Chapter 1

Introduction & Review of Literature
1.1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of clonogenic, hematologic disorders characterized by the somatic acquisition of genetic and epigenetic alterations in hematopoietic stem/progenitor cells that perturb normal mechanisms of self-renewal, proliferation, and differentiation. Heterogeneity of AML arises from the diversity of myeloid precursors that are susceptible to malignant transformation and the assortment of genetic events that can lead to this malignancy. Most subtypes of AML are characterized by subpopulations of leukemic stem cells that possess an unlimited self-renewal capacity and a hierarchical organization similar to normal hematopoietic stem cells (HSC).

Cytotoxic, combination chemotherapy consisting of the deoxycytidine analogue, cytarabine and an anthracycline antibiotic, daunorubicin has remained as the clinical mainstay for most of AML subtypes for past four decades. Acute promyelocytic leukemia (PML) is the only AML class, which usually assert best overall prognosis, wherein cytotoxic chemotherapy is eliminated altogether and cure can be achieved with arsenic and/or all-trans retinoic acid (ATRA). Considering AML to be an elderly malignancy, with majority of the affected patients being above 60 years, this standard approach has resulted in progression-free survival in younger patients (<60 years: 60–75% complete remission rates) alone. Elderly patients are more likely to have comorbid illness, poor performance status, and impaired organ function. These clinical features limit their ability to tolerate intensive cytotoxic chemotherapy and result in higher mortality of 30-40%. However, inspite of achieving remission, improvement of therapeutic outcomes in AML have generally been unsuccessful, owing to higher relapse rates throughout all age groups. AML relapses are primarily attributed to the persistence of a residual, quiescent leukemic stem/progenitor cell (LSPC) fraction. This LSPC population owing to their low mitotic index are resistant to the most aggressive chemotherapeutic regimens, which targets actively dividing cells alone. Additionally, emerging evidences have identified fervent involvement of aberrantly activated kinome and derailed epigenome in leukemogenesis. The clinical community has now realized that further manipulation
of standard cytotoxic chemotherapy, which is incapable of addressing the above compartments, is unlikely to improve response rates. For efficient, clinical management of AML, treatment strategies need to be suitably modified to eradicate the resilient LSPC population by addressing the heterogeneity of the malignancy at multiple levels including kinome and epigenome.

Recent perceptions about molecular mechanisms that drive leukemogenesis have identified several deregulated signal transduction pathways (STPs) that cause constitutive activation of protein kinases imparting proliferative and survival advantages to leukemic cells. Research on aberrant AML kinome have stemmed from the clinical success of the molecularly targeted drug, imatinib, attempted against chronic myeloid leukemia (CML). This was further fuelled by the certainty that inspite of the predominance of mutations and/or translocations, their molecular manifestation results from dysfunction of STPs at the protein level. Raf/MEK/ERK, PI3K/Akt/mTOR and Jak/STAT pathways are observed to be frequently activated in AML by chromosomal translocations, upstream mutations in cytokine receptors as well as other genetic mechanisms. Moreover, there exists significant cross-signaling between these survival cascades and are found to affect the prognosis adversely. Effective targeting of these pathways using small molecule inhibitors are ongoing and are supposed to ultimately suppress their erratic activation, induce apoptosis and inhibit leukemic progression. Of the many molecules designed for targeting Ras/Raf/MAPK, PI3K/Akt/mTOR and Jak/STAT pathways, everolimus and sorafenib have showed promising results in Phase II AML clinical trials. Everolimus (RAD001, Afinitor®, Novartis) functions by forming an inhibitory complex with mTOR, impairing cellular protein translation machinery thus exercising anti-proliferative effect. Sorafenib (BAY43-9006, Nexavar®, Bayer), is an established multi-kinase inhibitor capable of blocking pathways and transcription factors implicated in growth and evasion of apoptosis. Moreover, sorafenib is actively pursued as a potent inhibitor of STAT5 cascade, which is also implicated in the persistence of AML phenotype. However, genetic complexity of AML suggests that ablation of a single pathway is unlikely to produce sustained growth inhibition. Hence
a polypharmacological approach with the therapeutic interference of multiple, inter-related small molecule kinase inhibitors (SMI) have been assumed necessary to achieve successful clinical outcome. This combinatorial approach would also prevent possible acquired resistance to SMI. This necessitates simultaneous intracellular delivery of more than one drug at given time, to aid synergistic kinase inhibition mediated anti-leukemic activity. Conversely, simultaneous intracellular delivery seems as a challenge since most of these drugs are hydrophobic molecules having sub-optimal pharmacokinetics, which severely affects their stability and availability within the cell at the same time.

