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# Leukemia Therapy: Mechanisms of Drug Resistance and Investigational Strategies

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author AKP performed the literature search and wrote the first draft of the manuscript. Authors MZ and XH were involved in outlining the scope of the manuscript and edited it. All authors read and approved the final manuscript.*

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## ABSTRACT

Certain types of leukemia are amongst the first neoplasias to be "cured" with relatively high 5-year remission rates. This is largely due to targeted therapeutics. However, considerable resistance to tumor-specific targeting drugs has developed due to the presence of abundant cancer stem cells, profound genetic diversity, redundant growth/survival pathways and residual disease. Although these issues propose a challenge, they also provide the opportunity for novel innovations of therapy which currently include the development of multi-target kinase inhibitors, multiple drugs acting on multiple targets, key upstream targets covering multiple downstream targets and the future direction of hybrid molecules targeting two or more targets in different pathways.

*Keywords: Leukemia; targeted therapeutics; drug resistance; novel therapies; multi-target kinase inhibitors; hybrid molecules.*

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## 1. INTRODUCTION

The hematological malignancies consist of the various forms of leukemia, lymphoma and myeloma in addition to the related myeloproliferative diseases (thrombocythemia, polycythemia vera, idiopathic myelofibrosis) and myelodysplastic syndromes (clonal cytopenias, oligoblastic myelogenous leukemia). Many people suffer from hematological malignancies. Compiled data from the Leukemia and Lymphoma Society, American Cancer Society and the Surveillance, Epidemiology, and End Results (SEER) database of the National Cancer Institute showed that approximately 135,000 new cases occurred in 2007 alone and the prevalence of disease was calculated to be more than 800,000 in 2004[1]. In the UK, an average of 26,827 new cases of hematological malignancies were registered annually from 2004-2008 [2]. They account for 9% of all cancers and are the 4$^{th}$ most common cancer in both males and females in economically developed regions of the world [3]. Although many strides have been made in terms of therapeutics for hematological malignancies, there remain many pitfalls that have yet to be overcome.

Over the past 60 years, many significant breakthroughs have occurred ranging from the development of radiation therapy to a wide array of drug therapies i.e. nitrogen mustard, adrenocorticotropic hormone, cortisone acetate and anti-folic acid derivatives. Such advances in drug development have culminated today to include 14 classes of drugs and over 50 individual drugs. As of now, 70% of children with acute lymphocytic leukemia (ALL) are able to be "cured," with extremely high 5-year complete remission rates. Furthermore, prolonged remission rates are now high for other hematological malignancies i.e. several genetic variants of acute myelogenous leukemia (AML) in young adults and lymphomas in children and young adults. The development of imatinib for chronic myelogenous leukemia (CML) marked a major step in drug development as it was among the first tumor specific targeting drugs. Despite this remarkable progress, the majority of patients diagnosed with hematological malignancies face uncertain outcomes and shortened life spans. This is in part true because hematological malignancies exhibit significant genetic diversity in that they contain hundreds of unique genetic primary lesions; they also contain a significant population of stem cells within over 1 trillion disseminated cancer cells. Furthermore, the redundant growth and survival pathways defining cancer phenotypes make these malignancies conducive to the development of drug resistance, posing another major challenge in the battle against hematological malignancies [1]. Finally, residual disease remaining in areas where it is difficult for therapeutic drugs to accumulate such as the testicles, eyes and CNS tissue provides another major challenge in battling the hematological malignancies.

## 2. OVERVIEW OF DRUG RESISTANCE MECHANISMS

### 2.1 Drug Specific Induction of Resistance

Chronic myelogenous leukemia (CML) is defined by the presence of a reciprocal translocation of genetic material between chromosomes 9 and 22, producing what is known as the Philadelphia chromosome. The resulting breakpoint cluster region/Abelson (Bcr-Abl) oncoprotein is a constitutively active tyrosine kinase that is responsible for activating a number of signal transduction pathways that propagate uncontrolled cell proliferation and reduce apoptosis [4]. Imatinib, a drug developed to specifically target this Bcr-Abl modality revolutionized the treatment for CML; a phase III trial investigating its efficacy revealed significantly improved patient outcomes, response rates and overall survival when compared

to previous standards of chemotherapy. The survival rate, however, is not 100% due to the development of resistance and intolerance of its side effects in some patients [5-7]. In CML, primary or intrinsic resistance is defined by the National Comprehensive Cancer Network (NCCN) and Leukemia Net guidelines as failure to achieve complete hematological response (CHR) by 3 months, cytogenetic response (CyR) by 6 months, partial cytogenetic response by 12 months and complete cytogenetic response (CCyR) by 18 months. This study revealed that 16% of patients with newly diagnosed chronic phase CML failed to achieve at least a partial CyR after 12 months of treatment with imatinib and 24% failed to achieve CCyR after 18 months of treatment [7].

