approach in cancers which

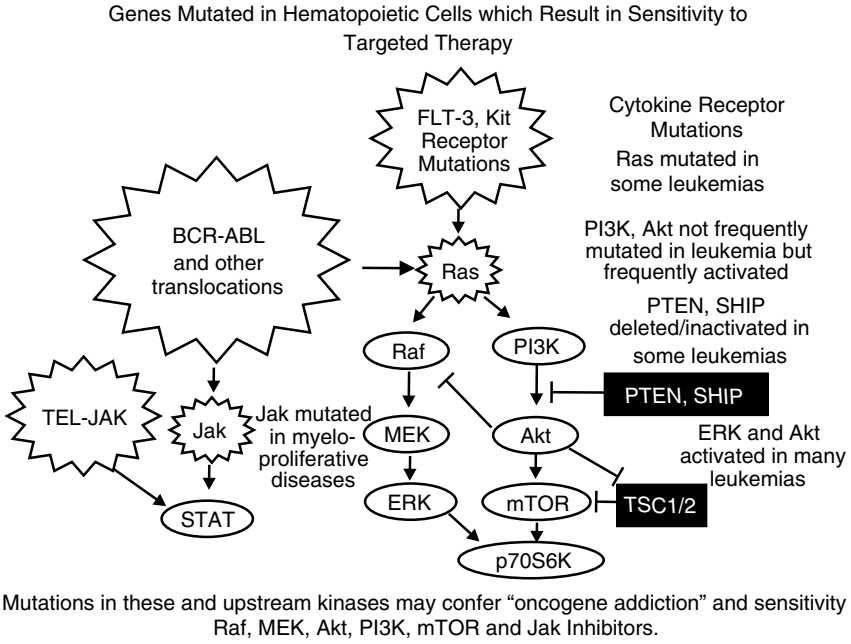

Figure 2 Genes mutated in hematopoietic cells, which result in sensitivity to targeted therapy. Activation of these molecules often results in dependence ('oncogene addiction') of the cells on the mutated gene for proliferation. This also confers an Achilles heel to treatment providing a specific inhibitor is available.

have WT p53. An overview of targeting the p53 and apoptotic pathways in combination with chemotherapeutic drugs is presented in Figure 3. These inhibitors may augment the effects of chemotherapy.

Aberrant regulation of apoptosis and drug resistance

Cell death following cytotoxic drug treatment is generally apoptotic as opposed to necrotic. Many chemotherapeutic drugs induce apoptosis by activating the intrinsic cell death pathway, which involves cytochrome *c* release and activation of the apoptosome-catalyzed caspase cascade. During apoptosis, activation of caspase family cysteine proteases occurs. Although various cytotoxic drugs differ in their mechanism of action, each ultimately relies upon built in apoptotic machinery to elicit cell death.[34–39] Caspase family cysteine proteases are responsible for proteolytic cleavage of cellular proteins C terminus to aspartate residues. Drug resistance has often been linked with the altered expression of proteins involved in the regulation of apoptosis (for example, Bcl-2, caspase 3). Many of the molecules may be regulated either directly or indirectly by p53 and phosphatase and tensin homologue deleted on chromosome ten (PTEN), which are often mutated or suppressed in drug-resistant cancers. Bcl-2 inhibitors have been developed which suppress Bcl-2 and Bcl-X_L but not Mcl-1.[32] Bcl-2 inhibitors are often referred to as BH3 (Bcl-2 homology region-3) mimetics which reflects their ability to target several antiapoptotic proteins.[32]

Therapeutic targeting of the Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR, Jak/STAT and apoptotic pathways

Small molecule inhibitors such as imatinib have proven effective in the treatment of CML and certain other cancers which proliferate in response to BCR-ABL, platelet-derived growth factor receptor (PDGF-R) and c-Kit[35,37,38,40–43] such as gastrointestinal stromal tumors (GIST). Raf and MEK inhibitors have been developed and some are in clinical trials.[34,38,43] We have determined that a consequence of chemotherapeutic treatment of hematopoietic, breast and prostate cancer cell lines is the induction of extracellular signal-regulated kinase (ERK).[44] This pathway is also induced after chemotherapy due to the induction of reactive oxygen species. Eliminating this deleterious side effect of these therapies may enhance their ability to kill drug-resistant cancer.

PI3K, PDK, Akt and mTOR inhibitors have been developed. mTOR inhibitors have been used for many years as immunosuppressive drugs in kidney transplant patients. A side effect of mTOR inhibitors is the inhibition of a negative feedback pathway which results in Akt activation.[45] Conflicting results have been obtained when mTOR inhibitors have been used in AML cells. In some cases, no PI3K/Akt upregulation has been reported.[44] However, another group has demonstrated that mTORC1 inhibition activates PI3K/Akt by upregulating insulin like growth factor-1 receptor (IGF-1R) signaling in AML.[46] An overview of the effects of Raf/MEK and PI3K/PDK/Akt/mTOR inhibitors is presented in Figure 4.

Raf inhibitors have been developed[47–51] and some (for example, sorafenib) are being evaluated in clinical trials for renal cell carcinoma and melanoma. They may be evaluated in leukemia clinical trials. Certain Raf inhibitors have been developed which are small molecule competitive inhibitors of the adinosinetriphosphate (ATP)-binding site of Raf protein. These inhibitors (for example, L-779,450, ZM 336372, Bay 43-9006, also known as sorafenib) bind the Raf kinase domain (KD) and therefore prevent its activity. Some Raf inhibitors may affect a single Raf isoform (for example, Raf-1), others may affect multiple Raf proteins, which are more similar (Raf-1 and A-Raf), and while other pan Raf inhibitors may affect all three Raf proteins (Raf-1, A-Raf and B-Raf). We have observed that the L-779,450 inhibitor suppresses the effects of A-Raf and Raf-1 more than the effects of B-Raf.[47] Like many Raf inhibitors, L-779,450 is not specific for Raf; it also inhibits the closely related $p38^{MAPK}$