In addition to deregulated pathways, aberrant recruitment of epigenetic factors by numerous oncogenic fusion proteins is also identified to confer differentiation blockade and modulation of self-renewal properties in AML stem/progenitor cells. Well-designed studies in primary leukemia samples, have demonstrated that AML blasts exhibit significantly low histone H3 acetylation (H3Ac) levels at >1000 genomic loci compared with CD34+ progenitor cells, suggesting that a large number of genes are epigenetically silenced by histone deacetylase (HDACs) in AML. Histone deacetylase inhibitors (HDACi) are observed to perform epigenetic reprogramming of AML transcriptome by altering acetylation status of core histones and subsequent modification of the chromatin structure, permitting re-expression of silenced tumor suppressor genes. They are shown to potentially function on a pleiotropic level, tackling the heterogeneity of AML more efficiently by inducing cellular differentiation and inhibition of proliferation and/or apoptosis even in dreaded LSPC. Vorinostat (suberoylanilide hydroxamic acid, SAHA; Zolinza™, Merck) is a strong HDACi approved by FDA for treatment of cutaneous T-cell lymphoma (CTCL), and currently pursued for the AML management. Pooled data from various vorinostat clinical trials, both as single agent and in combination with other agents, have emphasized on its anti-leukemic effect with acceptable safety and tolerability profiles. However, vorinostat exhibit poor aqueous solubility and adverse pharmacokinetic profile, which necessitates administration of high doses for observing appreciable anti-leukemic effects.
Owing to the hydrophobic nature of most of these molecules, development of their parenteral formulations, which ensures high bioavailability, is rendered difficult. With the emergence of nanotechnology, nanocarriers are being investigated as improvised drug delivery platforms, which facilitate delivery of significant amount of anti-cancer agents to specific target site, which can overcome toxicities of solvent-based delivery approaches. They improve the solubility of hydrophobic compounds and render them suitable for parenteral administration. Biodegradable and biocompatible materials are employed for nanosystem fabrication which minimizes the possibilities of hypersensitivity reactions and affords good tissue compatibility. Poly lactic-co-glycolic acid (PLGA) based nanoparticulate system is one of the most successful and interesting colloidal systems. PLGA is well established for sustained release of parenteral drugs owing to their versatile degradation kinetics, established safety, biocompatibility and high encapsulation efficiencies for hydrophobic payloads. Human serum albumin (HSA) is another promising nano-carrier with desirable PK properties due to its endogenous nature. Moreover, HSA nanoformulations itself has received special interest in drug delivery studies due to its inherent ability for passive targeting through enhanced permeation and retention (EPR) effect and active targeting through special albumin receptors on tumor vasculatures. These properties as well as its ready availability, biodegradability, and lack of toxicity and immunogenicity make it an ideal candidate for nanoparticulate drug delivery systems. Abraxane® (nanoparticle albumin-bound (nab)-paclitaxel) exemplified the gloriously successful clinical manifestation of the above points and turned out to be one of the most hugely successful anti-cancer nanomedicine.