The most common mechanism of Bcr-Abl dependent resistance is point mutations in the Abl kinase domain of the Bcr-Abl fusion protein. Over 90 separate resistance-conferring point mutations at 57 residues in the Abl kinase have been documented and generally occur in regions of the kinase domain, the ATP-binding loop (P-loop), the contact site, the SH2 binding site and the A-loop [8,9]. 30-40% of these point mutations are P-loop mutations which data has shown to correlate with poor prognosis for response and survival [10,11]. They tend to occur more frequently in advanced CML and their development has been associated with accelerated or blast crisis phase CML [12,13]. Kinase assays have shown imatinib to be 70-100 times less sensitive in treating CML when p-loop mutations are present [14]. They have also been shown to have higher transforming activity than wild-type Bcr-Abl [15].

Aurora kinases fall into the category of mitotic protein kinases and have recently been heavily investigated in the realm of anticancer drug development. It is known that mammals contain Aurora A, B and C kinases that act as essential regulators of mitotic events. Aurora A acts at the spindle pole to ensure the integrity of the centrosomes. Aurora B and C, on the other hand, function as part of the chromosomal passenger complex (CPC) that ensures proper segregation and alignment of the chromosomes [16-19]. Research has shown that Aurora kinases tend to be over-expressed in malignancies. Aurora A has been noted to be over-expressed in breast and bladder cancer, while Aurora B has been noted to be over-expressed in gastric cancer, glioblastoma multiforme, oral cancer and lung cancer. Most of the research involving Aurora kinases involves studying their inhibition. Most recent studies have focused on the Aurora kinase inhibitors Hesperadin, ZM447439 and MK-0457 (VX-680). Hesperadin inhibits Aurora B, ZM447439 inhibits Aurora A, B and C and MK-0457 inhibits wild-type and mutated BCR-ABL, including the T315I mutation in addition to FLT3 and JAK2. MK-0457 also inhibits Aurora A, B and C at low concentrations. Overall, Aurora kinase inhibitors do not inhibit cells from entering mitosis. Rather, they cause defects in chromosome segregation. Exposed cells exit mitosis but these cells are unable to divide, resulting in polyploidy cells that undergo apoptosis [20-22]. In research studies conducted by Dreier et al. it was determined that Aurora kinase inhibitors more efficiently killed cells lacking p53. ZM447439 induced DNA damage and upregulated p53 through a pathway relying on ATM/ATR protein kinases. The cells that avoided killing did not appear to be resistant to the drug. Although it is likely that prolonged exposure to Aurora kinase inhibitors and the use of stronger doses in the clinical setting may generate resistant cells, the data suggests that the resulting cells could be susceptible to subsequent treatments with the same agents if they behave in a similar manner to their behavior *In vitro* [23].

## 2.2 Cross Resistance

In applying the concept of cross resistance, a mutation that confers resistance against one drug likely does not confer resistance against any of the other drugs. Thus, the idea is that

combining two drugs will increase the probability of treatment success because strains that develop resistance to one drug should be susceptible to the other drug and vice versa in the combination treatment. It is the same concept that is applied in the treatment of HIV, requiring simultaneous use of multiple drugs to avoid the development of resistance. The hurdle to this concept is the development of cross resistance. For example, the T315I mutation in CML can confer cross resistance to all the drugs (imatinib, dasatinib and nilotinib) currently available to treat CML. Through the use of a computational mathematical model, Komarova et al. concluded that combining two cross-resistant drugs that do not inhibit the T315I cross-resistant mutant improves the chances of treatment success while combining more than two of these drugs does not improve treatment success beyond that of the two-drug combination. In addition, this study put forth the notion that once a drug that is effective against the T315I mutation is developed, combining it with two of the three presently existing cross-resistant drugs may be the optimal strategy for treatment of imatinib-resistant CML in an effort to synergistically overcome cross resistance [24].

### 2.3 Stress-induced Resistance

Although oxidative stress can itself play a part in killing malignant cells, it can also be involved in the upregulation of p53 and through this mechanism confer resistance to both normal and leukemic cells. It is thought that p53 upregulation is highly sensitive to reactive oxygen species (ROS) in that activation of the p53 system requires mitochondrial activity. Furthermore, it is thought that mitochondrial ROS act as second messengers between the respiratory chain and proapoptotic p53 machinery, thereby playing an important role in the development of chemoresistance [25].

Another stress-induced mechanism of resistance to imatinib has been linked to heat-shock protein 70 (Hsp-70). Pocaly et al. found no evidence of conventional mechanisms of resistance in generating the imatinib-resistant cell line K562-r. Rather, proteomic analysis showed only differential expression of Hsp-70. Hsp-70 levels were found to be three times as high in imatinib-resistant strains versus imatinib-sensitive strains. The increase in Hsp-70 was not linked to heat-shock transcription factor-1 overexpression or activation. In addition, RNA silencing decreased the expression of Hsp-70 by 90%, accompanied by a 34% reduction in cell viability in the presence of imatinib. This finding in vitro correlates to in vivo studies in that patients responding to imatinib have lower levels of Hsp-70 in blast cells compared to those who do not respond to imatinib [26]. At the molecular level, heat shock proteins conventionally act as molecular chaperones that bind to unfolded or mis-folded proteins to correct abnormalities in folding and prevent intracellular protein aggregation [27,28]. Expression of Hsp-70 has been linked to cellular stress. It is often increased in acute leukemia and contributes to mediating the antiapoptotic effect of Bcr-Abl by inhibiting apoptotic signals [29-32]. Furthermore, it is indirectly involved in preventing apoptosis by its interaction with the co-chaperone CHIP or BAG-1, leading to the proteasomal degradation of proapoptotic proteins [33,34]. These findings make Hsp-70 a potential marker and therapeutic target for CML.