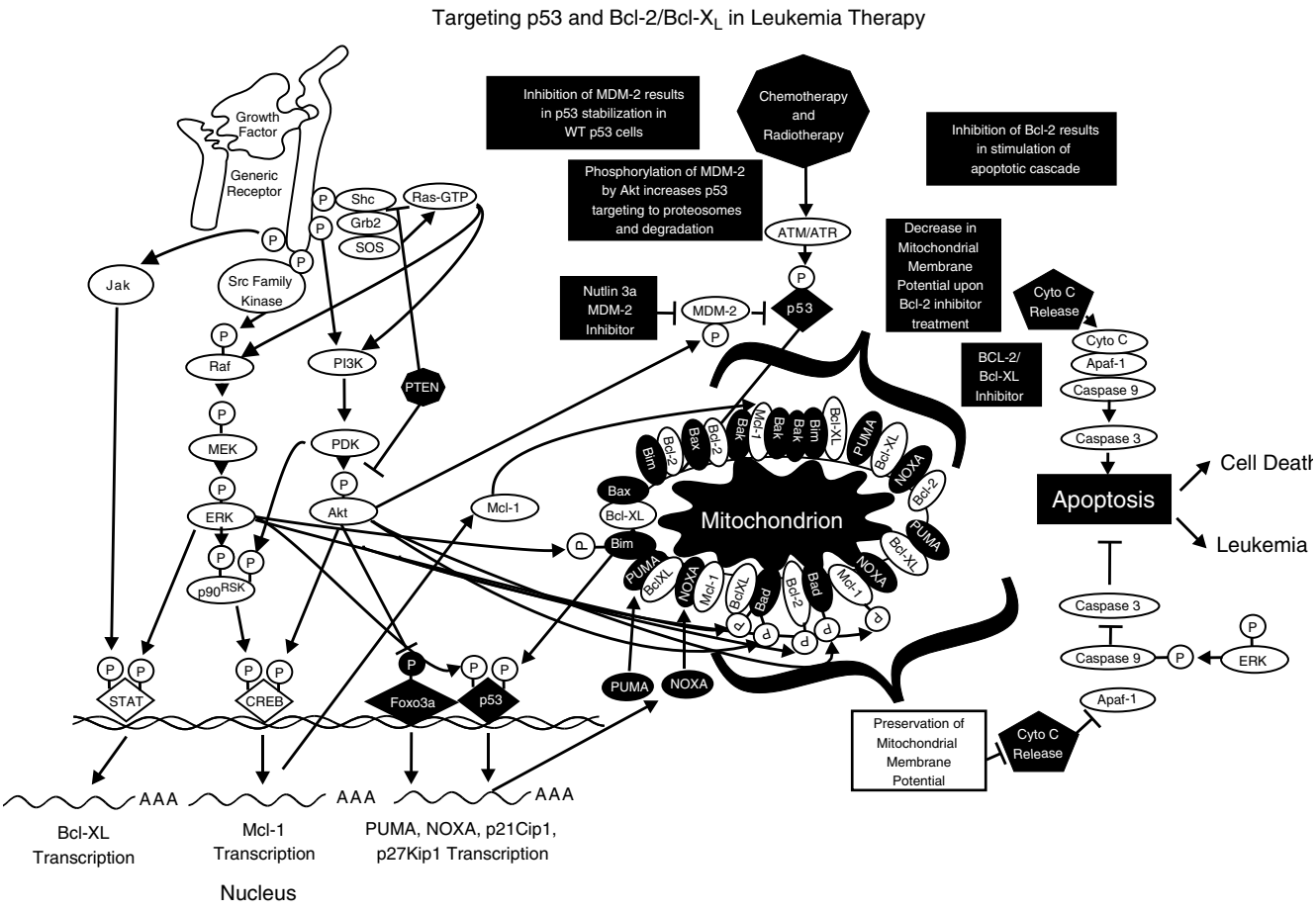

Figure 3 Targeting p53 and Bcl-2/Bcl-X_L in leukemia therapy. Targeting of p53 and Bcl-2/Bcl-X_L with small molecule inhibitors in combination with chemotherapeutic drugs may enhance leukemia therapy. For treatment with murine double minute-2 (MDM-2) inhibitors to be effective, p53 should be WT and therapy is often used in combination with a drug which induces p53.

Likewise, sorafenib inhibits other kinases besides Raf (for example, VEGF-II receptor, PDGF-R, Kit, Flt-3, Fms) and is more appropriately referred to as a multi-kinase inhibitor. Knowledge of the particular *RAF* gene mutated or overexpressed in certain leukemia may provide critical information regarding how to treat the leukemia patient; some patients which overexpress a particular *RAF* gene may be more sensitive to inhibition by agents which target that particular Raf protein. Inhibition of certain Raf proteins might prove beneficial, while suppression of other Raf proteins under certain circumstances might prove detrimental. Thus the development of unique and broad-spectrum Raf inhibitors may prove useful in leukemia therapy.

Chaperonin proteins such as 14-3-3 and Hsp90 regulate Raf activity.[52,53] Raf activity is regulated by dimerization. These biochemical properties result in Raf activity being sensitive to drugs which block protein–protein interactions such as geldanamycin.[52,53] Geldanamycin and its 17-allyamino-17-demethoxygeldanamycin (17-AAG) analogue are nonspecific Raf inhibitors as they also affect the activity of many proteins which are stabilized by interaction with Hsp90. Geldanamycin and 17-AAG are currently in clinical trials.[53–55] Modified geldanamycins may be useful in treatment of CMLs which are resistant to BCR-ABL inhibitors (see below). We often think of a single Raf protein carrying out its biochemical activity. However, Raf isoforms dimerize with themselves and other Raf isoforms to become active. Drugs such as coumermycin, which inhibit Raf dimerization and others such as geldanamycin, which prevent interaction of Raf with Hsp90 and 14-3-3 proteins suppress Raf activity.

An alternative approach to targeting Raf is to prevent Raf activation by targeting kinases (for example, Src, protein kinase C (PKC), PKA, p21-activated protein kinase (PAK) or Akt) and phosphatases (for example, protein phosphatase-2A (PP2A)) involved in Raf activation. Some Src kinase inhibitors such as dasatinib would be predicted to inhibit Raf activation as it should suppress Raf-1 activation by Src. It was stated previously that dasatinib is being used to treat some CMLs, which are imatinib-resistant. It is worth noting that some of these kinases normally inhibit Raf activation (Akt and PKA). A major limitation of this approach would be that these kinases and phosphatases could result in activation or inactivation of other proteins and could have diverse effects on cell physiology.