However, currently approved drug nanoformulations belong to the ‘single-carrier-single-payload’ category. Nanoconstructs could also be suitably designed to enable delivery of multiple drugs, in sequential or in concurrent fashion. Core-shell constructs could be exploited for efficient delivery of multiple drugs. Nano-construct surface should be appropriately modified to provide stealth properties, to prolong circulation times. Further conjugation with targeting ligands (small molecules,
peptides, antibodies, engineered proteins, or nucleic acid aptamers) would confer advantage of high local concentration at tumor sites. The most promising approach for intracellular delivery of cytotoxic agents or signal-pathway inhibitors is to target cell surface receptors that are preferentially expressed on tumor cells, as in the case of CD33 or CD123 in AML. Nanocarriers targeted to these extracellular receptors are generally, specifically internalized through receptor-mediated endocytosis, which ensures efficient intracellular delivery of therapeutic payloads. This mostly translates in to improving the therapeutic index of anti-cancer drugs, reducing non-specific toxicity to healthy cells ensuring improved therapeutic outcomes and better patient compliance.

1.2. Scope of the thesis
Considering the involvement of aberrant kinome, epigenome and leukemic stem cells in AML, this thesis focuses on the development of nanopolypharmaceuticals that can deliver rationally selected small molecule inhibitors (SMI) as single agents or in combination to address the above leads. In contrast to aggressive chemoregimens which ablate rapidly dividing leukemic and healthy cells, our developed nanomedicines are molecular targeted and hence presents a wider therapeutic index, exerting its activity specifically on clonal leukemic cells. Moreover, these kinome and epigenome targeted therapeutic strategies owing to their molecular specificity can possibly target the leukemic stem cell compartment effectively. Initially, to target the deregulated AML kinome we developed a polymer-protein core-shell nanomedicine to inhibit critically aberrant pro-survival kinases (mTOR, MAPK and STAT5) in primitive (CD34+/CD38−) AML cells. Subsequently, in an attempt to address deregulated histone deacetylation patterns in AML, we next developed a novel, protein-vorinostat nanomedicine and tested its anti-leukemic activity against heterogeneous population of AML patient samples and three representative cell lines. From observed cues of LSPC targeting potential of nano-vorinostat, we tested its specific anti-leukemic effect on CD34+ CD38− CD123+ fraction along with its hemocompatibility and synergism with another epigenetic drug, decitabine.
1.3. Review of literature

1.3.1 Acute Myeloid Leukemia

Hematopoiesis is the process by which all lineages of blood cells are generated in a hierarchical and stepwise manner from hematopoietic stem cells (HSC), which forms the self-renewing compartment, maintaining its quiescence. Hematological malignancies usually result from pathogenic events that disrupt this normal homeostatic balance.

Figure 1.1. Hierarchical model of normal hematopoiesis and human Acute Myeloid Leukemia (a) Self-renewing HSC give rise to multipotent progenitors that in turn give rise to lineage-committed progenitors that eventually produce terminally differentiated blood cells. (b) AML is organized as a hierarchy initiated by self-renewing leukemia stem cells that give rise to leukemic progenitors, which in turn give rise to the leukemia blasts (Image courtesy: Majeti Lab, Stanford School of Medicine, Division of Hematology & Stem cell biology and regenerative medicine)

AML is a malignant neoplasm of hematopoietic system characterized by clonal proliferation of myeloid precursors with an impaired capacity to differentiate into more mature cellular elements (Figure 1.1). Consequently there is an accumulation of immature myeloid precursors, leukemic blasts in the bone marrow, peripheral blood,
and occasionally in other tissues, with variable reduction in the production of normal red blood cells, platelets and mature granulocytes\textsuperscript{4}. The clonal accumulation of blasts often results in pancytopenia, anaemia, bleeding and an increased risk of infection. In most cases the disease involves the peripheral blood but it can involve any organ including the spleen, liver, and lymph nodes\textsuperscript{5-7}. The disease primarily occurs in adults, shows slightly higher frequency in males, and like many cancers, the incidence rises steeply with increasing age. Median age of AML diagnosis is 60 years\textsuperscript{8}. The etiology of leukemia is unknown; however, environmental factors such as ionizing radiation and chemical exposure have been associated with the disease\textsuperscript{9}. Furthermore, clinical observations have identified unusual AML susceptibility in monozygotic twins and in children with certain genetic diseases and congenital disorders\textsuperscript{10} mostly Down syndrome\textsuperscript{11}. However, like other leukemias and solid tumor malignancies, the etiology of AML likely involves an intricate combination of hereditary, genetic as well as environmental factors.