## 3. SELECTIVE SURVIVAL OF RESISTANT CANCER CELLS

### 3.1 Non-sensitive “Gifted” Cells

Cancer cells arise as a result of somatically acquired changes in the DNA of cells. The formation of cancer cells from normal cells is governed by the principle of driver mutations.

Driver mutations are causally implicated in oncogenesis and confer growth advantage to the cancer cell that has been positively selected in the microenvironment of the tissue in which the cancer has arisen. A passenger mutation, on the other hand, has not been selected, does not confer growth advantage and therefore does not contribute to cancer development. Passenger mutations are found within genomes because somatic mutations without functional consequences often occur during cell division [35].

One important subclass of driver mutations is that which confers resistance to cancer therapy. Often these mutations occur in cancers that were responsive to therapy but are now resistant. They often confer limited growth advantage to the cells in the absence of therapy. It has been noted that some of these mutations predate the initiation of treatment and exist as passenger mutations in minor subclones of the cancer cell population until the selective environment is changed by the initiation of therapy [36,37]. The passenger is then converted to a driver, allowing the resistant subclone to expand preferentially, manifesting as the recurrence. Further corroborating this concept is a study done by Roche-Lestienne et al. in which STI571-resistant mutated cells were present prior to therapy (STI571 is the investigational name for the drug now known as imatinib). Rather than being induced by STI571, it appears these cells resulted from secondary mutational events during the disease course pre-existent to STI571 treatment [37].

## 3.2 Mutated "Resilient" Cells

Mutations in genes may affect the activity of corresponding proteins. Often, activity is increased by mutations that alter life-time, substrate specificity, binding capacity or auto regulatory elements of the protein in question. There is some suggestion that the FLT3-RAS signaling pathway should be further explored as a potential target for novel inhibitory drugs. In childhood precursor B-ALL, activating mutations have been found in the fms-like tyrosine kinase receptor gene (FLT3) in approximately 8% of cases, particularly MLL-rearranged and hyperdiploid ALL [38,39]. The FLT3 gene activates signal transduction pathways involved in proliferation and survival of progenitor cells in the early stages of hematopoiesis. The FLT3 mutations have been shown to destroy the auto-inhibitory capacity of the juxtamembrane domain (FLT3-ITD mutation). They also result in constitutive activity due to single amino acid substitutions in the kinase domain (FLT3 835/836 mutations). Just a high level of FLT3 expression itself is sufficient to lead to phosphorylation of the FLT3 receptor in the absence of activating mutations. Not surprisingly, based on the concept outlined above, a high expression of FLT3 has been linked to poor prognosis of MLL-rearranged ALL in infants [40]. PKC412 and CEP-701 are two small-molecule inhibitors which are targeted toward activated FLT3. They interfere with the catalytic domain of the tyrosine kinase and thereby abolish further triggering of the downstream survival (AKT-mediated) and proliferation (RAS-MAPK-mediated) signaling cascades [41-43].

## 3.3 Fusion "Hybrid" Cells

There is some evidence that the fusion of cancer cells can generate new mechanisms of resistance. In one study the cell lines PL-11 and Hs766T were fused and resulted in full complementation of the hypersensitivity to DNA cross-linkers in all hybrids by complementing the defective Fanconi anemia pathway. Both cell lines separately have a phenotype of hypersensitivity to DNA cross-linking agents such as mitomycin c which causes cell cycle arrest in G2. A marked decrease in survival after treatment with mitomycin c has been noted in these cell lines when compared to other cell lines. This hypersensitivity

was thought to be due to mutations in genes of the Fanconi anemia pathway. It is known that exons 7-14 of the FANCC gene have been homozygously deleted in the FANCG gene in PL-11 while Hs766T has a homozygous nonsense mutation in the FANCG gene. In addition, PL-11 contains wild type FANCG and Hs766T has wild type FANCC. This suggests that fusing CIN cell lines to form mapped hybrids may offer new tools for positional cloning or classification of simple and complex cancer phenotypes such as mechanical defects and altered drug responses [44-46].

### 3.4 Tumor Stem "Quiescent" Cells

Hematopoietic stem cells (HSCs) maintain hematopoietic homeostasis throughout an organism's lifespan via self-renewal and differentiation into mature blood cells. This is done by the adoption of a quiescent state in which HSCs remain in the non-dividing G0 phase of the cell cycle. It was recently shown that maintenance of genomic integrity is crucial for the preservation of self-renewal capacity of HSCs. Literature suggests that low levels of reactive oxygen species (ROS) and adequate DNA damage responses (DDR) are of prime importance in maintaining genomic integrity and HSC function. Several studies have demonstrated that inappropriate ROS levels result from disruption of the Atm, PI3K-Akt or Mdm2-p53 pathways and thereby impair HSC function in vivo. Although murine HSCs have been found to be more resistant than progenitor cells to mild DNA damage *In vivo*, the surviving HSCs often acquire genetic aberrations that lead to leukemogenesis. One reason for this may be that non-dividing HSCs employ the error-prone non-homologous end-joining pathway of DNA repair to fix DNA breaks while progenitors undergo apoptosis. Furthermore, proliferative HSCs employ the high-fidelity homologous recombination mechanism. Thus, further investigation into HSC-specific mechanisms for the maintenance of genomic integrity may provide insight into leukemogenesis [47].