Currently it is believed that *MEK1* is not frequently mutated in human cancer. However, aberrant activation of *MEK1* is observed in many different cancers due to the activation of the Raf/MEK/ERK pathway by upstream kinases (for example, BCR-ABL) and growth factor receptors (for example, PDGF-R, Kit, Flt-3, Fms) as well as other unknown mechanisms. Specific inhibitors to MEK have been developed (PD98059, U0126, PD184352 (also known as CI1040), PD-0325901, Array MEK inhibitors (ARRY-142886 and others).[56] The successful

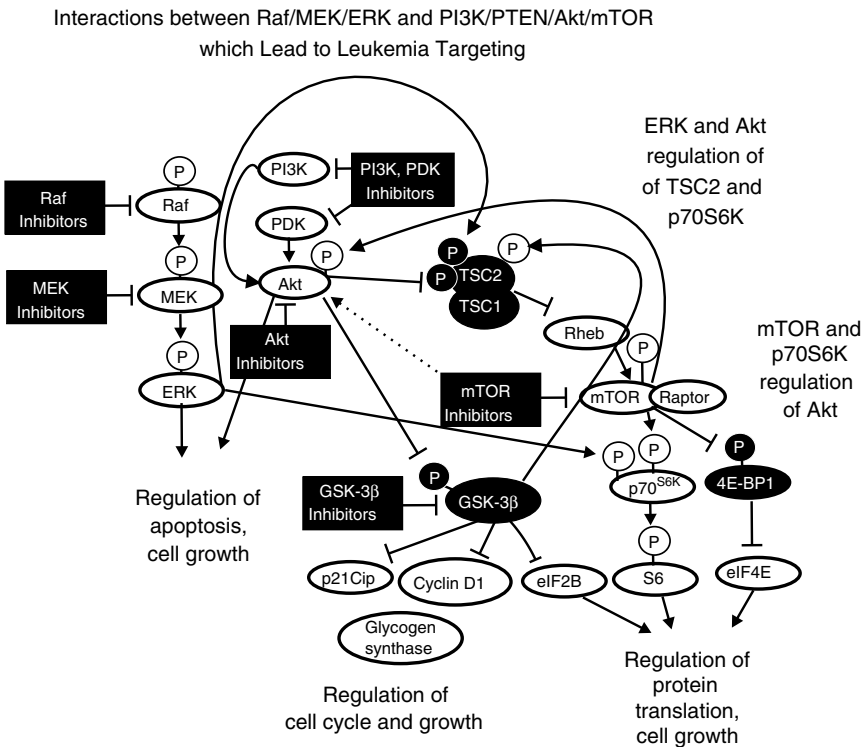

Figure 4 Interactions between Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR which lead to leukemia targeting. Targeting mTOR is complicated as it can result in Akt activation in some cells. However simultaneous targeting of Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR may prove effective in treatment of various leukemias where the mutation responsible for growth is not known. Furthermore targeting of both pathways may prove effective for treatment of drug-resistant cancer.

development of MEK inhibitors may be due to the relatively few phosphorylation sites on MEK involved in activation/inactivation. Furthermore, effective suppression of MEK1,2 is highly specific as ERK1,2 are the only downstream targets. An advantage of inhibiting the Raf/MEK/ERK cascade is that it can be targeted without knowledge of the precise genetic mutation, which results in its aberrant activation. This is important as the nature of the critical mutation(s), which leads to the malignant growth of at least 50% of AMLs and other cancers, are not currently known. In summary, an advantage of targeting MEK is that the Raf/MEK/ERK pathway is a convergence point where a number of upstream signaling pathways can be blocked with the inhibition of a single kinase (MEK). Some of the MEK inhibitors are being evaluated in clinical trials for breast cancer (for example, PD-0325901), they are also being examined in combination with inhibitors which target other signaling and antiapoptotic molecules to improve leukemia therapy.[56]

To our knowledge, no small molecular weight ERK inhibitors have been developed yet, however, inhibitors to ERK could prove very useful as ERK can phosphorylate many targets (Rsk, c-Myc, Elk and at least 150 more). There are at least two ERK molecules regulated by the Raf/MEK/ERK cascade, ERK1 and ERK2. Little is known about the different *in vivo* targets of ERK1 and ERK2. Some cell lines and even leukemia specimens from patients will show different levels of activated ERK1 and ERK2. The biological significance of this differential expression of the activated ERK isoforms remains unclear. ERK2 has been postulated to have pro-proliferative effects, while ERK1 has antiproliferative effects.[57] The phenomenon of different expression of MAPK isoforms is also observed with the JNK isoforms. Clearly, there must be biological significance to differential expression of MAPK isoforms. Development of specific inhibitors to ERK1 and ERK2 might eventually prove useful in the treatment of certain diseases.

The PI3K/PTEN/Akt/mTOR pathway may be inhibited with PI3K (LY294002, PX-866), PDK1 (OSU-03012, celecoxib), Akt (A-443654, perifosine, tricribine) or downstream mTOR inhibitors such as rapamycin and modified rapamycins (CCI-779 and RAD001).[58,59] Initially mTOR inhibitors showed much promise as PTEN is often deleted in various tumors. However, it has been recently determined that the mTOR pathway has a complicated feedback loop, which actually involves suppression of Akt, hence mTOR inhibitors would be predicted to activate Akt in some cells (Figure 4). This remains controversial as previously discussed.[44,46] Recent evidence has highlighted that mTOR can also be activated by Raf/MEK/ERK.[59,60] This may well be another relevant cross talk between the Ras/Raf/MEK/ERK and the PI3K/PTEN/Akt/mTOR pathways and might offer a further rationale for treatments combining drugs which inhibit both signaling networks. A diagram illustrating potential targets in these signal transduction pathways to target is presented in Figure 5.

Novel hematopoietic targets and resistance

Two recent frontiers in leukemia research are the emergence of imatinib-resistant CMLs[61–77] and the discovery of mutations at the Jak2 kinase in certain myeloproliferative diseases.[78–113] These topic areas document the importance of genetic approaches in leukemia and abnormal hematopoiesis research as they were discovered upon identification of the genetic mutations responsible for the hematopoietic neoplasia.