1.3.2. Classification of AML

1.3.2.1. French-American–British (FAB) classification

FAB classification system divides AML into eight subtypes, M0 through to M7, based on the origin cell type its degree of differentiation (Table 1.1.).\textsuperscript{12} This is analyzed using microscopy and/or cytogenetics, to characterize any underlying chromosomal abnormalities. Although more advanced WHO classification may be more useful, the FAB system is still widely used.

1.3.2.2. World Health Organization (WHO) classification

AML is currently classified using the WHO classification system based upon a combination of morphology, immunophenotype, genetics, and clinical features (Table 1.2.).\textsuperscript{13}
Table 1.1. French-American-British (FAB) Classification of AML

<table>
<thead>
<tr>
<th>French-American-British subtype</th>
<th>Incidence (%)</th>
</tr>
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<tbody>
<tr>
<td>M0: Minimally differentiated</td>
<td>5</td>
</tr>
<tr>
<td>M1: Myeloblastic leukemia without maturation</td>
<td>15</td>
</tr>
<tr>
<td>M2: Myeloblastic leukemia with maturation</td>
<td>25</td>
</tr>
<tr>
<td>M3: Hypergranular promyelocytic leukemia</td>
<td>10</td>
</tr>
<tr>
<td>M4: Myelomonocytic leukemia</td>
<td>25</td>
</tr>
<tr>
<td>M5: Monocytic leukemia</td>
<td>5</td>
</tr>
<tr>
<td>M6: Erythroleukemia</td>
<td>5</td>
</tr>
<tr>
<td>M7: Megakaryoblastic leukemia</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 1.2. World Health Organization (WHO) Classification of AML

<table>
<thead>
<tr>
<th>WHO subtypes</th>
<th>Includes:</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML with recurrent genetic abnormalities</td>
<td>AML1-ETO, PML-RARα, CBFβ-MYH11, MLL re-arrangements</td>
<td>11</td>
</tr>
<tr>
<td>AML with multilineage dysplasia</td>
<td>Following a myelodysplastic syndrome or myeloproliferative disorder or not</td>
<td>6</td>
</tr>
<tr>
<td>AML and myelodysplastic syndrome, therapy related</td>
<td>Due to exposure to alkylating agents and topoisomerase inhibitors</td>
<td>2</td>
</tr>
<tr>
<td>AML not otherwise categorized</td>
<td>Modified FAB sub-groups and others</td>
<td>81</td>
</tr>
</tbody>
</table>

It is recommended that patients with AML be classified according to this classification as it attempts to identify biologic entities with the intention that future work will elucidate molecular pathways that might be amenable to targeted therapies. According to FAB classification, diagnosis of acute leukemia is made when immature blasts count are at least 30% of all nucleated marrow elements. WHO
system requires blast counts greater than 20% of all nucleated marrow elements, to render a diagnosis of acute leukemia.\textsuperscript{14}

\subsection*{1.3.3. Current standard treatment for AML}

In the past four decades, clinical research aimed at improving the cure rate in AML has focussed primarily on increasing cytotoxic drug delivery to patients to maximize tumor cell kill.\textsuperscript{15} Both autologous and allogeneic stem cell transplantation, when feasible, have been considered mainly as tools enabling the use of otherwise myeloablative therapies, although the contribution of the graft-versus-leukemia (GVL) effect was recognized as important as early as 1990.