## 4. OVERVIEW OF CURRENT THERAPUETICS

There are several classes of drugs used in the treatment of hematological malignancies. A brief overview of the major drug classes follows. Antitumor antibiotics (i.e. bleomycin, daunorubicin, doxorubicin, idarubicin, mitoxantrone) interact directly with DNA in the nucleus of cells and thereby interfere with cell survival and are used to treat almost all of the hematological malignancies. Antimetabolites (i.e. cladribine, cytarabine fludarabine, hydroxyurea, mercaptopurine (6-MP), methotrexate) have very similar components to DNA and RNA but are different enough that when they are substituted for a component of DNA/RNA they do not allow the cell to form DNA/RNA respectively. By this mechanism cell growth is halted and cell death is accelerated. Biomodifiers (i.e. interferon-a, thalidomide, lenalidomide) are thought to have immune, cytotoxic and antiangiogenic effects but the exact mechanisms by which they work is still unknown. Bisphosphonates (i.e. pamidronate and zoledronic acid) block the resorption of bone and are thus used in the treatment of myeloma. DNA-repair enzyme inhibitors (i.e. etoposide, teniposide, topotecan) allow the DNA to become more susceptible to injury by blocking the proteins and enzymes in the cell nucleus that normally repair injury to DNA.

Certain drugs (vinblastine, vincristine, paclitaxel) act to block mitosis and are often used to treat lymphomas/lymphocytic leukemias. Histone deacetylase inhibitors modulate chromatic structure and gene expression by inducing growth arrest, cell differentiation and death of leukemia cells. In high doses, glucocorticoids (dexamethasone, methylprednisolone prednisone) also have a cytotoxic effect on malignant lymphocytes. Monoclonal antibodies

(rituximab, gemtuzumab) have been shown to better target and destroy cancer cells with fewer side effects than conventional chemotherapy while phototherapy drugs activated by UV light (psoralen) are employed in the treatment of skin lymphoma cells. Furthermore, cell-maturing agents which target the PML-RARa fusion gene (tretinoin or all-trans-retinoic acid (ATRA)) are used to induce maturation of chronic myelogenous leukemic cells to mature granulocytes which results in apoptosis; arsenic trioxides work by similar mechanisms and have been approved for use in ATRA-resistant acute promyelocytic leukemia. DNA-damaging drugs (busulfan, carboplatin, carmustine, chlorambucil, cisplatin, cyclophosphamide, ifosfamide, melphalan, etc.) react with cell DNA chemically and alter it to enhance cell death. Proteasome inhibitors (i.e. bortezomib) act to break down proteins in the proteasome which are vital to the function of the cell and are often used in the treatment of myeloma. Finally, tyrosine kinase inhibitors (i.e. imatinib mesylate, dasatinib, nilotinib) act to counteract mutant proteins that initiate malignant cell transformation and have been very effective in treating CML and certain acute leukemias.

In addition to these current standard therapies employed to treat hematological malignancies, there are many investigational drugs that show promise, some of which will be discussed below. Bone marrow transplant is also an option when chemotherapy is not sufficiently effective. The use of allogeneic hematopoietic stem cell transplantation (from an unrelated donor) has become widespread and can often cure lymphomas and leukemias [1].

## 5. INVESTIGATIONAL STRATEGIES IN DEVELOPMENT

### 5.1 Anti-CD20 Monoclonal Antibodies

Rituximab (IDEC-C2B8) is a highly specific mouse/human chimeric antibody engineered by grafting the variable regions targeting the CD-20 antigen from a murine anti-CD20 antibody (2B8) on to human constant regions [48]; it has shown promise in the treatment of non-Hodgkin lymphoma (NHL) as it has a longer half-life than the parent murine antibody and is less immunogenic. It barely elicits a human anti-mouse antibody (HAMA) response which can be a major complication of immunotherapy. It reacts only to B cells by binding complement C1q, thereby inducing antibody-dependent cellular cytotoxicity [49,50]. It also inhibits cell proliferation and directly induces apoptosis [51]. Through disruption of anti-apoptotic pathways, it has been shown to sensitize malignant B cells to chemotherapy and thus can be used in synergy with chemotherapeutic agents [52].