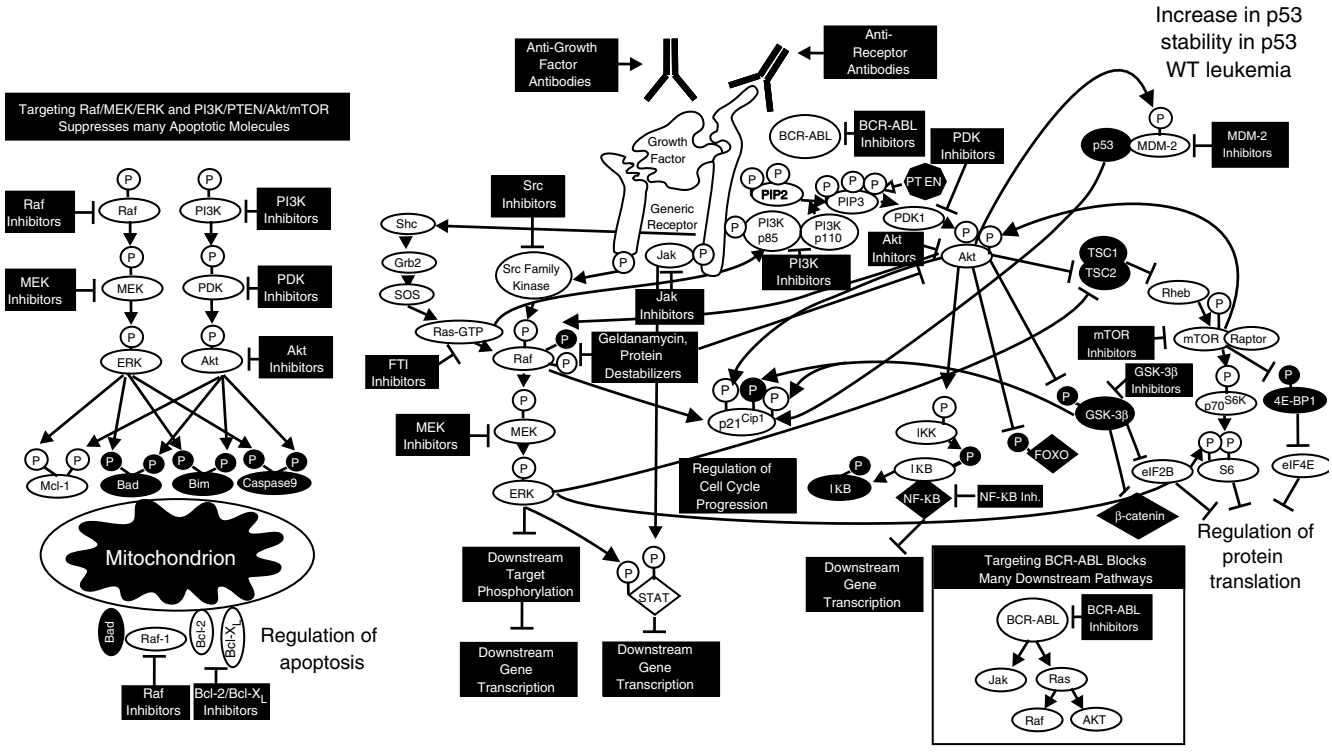

Figure 5 Targeting signal transduction pathways important in leukemia. Potential sites of action of small molecular weight inhibitors and cytotoxic antibodies are indicated. In some cases inhibitors will suppress growth, apoptotic and cell cycle regulatory pathways. This diagram serves to illustrate the concept that targeting Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT can have dramatic effects on many growth regulatory molecules. Proteins activated by phosphorylation are indicated by a black P in a white circle. Proteins inactivated by S/T phosphorylation induced by the PI3K/PTEN/Akt/mTOR pathway are shown in black circles with white P in a black circle.

In some cases, the precise gene responsible for driving proliferation of the abnormal cell is known (for example, *BCR-ABL* in CML, *FLT3* in some AMLs, Jak2 in some myeloproliferative disorders). However, even in the previously listed diseases, there may be additional genes, which are also critical for their altered growth properties. Treatment of some of these diseases with specific kinase inhibitors is often effective; however, resistance to the inhibitors may develop due to further mutations in the aberrant kinases which often prevent the signal transduction inhibitor from inhibiting the altered kinase (for example, additional mutations in *BCR-ABL* in imatinib-resistant CMLs; see below). In these novel 'drug-resistant' cases, additional therapeutic approaches are necessary. In some of these cases, it may be possible to inhibit the drug-resistant cells with novel inhibitors (for example, INNO-406 (also known as NS-187), MK0457, AMN107 (also known as nilotinib) which will suppress the resistant oncoprotein or combinations of MEK and PI3K/Akt inhibitors. Interestingly, the aurora kinase inhibitor MK0457 (also referred to as VX-680) is active also in CML patients harboring the T315I BCR-ABL mutation, which confers a particular bad prognosis, whereas both INNO-406 and AMN107 are ineffective (see below).

We have observed that imatinib-resistant hematopoietic cells (which have mutated *BCR-ABL* genes) are sensitive to MEK inhibitors. This result is not surprising as an Src inhibitor (dasatinib) is being used to inhibit imatinib-resistant cells as they often have overexpression of an activated Src family kinase, such as Lyn, which likely functions by inducing the Raf/MEK/ERK cascade.

Targeting imatinib-resistant CML

Although, imatinib has been a modern day wonder drug in the therapy of CML, imatinib-resistant clones develop, often due to point mutations in the KD of the BCR-ABL gene, which prevent imatinib from suppressing the chimeric kinase activity and subsequent uncontrolled growth. As mentioned previously, some BCR-ABL mutations have been shown to be sensitive to the second-generation BCR-ABL inhibitors dastatinib (BMS-354825, sold under the trade name Sprycel, an Src and BCR-ABL inhibitor) and nilotinib (AMN 107, sold under the trade name Tasigna a BCR-ABL inhibitor). However, patients with the T315I BCR-ABL mutation, are not sensitive to imatinib, dasatinib or nilotinib.[114] Structural biophysical studies have revealed that the ATP-binding site in T315I mutation in BCR-ABL cannot accommodate imatinib.[115] It is through three-dimensional X-ray crystallographic studies of the interaction of drugs such as imatinib with the KD of BCR-ABL that additional inhibitors (for example, nilotinib) have been developed, and we are better able to understand the mechanisms by which inhibitor-resistance develops.