\subsubsection*{1.3.3.1. Induction therapy}

The goal of induction therapy is to rapidly restore normal bone marrow function and to reduce the leukemic cell burden from approximately $10^{12}$ to $10^9$ cells (below cytological detectable levels) to achieve remission. Various acceptable induction regimens are available out of which the standard approach is combination of cell cycle–specific, deoxycytidine analog cytarabine and non–cell-cycle–specific anthracycline antibiotic, such as daunorubicin or idarubicin. Cytarabine is continuously infused for 7 days at 100 mg/m\textsuperscript{2} and daunorubicin is administered as an i.v. push at 45 to 60 mg/m\textsuperscript{2} for 3 days.\textsuperscript{16-17} To improve the CR rate, studies have tested alternative and higher doses of anthracyclines, like idarubicin\textsuperscript{17} or the anthracenedione, mitoxantrone, which inhibit the enzyme topoisomerase IIa,\textsuperscript{18} new agents combined with cytarabine and daunorubicin such as etoposide,\textsuperscript{19} the purine analog fludarabine\textsuperscript{20} or the camptothecin topotecan\textsuperscript{20} or sequential standard therapy followed by high doses of cytarabine\textsuperscript{21} Despite theoretic advantages,\textsuperscript{22} none of these approaches is definitively better than the standard regimen.

\subsubsection*{1.3.3.2. Post-remission consolidation therapy}

Various strategies have been explored to eliminate minimal residual disease not apparent in the bone marrow of patients in complete remission, which could contribute to relapse. Such strategies have included intensive consolidation therapy,
high-dose chemotherapy, or chemo-radiotherapy with either allogeneic or autologous hematopoietic stem-cell transplantation (HSCT).

1.3.3.3. Intensive consolidation chemotherapy

Several studies have evaluated the role of intensive postremission consolidation with high-dose (3 gm/m²/dose) cytarabine (HiDAC). A prospective study by the Cancer and Leukemia Group B (CALGB) demonstrated that 4 courses of HiDAC are significantly better than 4 courses of intermediate- (400 mg/m²/dose) or standard-dose cytarabine (100 mg/m²/dose), confirming a dose response effect in younger patients and a benefit in patients with good-risk cytogenetics.\textsuperscript{23} Cerebellar dysfunction, particularly in older adults and in those with hepatic or renal dysfunction, is an important toxicity.

1.3.3.4. Hematopoietic stem cell transplant

Hematopoietic stem-cell transplantation refers to the administration of very intensive chemotherapy with or without radiation and infusion of previously collected hematopoietic stem cells harvested from either the patient (autologous), or a human leukocyte antigen (HLA)–matched donor (allogeneic). Autologous HSCT is limited by the lack of the immunologic reaction referred to as graft-versus-leukemia (GVL) effect present in patients undergoing allogeneic HSCT, in which the donated allogeneic cells recognize the recipient’s leukemic cells as foreign. Furthermore, there is a theoretic risk of infusion of occult residual leukemic cells.\textsuperscript{24} To decrease toxicities associated with such intensive doses of chemotherapy, lower doses have been explored, relying more on the immunologically mediated GVL effect to eradicate the disease.\textsuperscript{25} A potential limitation is the 3 to 9 months required for the immunologic GVL effect to develop in the presence of rapidly proliferating leukemia cells. Allogeneic HSCT for patients with AML in first CR is associated with the lowest relapse rate and provides the best anti-leukemic potential, but it is associated consistently with a higher risk of treatment-related mortality than either autologous HSCT or consolidation chemotherapy.\textsuperscript{26,27} As a result, the benefit of a lower relapse rate is offset by a higher treatment-related mortality.
1.3.4. Prognosis and genetics of AML

Remission rates with current standard induction chemotherapy range from 50% to 75%.$^{15}$ However, majority of patients relapse and succumb to the disease within 2 years of achieving a remission. Remission rates and overall survival depend on a number of features, including age of the patient, intensity of postremission therapy, biologic characteristics of the disease and most importantly, cytogenetics at presentation.$^{22}$ Prognosis and genetics of AML are tightly linked. A risk stratification method based on cytogenetics divides patients into three main groups - favorable, intermediate, and adverse cytogenetics. Although specific chromosomal aberrations within each group are not entirely consistent among all studies, a general consensus exists (Table 1.3.).$^{28}$