Rituximab can be used as a single agent therapy in lymphoma but has also worked well in treating aggressive/indolent lymphomas, chronic lymphocytic leukemia and in combination with chemotherapy. In one important large multi-institutional study, 166 patients with relapsed or refractory low-grade or follicular B-cell lymphoma were treated with mono therapy rituximab. The overall response rate (RR) in the intent-to-treat analysis was 48% with 6% complete responses (CRs); the projected median time to progression (TTP) was 12.5 months [53]. Another important study evaluated single-agent therapy in patients with aggressive lymphoma such as diffuse large cell lymphoma (DLCL), mantle cell lymphoma (MCL) and other intermediate or high-grade lymphomas. The study included 54 patients (mostly in first or subsequent relapse or with refractory disease or progression after initial response); nine elderly patients were previously untreated. The overall RR was 31% (CR 9%, PR 22%). DLCL had a RR of 37% and MCL had a RR of 33%. This study showed that rituximab is an active agent in aggressive lymphomas and suggested that it may have

therapeutic potential in combination with chemotherapy in treating these subgroups of lymphomas [54].

The combination of rituximab with CHOP chemotherapy was proven to be extremely effective for the treatment of indolent lymphoma in a phase II trial done by Czuczman. In this study, 40 patients (of which 31 had been previously untreated) received 6 cycles of a combination of rituximab and standard CHOP chemotherapy. On intent-to-treat analysis, the RR was 95% while 55% achieved a complete response and 44% achieved a partial response. Some patients also converted from PCR positive bcl-2/lg gene sequence to PCR negative for this gene sequence. Furthermore, the toxicity profile remained the same as with either rituximab or CHOP given alone, while the addition of rituximab did not compromise CHOP dose intensity [55].

Multiple other studies have investigated the application of rituximab in combination with other multi-drug regimens in indolent lymphoma. In one such phase II single institution trial of rituximab and fludarabine given together in 40 patients with treated and untreated low-grade lymphoma, the RR was reported to be 90% with a CR/CR-unconfirmed rate of 82.5% with median duration of response not reached at 15+months. Toxicity was deemed acceptable but was notable for increased cytopenia (mainly neutropenia) and increased herpes simplex/zoster infections. Increased incidence of bacterial or opportunistic infections was not noted [56].

The combination of rituximab with other monoclonal antibodies that target alternative lymphocyte antigens is another area of study. Alemtuzumab (CAMPATH-1H) is a monoclonal antibody that binds to CD52 antigen which is found on the majority of T and B lymphocytes. It is used in fludarabine-refractory B-CLL. The combination of rituximab with alemtuzumab has been studied for use in relapsed/refractory B-CLL. Studies suggest this combination is safe/feasible and has shown encouraging activity in this poor-risk patient group [57,58]. Rituximab in combination with other agents such as interferon-alpha (IFNa) is also under investigation. In one randomized phase II study, 124 patients with follicular grade 1-2 CD20+B-cell NHL who had received no chemotherapy or <6 months of chlorambucil received weekly rituximab for 4 weeks. 11% of patients achieved CR and received no further therapy while those who remained were randomized to two groups; one group received another 4 weeks of weekly rituximab and the other group received rituximab and INFa. The RR in the rituximab and INFa group was found to be 94% with 48% CRs compared to a RR of 77% with 22% CRs in the rituximab only group [59].

In aggressive lymphomas, CHOP results in clinical response approximately 80% of the time but is only curative 40% of the time. However, it has remained the gold standard in treatment of the aggressive lymphomas as there are few other options for effective treatment [60]. The combination of CHOP and rituximab has shown promise in the treatment of diffuse large B-cell lymphoma (DLBCL) in the elderly. In one randomized clinical trial, patients were randomized to receive either 8 cycles of CHOP or 8 cycles of CHOP with rituximab given on day 1 of each cycle. CR was higher in the combination group, 76% versus 63%. 2-year event-free and overall survival were also greater in the combination group. Overall toxicity of the 2 groups was similar, although the combination group did have a higher incidence of herpes zoster. This was a seminal study as it demonstrated the first improvement in overall survival for any regimen compared with CHOP and established the addition of rituximab as an agent to be combined with standard chemotherapy for front-line treatment [61].

## 5.2 Heat Shock Protein 90 (Hsp90) Inhibitors

Heat shock protein 90 is an essential molecular chaperone that accounts for 1-2% of all protein in cells and has been identified as a potential molecular target for cancer therapeutics for a number of reasons. First, it is key to the stability and function of a host of proteins important to the cancer cell i.e. BCR-ABL, ERB-B2, EGFR, CRAF, BRAF, AKT, MET, VEGFR, FLT3, androgen receptors, estrogen receptors, (HIF) 1-alpha and telomerase [62]. These proteins are essential to the traits of cancer which include growth factor independence, resistance to antigrowth signals, unlimited replicative potential, avoidance of apoptosis, sustained angiogenesis and tissue invasion and metastasis [63]. It is for this reason that therapies that selectively target a single tyrosine kinase have failed since multiple parallel transduction signals are simultaneously activated. There is hope that this problem can be overcome by targeting HSP90 because its inhibition will lead to the inhibition of multiple signal transduction pathways. There are a number of reasons inhibition of HSP90 should theoretically work to inhibit cancer. Inhibition of oncoproteins which drive the malignant process and are more prevalent in cancer cells than in normal cells is key [64]. In addition, oncoproteins are often expressed as mutant forms which require HSP90 for stability compared to their wild-type counterparts [65,66]. Finally, cancer cells are subject to intratumoral acidosis, hypoxia and deprivation of nutrients which creates an environment of cellular stress that requires chaperone machinery to a greater extent than their normal counterparts [67]. The focus of drug development has been on inhibition of ATP-binding at the NH2-terminal domain of HSP90 because normally when ATP binds to this site it adopts a closed conformation and becomes a mature complex required for client protein folding and stabilization [68].