In a retrospective study analyzing the predictive impact of 94 BCR-ABL KD mutations, 18 were found to be mutated at T315I, 26 in the P-loop (which binds the phosphate in ATP) and 50 at

other sites. The T315I and P-loop mutations were preferentially observed in the accelerated phase of CML and the survival of patients with these mutations was significantly worse than patients harboring other mutations.[116] These BCR-ABL mutations may be better detected in the clinic using circulating plasma RNA as apposed to bone marrow RNA.[117]

The BCR-ABL T315I (threonine to isoleucine) mutation does not confer a growth advantage in the absence of imatinib but clearly does bestow imatinib, dasatinib and nilotinib resistance.[118] In one study, which examined the diversity of BCR-ABL KD mutations,[119] in persistent CML patients who received a second-generation BCR-ABL inhibitor after imatinib failure, 67 different KD mutations were observed before the start of therapy with the second BCR-ABL inhibitor, 15% of them had the T315I mutation. Upon treatment with dasatinib, nilotinib or a combination of dasatinib, nilotinib, and imatinib 26% of the patients developed additional BCR-ABL KD mutations. These results indicate that additional BCR-ABL KD domain mutations can occur upon sequential treatment with additional BCR-ABL inhibitors.

Different therapeutic approaches may prove effective in suppressing the effects of the T315I BCR-ABL mutation. It has been shown recently that certain aurora kinase inhibitors (MK-0457 (also known as VX-680), PHA-739358) suppress the growth promoting effects of the T315I mutation.[120–122] Crystallographic studies have indicated that the PHA-739358 aurora kinase inhibitor associates with the active conformation of the BCR-ABL KD in the ATP-binding site. The T315I mutation lacks the steric hindrance induced by the mutation of T to I.[122] Novel Hsp 90 inhibitors, such as IPI-504 will decrease the expression of the mutant BCR-ABL protein, although it should be kept in mind that the Hsp 90 inhibitors affect many proteins including Raf and Akt.[123] Combinations of MEK and dasatinib inhibitors may be effective in the treatment of imatinib-resistant patients harboring certain BCR-ABL mutations (for example, E225K, M351T). Unfortunately, this combination was not effective against imatinib-resistant cells with the T315I mutation.[124] Targeting the 14-3-3 scaffolding proteins, which also have important roles in regulation of signaling molecules with inhibitors such as R18, may sensitize native and mutant BCR-ABL proteins to inhibition with MEK (U0126), mTOR (rapamycin) and Bcl-2 (GX15-070) inhibitors.[125] These 14-3-3 inhibitor and MEK, mTOR or Bcl-2 inhibitor combinations caused reactivation of Foxo3a, which is normally inhibited by Akt. The activated Foxo3a was then able to induce the expression of downstream targets such as $p27^{Kip1}$ and Bim1, which have critical roles in the cell cycle regulation and apoptosis. Thus novel therapeutic approaches have been developed to treat CML patients who become imatinib-resistant. We must be one step ahead of the genetic mutations which result in imatinib-resistance and develop novel approaches to treat this therapeutic resistance. As we obtain more information about signaling pathways as well as the mechanism of action of key kinases in the pathways, we may be able to use our knowledge to develop more effective treatments.

Other genetic mutations responsible for therapeutic resistance

An additional hot spot in leukemia research is the identification of genetic mechanisms, which result in drug resistance by genomic and proteomic approaches. Many different types of drug-resistant leukemias have been examined by genomic approaches to identify genes involved in drug resistance.[71,126–132] Some of the genes implicated in the drug resistance include genes involved in: apoptosis caspase 9 (*CASP9*), signaling (*HA-RAS)*, tumor necrosis factor receptor-associated protein-1 (*TRAP1*), fibroblast growth factor receptor-1 (*FGF-R1*), *IGF-1*, Rab protein-11 **(***RAB-11*), phosphoprotein c-abl-regulated kinase-ligand (*P-Crk-L*), sphingosine kinase-1 (*SPHK1*), Burton's tyrosine kinase (*BTK*) and protein tyrosine phosphatase nonreceptor type 22 (*PTPN22*). Genes implicated in drug resistance are also those involved in drug metabolism such as cyclooxygenase-1 (*COX-1*) and drug transporters including ATP-binding cassette (ABC) subfamily b, (*MDR/TAP*), member 1 (*ABCB1)*, ABC subfamily b, member 2 (*ABCG2)* and multidrug-resistant protein 1 (*MRP1)*. Furthermore heat shock proteins (HSP) may be involved in drug resistance such as HSP 70 kDa (*HSP70)*. Genes involved in DNA repair which are likely involved in drug resistance include mutS homolog 3 (*MSH3)* and damage-specific DNA-binding protein 2 (*DDB2)*. Genes involved in oxidative stress protection which may be involved in drug resistance include: glutathione synthetase (*GSS*), paraoxonase 2 (*PON2*) and vanin 1, (*VNN1*). Centromere genes may also be involved in drug resistance. Furthermore genes involved in cell adhesion such as *SCAM-1* and *CAMPATH-1* antigen may be involved in drug resistance. From these genomic approaches, a single set or type of genes involved in drug resistance has not emerged. These and other studies document the multifaceted mechanisms of drug resistance and suggest that targeting single protein in these drug-resistant leukemias will be ineffective. Targeting upstream kinases, which regulate the expression of these proteins involved in drug resistance may be more effective than attempting to suppress the individual proteins.

Combining signal transduction inhibitors

An approach, which we have been investigating recently, is to determine whether inhibition of two signal transduction pathways is a more effective means to induce apoptosis and cell death than inhibiting a single-signal transduction pathway. Many transformed cells have elevated Raf/MEK/ERK and/or PI3K/PTEN/Akt/mTOR signaling. These two pathways play prominent roles in the promotion of growth and the prevention of apoptosis. We have observed that inhibition of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways is usually more effective in inducing apoptosis and synergy between inhibitions of both pathways is often observed.[133] This is often the case in drug-resistant leukemias, as the drug-resistant cells often display elevated signaling of both pathways.

Classical chemotherapy often remains the most used anti-cancer therapy for many different types of cancer treatment. Drugs such as doxorubicin are effective in the treatment of many cancers, even though in some cases drug resistance clones emerge after prolonged treatment. Doxorubicin targets cellular events such as DNA replication which are downstream of the targets of signal transduction pathway inhibitors. Thus by combining classical chemotherapy with targeted therapy, it may be possible to enhance toxicity while lowering the effective concentrations of classical chemotherapeutics necessary for effective elimination of the particular tumor.

Although the precise targets of farnesyltransferase inhibitors remain controversial, treatment with farnesyltransferase inhibitor R115777 (zarnestra) lead to disease stabilization in 64% of multiple myeloma (MM) patients in a phase 2 clinical trial.[134] Furthermore R115777 synergized with paclitaxel and docetaxel

but not with doxorubicin, 5-fluorouracil, cisplatin, melphalan, mitoxantrone and dexamethasone in this MM trial. Currently it is not clear what the future of FTIs is in cancer therapy. As mentioned previously, they have not yielded promising results as single agents in some phase II leukemia trials.[28,135] They have shown much progress and probably they will reemerge at some point in the future as we learn more about their multiple targets.