Table 1.3. Cytogenetic risk group and prognosis

<table>
<thead>
<tr>
<th>Risk category</th>
<th>Abnormality</th>
<th>5-year survival (%)</th>
<th>Relapse rates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Favourable</td>
<td>t(8;21), t(15;17), inv(16)</td>
<td>75</td>
<td>33</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Normal, +8, +21, +22, del(7q), del(9q), 11q23 re-arrangements, all other structural or numerical changes</td>
<td>48</td>
<td>50</td>
</tr>
<tr>
<td>Adverse</td>
<td>Complex, -5, -7, del(5q), abnormal 3q</td>
<td>15</td>
<td>78</td>
</tr>
</tbody>
</table>

Age and cytogenetics have a close relationship. Old age is recognized as a risk factor for both major causes of therapeutic failure in AML: treatment related mortality and resistance to therapy.$^{29,30}$ Older individuals tolerate less well aggressive therapies due to poor performance status, presence of comorbid disease, decreased ability of clearance of chemotherapy and poor tolerance of systematic bacterial and fungal infections.$^{31}$ Moreover, elderly patients shows an increased proportion of unfavorable karyotype (abnormalities of chromosomes 5 and 7 or complex chromosomal aberrations),$^{30-31}$ emergence of AML from an antecedent haematological disorder (AHD),$^{31,32}$ presence of dysplastic changes,$^{30,33}$ frequent expression of multidrug resistance (MDR) phenotype$^{32}$ and the involvement of more
primitive progenitors in the leukemic process, all of the above associated with increased resistance to treatment.

1.3.5. Emerging therapeutic strategies in AML

Conventional treatment regimens for AML are mainly based on cytotoxic chemodrugs, which block cell division by inducing DNA damage affecting both leukemic cells and normal cells. Moreover, cytotoxic drugs target proliferating bulk leukemia cells alone, which succeed in reducing the leukemic burden eventually leading to remission. However, these chemodrugs always miss their strike on leukemic stem/progenitor cells (LSPC) which reside in the quiescent G0/G1 phase, contributing to minimal residual disease in AML. These cells thus remain intact even after the most aggressive chemotherapy, and repopulate the leukemic blasts to cause relapse. Thus LSC model has significant implications for the design of therapies for AML, to bring in efficient therapeutic outcomes in AML. Furthermore, current perception about molecular mechanisms that drive leukemogenesis in AML implicates involvement of many derailed survival and proliferation signal transduction pathways and aberrant recruitment of epigenetic modulators like histone deacetylases. Targeting these derailed signalling cascades and epigenetics in AML are believed to significantly improve therapeutic outcomes with less non-specific toxicities.

1.3.5.1 Aberrant AML kinome

Recent molecular investigations have found disruption of normal signalling of JAK/STAT, RAS/Raf/MEK/ERK, and PI3K/AKT pathways in AML occurring as a result of either the mutation of pathway components or alterations in the internal and external (from chemokines, cytokines, or stroma) signals received, contributing to leukemogenesis by perturbing the rates of proliferation, differentiation, and apoptosis. Research has documented that a single genetic abnormality may not be sufficient to promote leukemogenesis. Rather, multiple genetic abnormalities may be necessary for the development of overt leukemia.
1.3.5.1.1. Aberrant activation of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway in AML

The phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signalling axis plays a central role in cell proliferation, growth, and survival under physiological conditions. However, aberrantly activated PI3K/Akt/mTOR signaling has been implicated in acute myelogenous leukemia (AML) and associated with a poor prognosis.\textsuperscript{43,44} Mutations in growth regulatory genes such as FLT3 (Fms-Like Tyrosine kinase 3), Ras, and c-Kit are common in AML patients, resulting in activation of multiple signal transduction pathways which include PI3K/Akt/mTORC1.\textsuperscript{43-47} The pathway is activated when threonine 308 is phosphorylated by PDK1 and serine 473 (thought to be more crucial for full activation) is phosphorylated by PDK2.\textsuperscript{47} Recent articles have revealed effects of PI3K/Akt/mTORC1 signaling activation in HSCs in relation with development of leukemias.\textsuperscript{48-50} Therefore, further identification of specific PI3K/Akt/mTOR substrates and of their roles in the quiescence, proliferation, survival, and differentiation of HSCs and LSCs could provide innovative pharmacological treatments for patients with malignant hematological disorders.