Inhibition of HSP90 may prove to be especially important in the development of therapies for many of the hematological malignancies i.e. CML, multiple myeloma, CLL and AML. BCR-ABL is a known client protein of HSP90 and its inhibition may prove to be a valid treatment in CML resistant to kinase inhibitors such as imatinib. In multiple myeloma, there is evidence that HSP90 is over expressed and its inhibition has been associated with inhibition of signaling pathways induced by cytokines (i.e. IL-6) via inhibition of downstream signaling inhibitors (i.e. AKT) [69,70]. Furthermore, HSP90 is also associated with activation of the unfolded protein response [71]. ZAP-70 is a known client of HSP90 in a subset of aggressive CLL and inhibition of HSP90 has been associated with decreased ZAP-70 activity [72,73]. In AML, FLT3 is a known client protein of HSP90. FLT3 is activated in one third of AMLs and functions to activate downstream signal transduction pathways. Thus, inhibition of HSP90 should lead to inhibition of FLT3 and may provide therapeutic benefit in AML [74].

## 5.3 Multi-Targeted Kinase Inhibitors

In developing drugs that may be used in the event of resistance, one concept that has shown promise is the use of multi-targeted kinase inhibitors. Dasatanib, a potent orally bioavailable dual Bcr-Abl/Src kinase inhibitor is one such drug. It is the first tyrosine kinase inhibitor to be approved in the United States and Europe for all imatinib-resistant and imatinib-intolerant patients across all phases of CML in addition to Philadelphia-chromosome positive acute lymphoblastic leukemia. Although it also targets the Bcr-Abl modality, dasatanib is structurally unrelated to imatinib and binds multiple conformations of the Abl kinase domain [75-78]. *In vitro*, dasatanib demonstrated 325 times more activity against native Bcr-Abl compared to imatinib; it also showed efficacy against all imatinib-resistant Bcr-Abl mutations with the exception of T315I [76,77]. Furthermore, it was found to exhibit

significant activity against multiple targets i.e. SFKs, c-Kit, platelet-derived growth factor receptor (PDGFR) and ephrin A receptor [75,79-81].

Recent findings indicate that drugs that target multiple kinases may be less toxic than previously thought and that targeting only a single kinase with a high degree of selectivity has significant downsides. One such group of kinase inhibitors that has been studied is Janus kinases (Jaks). Type I and II cytokine receptors lack receptor-intrinsic tyrosine kinase activity and instead transmit their signals through receptor-associated Janus kinases (Jak1, Jak2, Jak3 and Tyk2). The hormone-like cytokines growth hormone, prolactin, erythropoietin, thrombopoietin, GM-CSF, IL-3 and IL-5 all signal through Jak2 while IL-6, IL-10, IL-11, IL-19, IL-20, IL-22 and interferon-gamma signal through Jak1 and Jak2. Jak3 is the only Jak family member that associates with just one cytokine receptor subunit (the common gamma-chain) that is used exclusively by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. These cytokines are involved in T and NK cell development in addition to B cell function. Furthermore, these cytokines are not important for the development or function of organs or tissues outside the immune system. Thus, in theory, a Jak3-specific inhibitor should have limited but precise effects on immune system cells without affecting other cell types. This makes Jak3 an ideal inhibition target. A Jak3 inhibitor that is efficient and safe could prove to be revolutionary in the therapy of autoimmune diseases and transplant rejection. CP-690550, initially thought to be a selective Jak3 inhibitor, has been found to be efficacious in transplant rejection in animal models as well as against inflammatory diseases. The drug causes a reduction is NK cells, interferon-gamma and IL-6; T and B cells are not affected. However, the fact that it has also been noted to cause anemia suggests involvement of Jak2 inhibition as Jak2 is essential for the actions of erythropoietin and its inhibition is the most likely cause of anemia. To further support this theory, one study did successfully show that CP690550 binds Jak2 and Jak3 with approximately equal affinity even though it does affect a very restricted number of targets within the human kinome [82]. This suggests that multiple target kinase inhibition may prove to be safe and more effective than single target kinase inhibition [83].

Furthermore, a recent study by Wen and colleagues used high-content image-based screening to show that dimethylfasudil (diMF, H-1152P) selectively increases polyploidization, mature cell-surface marker expression and apoptosis of malignant megakaryocytes via its role as a broad kinase inhibitor. It could therefore be a promising treatment for acute megakaryocytic leukemia (AMKL). Aurora kinase A was identified to be a major target of diMF via the integrated target identification approach employing proteomic and shRNA screening. DiMF was also shown to inhibit proliferation/induce polyploidization and upregulation of megakaryocyte markers of cells with GATA1 and MPL mutations in addition to cells harboring+21 or the (1;22) translocation [84].