Inhibition of Ras/Raf/MEK/ERK and mTOR pathways has been a target in cancer therapies due to the critical involvement of both pathways in the promotion of proliferation. The effects of the combination of the Raf inhibitors sorafenib and mTOR inhibitors are being evaluated in clinical trials to treat melanoma.[136] Similar trials in leukemia are planned, but results have not been presented.

MEK inhibitors have been observed to synergize with UCN-01 and induce apoptosis in MM cells.[137] Part of the synergy may be due to UCN-01 inducing ERK activation, which is suppressed by the MEK inhibitor.

MEK inhibitors have also been observed to synergize with ATO to induce apoptosis in APL and AML cells.[138,139] The p53-related gene p73 is a molecular target of the combined therapy. ATO modulates the expression of the p73 gene by inducing the proapoptotic and antiproliferative 73 isoforms. p53 requires p63 and p73 for the induction of apoptosis in response to DNA-damaging drugs. p73 exists as multiple transactivation competent (TA) proapoptotic and antiproliferative p73 COOH-terminal splicing isoforms (α, β, γ, δ, ε, ζ), of which the two major forms are p73α and p73β. Dominant-negative (ΔN) p73 variants are expressed from a second promoter. These DN ΔNp73 variants lack the N-terminal TA domain, act as trans-repressors of p53- and p73-dependent transcription, and have antiapoptotic and proproliferative potential. Treatment of APL cells with the PD184352 MEK inhibitor reduced the level of ΔNp73 and decreased the ATO-mediated upregulation of ΔNp73, thus causing an increase in the TA/ΔNp73 ratio of dual-treated cells. High doses of ATO induced p53 accumulation in 11 of 21 patients. Combined treatment resulted in the induction of the proapoptotic p53/p73 target gene *p53AIP1* (p53-regulated apoptosis-inducing protein 1) and greatly enhanced the apoptosis of treated cells.[139] Thus this study documented the effectiveness of combining ATO with MEK inhibitors in the treatment of APL and identified the molecular mechanism responsible for the observed synergism.

It should be pointed out that the combination of MEK inhibitors and a chemotherapeutic drug may not always result in a positive interaction and in some cases combination therapy results in an antagonistic response. For example, combining MEK inhibitors with betulinic acid, a drug lethal for melanoma cells, antagonized the effects that betulinic acid normally has on apoptosis.[140] Furthermore, the precise timing of the addition of two drugs is important as they may differentially affect cell cycle progression; therefore, one drug may need to be added before the other for a synergynistic response to be observed and perhaps to prevent an antagonistic response.[139–141]

Combinations of the mTOR inhibitor rapamycin and the cell cycle check point kinase (Chk1) inhibitor UCN-01 also resulted in a synergistic induction of apoptosis in human leukemic cells that was regulated by the Raf/MEK/ERK, Akt and JNK signal transduction pathways.[142] Co-administration of UCN-01 and rapamycin reduced the levels of Mcl-1, Bcl-X_L, cyclin D1 and $p34^{cdc2}$. Similar studies were performed with the farnesyltransferase inhibitor L744832 and UCN-01 which also revealed a synergistic interaction in terms of the induction of apoptosis and interruption of both Akt and MEK/ERK pathways and activation of SEK1/JNK.[143] L744832 blocked the induction of ERK normally stimulated by UCN-01.

The effects of combining PI3K and mTOR inhibitors with the chemotherapeutic drug fludarabine have been examined in human leukemia cell lines.[144] Combination of fludarabine and either PI3K or mTOR inhibitors resulted in increased apoptosis compared to what was observed after fludarabine treatment alone.

Perifosine is an oral bioactive novel alkylphospholipid that inhibits Akt. Perifosine enhanced dexamethasome-, doxorubicin-, melphalan- and bortezomib-induced MM cytotoxicity.[145] Furthermore perifosine synergistically increased the effects of etoposide on the induction of apoptosis in human T-ALL cells.[141] Additional Akt inhibitors have been developed. An Akt inhibitor developed by Abbott (Chicago, IL, USA) (A-443654) augmented the effectiveness of paclitaxel and rapamycin in suppressing tumor growth in xenograft models.[146] We have recently observed that this Akt inhibitor synergizes with etoposide inducing the death of ALL cells (submitted). A problem associated with the Abbott Akt inhibitor is altered glucose tolerance. Unfortunately, there are also toxicity problems associated with the PI3K inhibitor LY294002 and pharmacological problems with some of the MEK inhibitors (CI1040) preventing their usage in human cancer patients.

Myelotarg (gemtuzumab ozogamicin), which targets the CD33 epitope of AML blasts, is a conjugate of the humanized anti-CD33 antibody and the intercalating drug calicheamicin and enables antibody-directed chemotherapy. CD33 is expressed on most AML blasts and also leukemic stem cells.[147,148] In contrast to the disappointing results of the unconjugated antibody, myelotarg is effective as monotherapy and part of a salvage regimen in relapsed AML and is evaluated during first-line treatment of AML.[148,149]

The effects of combining antibodies directed to cytokine receptors expressed on leukemic cells with signal transduction pathway inhibitors should be evaluated in more detail. We have observed that combining an antibody which inhibits the IGF-1R which is expressed on many leukemic cells, with MEK, PI3K or mTOR inhibitors resulted in synergy in terms of the extent of induction of apoptosis.[150] Targeting other cell surface receptors expressed on leukemic cells in combination with Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathway inhibitors maybe an effective leukemia treatment.