1.3.5.1.2. Aberrant activation of Ras/Raf/MAPK kinase/extracellular signal regulated kinase pathway (Ras/Raf/MEK/ERK) in AML

The Ras/Raf/MAPK kinase/extracellular signal regulated kinase pathway (Ras/Raf/MEK/ERK) pathway is a central signal transduction pathway, which transmits signals from multiple cell surface receptors to transcription factors in the nucleus.\textsuperscript{51} This pathway is frequently referred to as the MAPK pathway which stands for mitogen-activated protein kinase indicating that this pathway can be stimulated by mitogens, cytokines and growth factors. The pathway can be activated by Ras stimulating the membrane translocation of Raf and are also found to interact with PI3K/Akt/mTOR and JAK/STAT pathways.\textsuperscript{52} It has been reported that a high frequency of AML cases (50\%) displays constitutive activation of the Raf/MEK/ERK pathway in absence of any obvious genetic mutation and is associated with poor
outcomes. Activating mutations of FLT3 (FMS like tyrosine kinase-3) kinase internal tandem duplication (ITD) and point mutations (D835) present in 20-30% of AML cases are observed to be accompanied with atypical MAPK signalling. Similarly, elevated expression of ERK in AML is associated with a poor prognosis. It has been demonstrated that conditional activation of this pathway in murine hematopoietic cells results in an AML-like phenotype. Mutations of various genes in the receptor tyrosine kinase (RTK)/RAS-BRAF signal transduction pathway have been detected in therapy related myelodysplasia and AML. In addition, there exist cross-talk between Raf/MEK/ERK and PI3K/AKT resulting in concomitant activation of both pathways in individuals with AML, related to a worse prognosis than those with the activated single pathway. Studies using conditionally active kinase constructs indicate a requirement for the activation of the PI3K pathway during MEK-mediated transformation of certain hematopoietic cells and provide evidence that expression of activated Raf/MEK/ERK and PI3K/AKT pathways could synergize and result in the abrogation of cytokine dependence of hematopoietic cells. These observations suggested that MEK/ERK as well as AKT/mTOR signaling is involved in leukemogenesis and proliferation of leukemia cells, and these prosurvival signal pathways may be promising molecular targets for AML treatment.

1.3.5.1.3. Aberrant activation of Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway in AML

The Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway involves signaling from the cytokine receptor to the nucleus. Constitutive STAT activation occurs in approximately 70% of AML samples and is associated with a low degree of spontaneous apoptosis. In majority of cases, this activation results from either mutations in upstream receptor tyrosine kinases such as FLT3 and c-KIT, or autocrine growth factor production.
Figure 1.2. Signaling cascades that are frequently deregulated in AML which form critical targets for small molecular inhibitors in the treatment in AML.

From a therapeutic perspective, the finding that activation of each of these pathways is both common and affects prognosis makes them highly attractive targets for emerging therapies directed at preventing or reversing aberrant signal transduction. It is to be noted that effects of agents that target upstream activators of these STPs are likely to be blunted by the downstream cross-activation of other members of same pathway, by activated signaling proteins in different pathways. Thus, combinations of agents targeting separate STPs are more beneficial. In summary, leukemia treatment targeting multiple signal transduction pathways may be more efficacious than therapy aimed at inhibition of a single pathway.
1.3.5.2. Small molecule kinase inhibitors for targeting aberrant kinases

The emerging use of molecularly targeted agents, which disrupt specific oncogenic signalling processes, has provided an opportunity to elucidate the molecular basis for differential clinical sensitivity and, possibly, to implement strategies that match individual patients with cancer to specific drug therapies to which they are more likely to respond.66-69