## 5.4 Mammalian Target of Rapamycin (mTOR) Inhibitors

Another potential therapeutic target is mammalian target of rapamycin (mTOR) which is a serine/threonine kinase that functions as a key regulator of cell growth, protein synthesis and cell-cycle progression through interactions with a number of signaling pathways such as PI3K/AKT, ras, TCL1 and BCR/ABL. As many hematological malignancies have aberrant activation of mTOR and related signaling pathways, mTOR inhibitors may be useful in terms of therapeutics. They were originally developed as immunosuppressive agents and one attractive feature is that they are well tolerated and safe (i.e. sirolimus and second-generation mTOR inhibitors temsirolimus and everolimus). Early clinical data holds promise

for the use of mTOR inhibitors in ALL, CML, mantle cell lymphoma, anaplastic large cell lymphoma and lymphoproliferative disorders [85].

## 5.5 Proteasome and HDAC Inhibitors

The proteasome has proven to be a successful target in multiple myeloma and mantle cell lymphoma as evidenced by the effective use of bortezomib to treat these diseases. The theory is that malignant cells accumulate more misfolded/mutated/damaged proteins which are disposed of by the proteasome so these cells are more dependent on proteasome activity to maintain cell function [86]. Inhibition of the proteasome induces apoptosis. However, in other hematological malignancies the proteasome inhibitor bortezomib has proven to be less successful as a single agent. One novel proteasome inhibitor that has shown promise in leukemia model systems is NPO-0052. Unlike bortezomib which only inhibits chymotrypsin-like activity of the proteasome, NPO-0052 was shown to inhibit all 3 proteolytic activities (chymotrypsin-like, trypsin-like and caspase-like) associated with the beta subunit of the purified human erythrocyte 20S proteasome [87]. It was also found to induce DNA fragmentation in leukemic and mononuclear cells from a Ph+ lymphoblastic leukemia (ALL) patient. In terms of mechanism, it relies on a caspase-8-dependent pathway to induce apoptosis and there is some suggestion that oxidative stress is involved since the presence of N-acetyl cysteine (NAC) proved to be protective against apoptosis. In mice, it was found to reduce total white blood cell burden over 35 days. Furthermore, when used in combination with HDAC inhibitors such as valproic acid and MS-275, it rendered greater levels of cell death than the combination of bortezomib and HDAC inhibitors [88].

## 5.6 Notch Inhibitors

NOTCH inhibition is another molecular target under investigation for therapeutic benefits. T-cell acute lymphoblastic leukemia (T-ALL) results from malignant transformation of immature T-cell progenitor cells caused by human T-cell leukemia virus type 1 (HTLV-1) and affects peripheral T lymphocytes [89,90]. It initially had a very poor prognosis but cure rates are now around 80% for children and 50% for adults due to aggressive chemotherapy protocols [90-92]. However, relapse associated with acquired chemotherapy resistance still poses a problem. In over 50% of human T-ALL, activating mutations are present in the NOTCH1 gene which makes it the most prominent oncogene specifically involved in the pathogenesis of the disease [93]. Thus, inhibition of NOTCH1 signaling with gamma-secretase inhibitors (GSIs) has been studied in T-ALL. NOTCH1 requires proteolytic processing by the presenilin/gamma-secretase complex for activation so inhibition of this complex by GSIs inhibits NOTCH signaling [94,95]. However, GSIs appear to have limited activity against human T-ALL and are associated with severe gastrointestinal toxicity due to the inhibition of NOTCH signaling in the gut which leads to the accumulation of goblet cells in the intestine. Inhibition of NOTCH1 signaling in glucocorticoid-resistant T-ALL did restore glucocorticoid sensitivity and co-treatment with glucocorticoids inhibited GSI-induced gut toxicity. This is paramount as glucocorticoids play a fundamental role in the treatment of all lymphoid tumors because of their capacity to induce apoptosis in lymphoid progenitor cells. The mechanism for glucocorticoid resistance in T-ALL remains unclear, although there is speculation that it has to do with upregulation of glucocorticoid receptor expression in response to glucocorticoids. The combination of GSIs and glucocorticoids shows promise for the use of anti-NOTCH1 therapies in human T-ALL and is an area that needs to be further studied [96].

## 5.7 Hybrid Molecules

In addition to NOTCH1 signaling pathways, T-ALL cell lines also exhibit PI3K/mTOR pathway activation. There is some evidence that targeting of the mTOR and NOTCH1 pathways simultaneously may have added efficacy. In one study done by Cullion et al. GSIs were administered to inhibit NOTCH1 signaling in conjunction with rapamycin which inhibits mTOR kinase activity. The result was induced apoptosis. Furthermore, this combination therapy was shown to inhibit human T-ALL cell growth and extend survival in a mouse xenograft model. If a hybrid molecule could be developed which works simultaneously to inhibit both pathways, this could revolutionize treatment for T-ALL and improve the poor outcomes/prognosis associated with the disease [97].