Targeting the bone marrow microenvironment

Normal hematopoiesis is maintained by dynamic interactions between hematopoietic cells and the bone marrow microenvironment (BMM). Several lines of evidence suggest that in hematological malignancies, interactions between malignant cells and BMM cells play important roles in aberrant activation of several signal transduction pathways. BMM cells include fibroblasts, mesenchymal stem cells, osteoblasts, macrophages, endothelial cells and adipocytes.[151,152] A large number of soluble mediators are secreted by BMM cells that can influence the activity of tumor cells. They include: interleukin (IL)-6, IL-11, leukemia inhibitory factor (LIF), M-CSF, SCF, G-CSF, GM-CSF, stromal-derived factor-1 (SDF-1), leptin, transforming growth factor-β (TGF-β), VEGF, bFGF, hepatocyte growth factor (HGF). The most well studied of these mediators is IL-6, which activates both Jak/STAT and Ras/MEK/ERK pathways.[153] It has been suggested that angiogenesis induction induced by VEGF is involved in the pathogenesis of hematological disorders in a way similar to the angiogenic switch that has been proposed to

be responsible for the growth of solid tumors. Consistently, VEGF promotes hematopoietic cell survival and inhibits apoptosis in leukemic cells after exposure to chemotherapeutic drugs by inducing Mcl-1.[154]

In AML, BMM cells protect leukemic cells from cytarabine-induced apoptosis via the induction of Bcl-2 and Bcl-X_L. Integrins (β1 and β2, or α4β1) on leukemic cells bind to BMM cell fibronectin and this activates PI3K/Akt/Bcl-2 signaling in leukemic blasts.[155] A central regulator of integrin signaling is integrin-linked kinase (ILK). ILK is constitutively active in AML blasts and its downregulation, by means of small molecule inhibitors, resulted in apoptotic cells death of leukemic cells co-cultured with BMM cells.[156] An important antiapoptotic role played by interactions between integrins (α4β1) on leukemic cells and adhesion molecules (VCAM-1 and fibronectin) on BMM cells has also been documented in B-ALL. Fibronectin stimulation inhibits caspase-3 activation and increases the expression of antiapoptotic proteins which include XIAP and survivin.[157] Conversely, treatment of cells with monoclonal antibodies directed to α4β1 on leukemic cells or VCAM-1 on BMM cells leads to apoptotic cell death of leukemic cells.[158] Taken together, these findings suggest that targeting the leukemia cell surrounding microenvironment could be a novel and effective therapeutic strategy for improving patient outcome in these malignant disorders.

Targeting the leukemic stem cell

Perhaps the holy grail in cancer today is the cancer stem cell or the cancer-initiating cells. Tumors have been shown to possess a minor faction of cancer stem cells, which maintain the propagation of the disease. In our case, we discuss the leukemia stem cell or the leukemia-initiating cell. Leukemic stem cells proliferate slowly and retain their self-renewal capacity through interactions with their cellular environment.[159] Due to their slow proliferative rate and their quiescent-like properties, leukemia stem cells are resistant to conventional chemotherapeutic approaches that target rapidly proliferating cells.[160]

Leukemic stem cells were first identified approximately 10 years ago in AML cells.[161–163.] These leukemic stem cells were often characterized and isolated by their unique side scatter population after fluorescence-activated cell sorting analysis after staining with certain dyes such as Hoechst 33342 (Frankfurt, Germany) and also by altered expression of drug transporter proteins. AML leukemia stem cells also often had the markers $CD34^+/CD38^-$, although some leukemic stem cells have been $CD34^-$, which gave rise to $CD34^+$ cells. Furthermore, these leukemia stem cells showed the unique ability to induce leukemia by after transplantation into nonobese diabetic/severe combined immunodeficiency mice and displayed long-term repopulating activity as well as lineage-marker activity.

The ABC transporters are evolutionary conserved transmembrane proteins, which are highly expressed in hematopoietic stem cells. These ABC transporters may serve as therapeutic targets in leukemic stem cells,[164] however it has been notoriously difficult to effectively target these molecules as they also serve essential functions in other tissue types.

Leukemic stem cells may also express elevated aldehyde dehydrogenase activity.[165] Aldehyde dehydrogenase activity is used to define hematopoietic stem cells but its role in leukemic stem cells in AML is not well defined. Recent studies have indicated that some leukemic AML stem cells express elevated aldehyde dehydrogenase, which is associated with a poor prognosis.[165] The targeting of aldehyde dehydrogenase may be difficult as it likely plays important functions in many different tissue types, not just leukemia stem cells.

AML stem cells were also shown to express high levels of the IL-3R but surprisingly, activation of the MAPK, Akt and STAT5 pathways were not detected in response to IL-3 stimulation.[166] Nevertheless, the IL-3R remains a potential target for AML stem cells. Different approaches have been devised to target cytokine receptors such as IL-3. One targeting approach deals with diphtheria toxin molecules conjugated to IL-3. This targeting approach does show specificity.[167,168]

CML stem cells are more resistant to targeted therapy with imatinib.[169] Some of these CML stem cells may display elevated BCR-ABL expression and changes in expression of IL-3, G-CSF and ABC1/MDR1, ABCG2, and the transcription factor Oct-1 occur upon culture with reduced levels of growth factors.[169] These results point to potential targets in CML stem cells, including BCR-ABL. Additional studies by this same group demonstrated that >70 different BCR-ABL mutations were present in the progeny of cultured CML stem cells.[170] This group has hypothesized that CML patients possess leukemic stem cells which already have BCR-ABL kinase mutations before the advent of BCR-ABL inhibitor therapy; hence the patients already have some resistant CML stem cells. As the CML stem cells proliferate slowly, the patient might initially respond to therapy, then given time, the CML stem cells, which are resistant to BCR-ABL inhibitors will emerge. This is one of the lingering problems in the treatment of CML, the emergence of the resistant cell, perhaps from preexisting CML stem cells, which already had mutations in BCR-ABL which would confer resistance to BCR-ABL-directed therapy. These CML stem cells are proliferating slowly, or in a quiescent-like state. Some investigators have proposed that a means to target these cells is to stimulate their proliferation and then treatment with BCR-ABL inhibitors.[171] However, this therapeutic approach will only be appropriate if the CML stem cell does not contain the T315I BCR-ABL or a similar mutation which confers resistance to BCR-ABL inhibitors.

Recently is has been observed that the Wnt signaling pathway may be elevated in leukemic stem cells.[172] This may occur due to mutations in Flt-3 and chimeric transcription factors such as promyelocytic leukemia gene fused to the Promyelocytic leukemia-retinoic acid receptor-α gene (PML-RARα) and AML-ETO. ITDs of the Flt-3 gene have been detected in leukemia stem cells.[173] An end result of these mutations is increased expression of the Wnt-signaling pathway, which may result in increased growth of the leukemic stem cell. The Wnt pathways also result in increased expression of β-catenin in leukemic AML stem cells. Elevated β-catenin expression is also associated with a poor event-free survival and shortened overall survival.[174] Targeting of various components in this pathway (for example, Flt-3) may inhibit leukemic stem cell growth. Indeed the Flt-3 inhibitor CEP-701 inhibited the engraftment of FLT3/ITD stem cells.[173] Alternatively various investigators and pharmaceutical companies are attempting to develop Wnt and Notch inhibitors (see below).