## 5.8 HH Signaling Inhibitors

Although the use of Hedgehog (Hh) signaling is well established in embryonic pattern formation [98], its functions in adult tissue maintenance/renewal and relationship to cancer development are still unclear. One study by Zhao et al. showed that the loss of Smoothened (Smo) which is an essential component of the Hh pathway [99] impairs hematopoietic stem cell renewal and decreases induction of CML by the BCR-ABL1 oncoprotein. The loss of Smo appears to cause depletion of CML stem cell numbers [100]. One possible proposed mechanism of action for this effect relates to increased Numb in the absence of Smo. Numb is a cell fate determinant that works by depleting CML stem cells. In addition, it was determined that Hh signaling impairs the propagation of CML driven by wild-type BCR-ABL1 as well as the growth of imatinib-resistant mouse and human CML. Thus, there is some suggestion that drug resistance and disease recurrence associated with imatinib treatment of CML can be avoided by targeting this essential stem cell maintenance pathway [101,102] and needs to be further investigated [103].

## 5.9 Alpha Radio Immune Conjugates

HuM195 is a humanized anti-CD33 monoclonal antibody that selectively targets myeloid leukemia cells and has activity against minimal disease. It has been used in relapsed acute myeloid leukemia (AML) but has only resulted in minimal overall response with complete response only in patients with low burden of disease [104]. Its potency has been shown to increase if conjugated with targeted radiotherapy which helps to overcome tumor antigen heterogeneity. When it is labeled with beta-emitters (131)I and (90)Y, it can result in major target cell killing but also kills normal bystander cells which can result in more toxicity, namely prolonged myelosuppression [105,106]. In a phase I trial Sgouros et al. found that (213) Bi-HuM195 appeared to localize to and was retained in areas of leukemic involvement i.e. the bone marrow, liver and spleen. Absorbed dose ratios between these sites and the whole body were found to be 1000-fold greater than the absorbed dose ratios for beta-emitting radionuclide used for immunotherapy [107].

In one study, 17 patients with AML and one patient with chronic myelomonocytic leukemia (CMML) were treated with escalated radioactivity doses from 10.36 to 37.0MBq/kg of (213)Bi-HuM195 and were assessed with bone marrow biopsy 7-10 days and 4 weeks after treatment. Fourteen out of 15 evaluable patients (93%) had reduction in peripheral blood leukemia cells and 14 of the18 patients (78%) had reduction in bone marrow blasts. The most significant toxicity was grade 3 or 4 leucopenia in 13 patients (76%). Grade 3 leucopenia was noted in 2 patients and grade 4 leucopenia was noted in 11 patients.

However, in 11 of these patients (85%) the leucopenia could be accounted for by substantial clearing (>95%) of circulating blasts. One patient who had relapsed after allogeneic bone marrow transplant was treated at the 37.0MBq/kg dose and experienced dose-limiting toxicity which was defined as grade 4 leucopenia for more than 35 days from the start of therapy. This study supported the feasibility, safety and antileukemic effects of (213)Bi-HuM195 and is the first proof-of-concept for systemic targeted alpha particle immunotherapy in humans [107].

In a subsequent study by Rosenblat et al. (213) Bi-HuM195 at doses ranging from 18.5 to 46.25MBq/kg was used after partially debulking AML with cytarabine 200mg/m2 daily for 5 days. 31 patients were included in the study. 13 had untreated AML (5 had de novo AML and 8 had secondary AML), 15 had relapsed AML and 3 had primary refractory AML. Bone marrow biopsies were done 4 and 8 weeks after treatment. 20 patients (77%) had >20% decrease in marrow blasts. However, patients who received <37MBq/kg had no response. Of the 11 patients with untreated AML, 2 achieved complete response, 1 achieved complete response with incomplete platelet recovery and 2 achieved partial response. None of the 7 patients with primary refractory AML or multiply treated relapsed disease responded to treatment. Myelosupression was the most common toxicity with development of grade 3-4 neutropenia in 9 patients. 20 patients (65%) developed bacterial infections and 19 patients (61%) developed presumed fungal PNA. Mortality due to infectious complications occurred in 2 of 21 patients (10%) treated with 37MBq/kg and 1 out of 4 (25%) patients treated with 46.25MBq/kg. 5 patients (16%) were found to have transient grade 3/4 liver function abnormalities [108]. Overall, this study suggested that the most pronounced benefit of using (213)Bi-HuM195 may be in the setting of initial debulking of AML [106].

## 6. CONCLUSION

Overall, the hematological malignancies remain prevalent at present. Although a number of advances in therapeutics have been made in the recent past, there remains a 'key pitfall' which needs to be addressed as discussed above. This key pitfall pertains to overcoming the array of different mechanisms of tumor resistance to current therapies. Targeting resistance mechanisms is at the heart of leukemia therapy. There are a number of therapies and strategies such as multi-target kinase inhibitors, multiple drugs acting on multiple targets, key upstream targets covering multiple downstream targets, hybrid molecules targeting two of more targets in different pathways and alpha radio immune conjugates that have shown promise in the treatment of hematological malignancies. However, they all require further investigation to determine their safety and efficacy for future use in humans. It is very important that we continue to work to develop new drugs and treatment methods as the prognosis of many of the hematological malignancies is still poor and any advances in treatment could prove to be beneficial for the millions of people suffering from these neoplasias.

## CONSENT

Not applicable.

## ETHICAL APPROVAL

Not applicable.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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