The Notch pathway may also be deregulated in leukemic stem cells. Genomic analysis from AML stem cells determined that the Jagged-2 gene, a Notch ligand is overexpressed in leukemic stem cells. Inhibition of γ-secretase by *N*-(*N*-(3,5-*di*fluorophenacetyl)-L-*a*lanyl)-*S*-*p*henylgly cine *t*-butyl ester (DAPT) inhibits leukemic stem cell growth. Gamma-secretase is a protease that is involved in Jagged and Notch signaling.[175] Likewise, this group also determined that certain genes are detected at lower levels in leukemic stem cells, this include genes involved in DNA repair, signal transduction and cell cycle

genes which are consistent with the quiescent nature of leukemic stem cells.

The Wilms' tumor (WT1) gene has also been observed to be expressed at elevated levels in leukemic stem cells.[176] In normal hematopoietic stem cells, WT1 was not detected at high levels in long-term hematopoietic stem cells or in multipotent progenitor cells. In contrast, in AML1-ETO + TEL-PDGFRβR or BCR-ABL murine leukemias, WT1 was expressed in approximately 50% of the transplantable leukemic stem cells.[176] Hence the WT1 protein may be a target for the successful treatment of leukemic stem cells.

Additional transcription factors have been implicated in regulating the leukemic stem cell. The Hox genes have been suggested to be critical for induction of leukemic stem cells and their maintenance. Meis 1 has recently been shown to be an essential and rate-limiting regulator of MLL leukemia stem cell potential.[177] This group demonstrated that Meis plays a major function in establishing leukemic stem cell potential by regulating self-renewal, differentiation arrest and cycling. In contrast, other transcription factors, (for example, PU.1 and JunB) may display reduced expression in AML stem cells.[178,179] Targeting of transcription factors has proven problematic, however, it is an area which has great potential.[180]

In summary specific therapeutic targeting of the leukemic stem cells is a field in its infancy. As we learn more about the leukemic stem cell, it will undoubtedly result in novel ways to target it. The leukemic stem cell remains a challenging target. It is a slow proliferating cell with quiescent-like properties. This makes this cell resistant to classical chemotherapeutic approaches. Furthermore, there may be preexisting mutations in the leukemia stem cell, which will confer resistance to certain targeted therapies. Thus we must be clever in our approaches to design therapies that will be effective to eliminate this cell responsible for initial tumor formation.

Concluding remarks

The development of selective small molecule inhibitors has been of the biggest advances in cancer research and therapy in the past decade. Certain leukemias such as CMLs were originally considered a death sentence 15 years ago. However, due to the discovery of imatinib that selectively targets the BCR-ABL, c-Kit and PDGF-R kinases, successful treatment of CML as well as some other cancers (for example, GIST) that proliferate in response to these activated oncoprotein, successful therapies have emerged. Although resistance to inhibitors like imatinib may occur, additional novel inhibitors have been developed, which will suppress the mutant kinase or pathway in resistant cells.

Leukemias such as CMLs are examples of a cancer which proliferates in response to unique defined mutations; in this case, the BCR-ABL chromosomal translocations. APL is another example of leukemia that can be effectively treated by drugs which induce differentiation, such as retinoids and ATO. Certain MMs may be sensitive to proteosome inhibitors and ATO. However, in most cases the precise mutation, which is driving the proliferation of the leukemia is not known. Alternatively, the mutation responsible for proliferation may be known, but there may be no effective inhibitor to suppress that aberrant protein. Also karyotype evolution during therapy due to the outgrowth of resistant cells with additional or other mutations (for example, imatinib resistance) may occur which forces sequential inhibitory treatment, if available. A fourth possibility is that more than one protein (mutation) may be required for driving the proliferation of the cells. This may require targeting by more than one inhibitor. There may be an effective inhibitor for one protein but not the other. In this case, which may be frequently observed in leukemia, treatment with an inhibitor and relatively nonspecific chemotherapy may be appropriate. Although chemotherapeutic drugs are relatively nonspecific and they do have significant detrimental side effects (for example, cardiotoxicity associated with doxorubicin therapy), they can be effective in certain leukemia therapies and their usefulness may be enhanced by the administration of appropriate inhibitors. Recently it has been shown, that in some cases, the effectiveness of the inhibitors and chemotherapy may be increased (synergic interaction) or decreased (antagonistic interaction) by the sequence of treatment. This phenomenon may be a result of the position in cell cycle progression the particular inhibitor or chemotherapeutic drug inhibits.

The development of toxic antibodies to receptors expressed on the cell surface (for example, myelotarg) and inhibition of leukemic cell proliferation is another novel aspect in effective leukemia therapy. The effective use of these toxic antibodies may be enhanced by chemo-targeted and in some cases hormonal therapies.

As stated previously, often the precise mutation driving the proliferation of the leukemia is not known or there is no effective inhibitor available. In these cases, it may be appropriate to target either the Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR or Jak/STAT pathways as these pathways are often activated by upstream mutations and they are responsible, at least in part, for driving the proliferation of the cells. Alternatively the apoptotic pathways are often overexpressed or aberrantly expressed in leukemia cells. The development of apoptotic specific inhibitors is more difficult as these molecules are also expressed in normal cells at significant levels. Mutation-specific kinase inhibitors are also being developed which could be very effective in the therapy of leukemias which contain the precise mutation.

A consequence of diverse cancer therapies (for example, chemotherapy, radiotherapy and hormonal therapy) is the induction of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways which may in some cases evoke survival functions. The mechanism of induction of these pathways may be in part in response to the reactive oxygen species generated by the different therapies. Thus in some cases it may be appropriate to combine these conventional therapies with small molecular weight inhibitors which target these pathways.

In summary, leukemia therapy has been significantly enhanced over the past 20 years by the discovery of how oncogenes regulate cellular proliferation and how small molecule cell membrane-permeable inhibitors may suppress the proteins critically required for the growth of the leukemic cells. Targeted therapy has increased the effectiveness of chemo-, radio- and hormonal-based therapies. Clearly more basic research and clinical trials need to be performed to improve leukemia therapy, but many advances have been made in leukemia therapy by insight from signal transduction research and pharmacology.

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