Inhibition of PI3K/Akt/mTOR module in AML: As direct inhibitors of AKT and PI3K inhibitors remain in early development, mTOR inhibitors are by far the most developed class of compounds which target the PI3K/Akt/mTOR pathway in AML.70, 57 Afinitor (Everolimus; RAD001; Novartis) is an orally active derivative of rapamycin that inhibits the serine/threonine kinase, mTOR, approved by FDA for treatment of renal cell carcinoma (RCC).71 Everolimus binds to FKBP12, forming a complex that inhibits mTOR kinase activity, and reduces the activity of the downstream effectors S6 ribosomal protein kinase (S6K1) and eukaryotic elongation factor 4E-binding protein (4EBP).72,73 Evidence of inhibition of mTOR signalling by everolimus was documented in patient samples with clinical responses in clinical trials. mTOR inhibition was also associated with transcriptional down-regulation of D-type cyclins (either cyclin D1 or cyclin D2) and a decrease in Glut1 mRNA levels in a subset of patients, suggesting that, in leukemic cells, everolimus attenuates transcription of these genes.74 However, mTOR inhibition was not observed as clinical response in a small subset of patients. This modest antitumor activity of mTOR inhibitors could be associated with activation of alternative pro-survival signalling pathways, such as MAPK/ERK.75-78

Inhibition of MAPK and STAT5 module in AML: Inhibition of MAPK signaling not only results in growth arrest of leukemia cells but also in activation of the apoptotic pathway through upregulation of pro-apoptotic proteins like Bim,79 and enhanced proteasome mediated destruction of anti-apoptotic Mcl-1.80 Sorafenib (Nexavar; Bayer/Onyx) is an oral multi-kinase small molecule, originally designed as
an inhibitor of Raf-1 kinase targeting the MAPK pathway; it has inhibitory properties against a number of other kinases including FLT3 and vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR) and c-kit.\textsuperscript{81-83} It has been approved by FDA for the treatment of renal cell and hepatocellular carcinoma (HCC).\textsuperscript{84} In preclinical studies, sorafenib induced dephosphorylation of MEK1/2 and ERK and induced apoptosis in AML cells.\textsuperscript{85} Besides, sorafenib is also currently pursued as a potential STAT5 inhibitor after studies showed induction of apoptosis in human leukemia cells, through inactivation of ERK1/2, inhibition of STAT5 and down-regulation of Mcl-1.\textsuperscript{86-89}

**Challenge:** From above literature, it could be hypothesized that combination of mTOR inhibitor, everolimus and the multi-kinase inhibitor, sorafenib may exert its complementary, pharmacologic inhibitory effects along three signaling pathways, efficiently handling the complex cross-talk between them and result in an effective AML therapeutic outcome. However, these two molecules are hydrophobic, which severely affects their bioavailability. Moreover, to exert a combinatorial effect, both the drugs should be within the cell at the same time. Pawaskar et al\textsuperscript{90} demonstrated that after concurrent oral administration of everolimus (1 mg/kg) and sorafenib (20 mg/kg), the PK parameters were significantly different. Similar observations have been derived from human clinical studies too.\textsuperscript{91} This point towards the necessity to develop novel drug delivery systems to achieve desired concentration of both drugs, intracellularly, at the same time.

1.3.6. **Aberrant AML epigenome**

Epigenetics refers to a stable, mitotically perpetuated regulatory mechanism of gene expression without an alteration of the coding sequence, where mechanisms mainly include DNA methylation and histone tail modifications. Among both, the latter is responsible for epigenetic alterations of the acetylation status of histones and play a prominent role in tumorigenesis.\textsuperscript{92} The extent of acetylation and deacetylation on different positions of core histones is determined by the antagonistic activity of histone acetylases (HAT) and histone deacetylases (HDAC) and alters the
nucleosomal conformation of both transformed and non-transformed cells. Deacetylation of histones by HDACs hinders the accessibility of DNA to transcription factors that are involved in determining malignant cell behaviour, thereby changing their activity, subcellular localisation and interaction partners. In addition, acetylation is an important post-translational modulation of a wide range of nuclear and cytoplasmic proteins involved in the regulation of a multitude of cellular functions (e.g., p53, tubulin, heat-shock protein 90). So far, 18 human genes that encode proven or putative HDACs have been identified, which can be classified into four distinct families. Class I, II and IV are zinc-dependent deacetylases, while Class III is NAD$^+$-dependent (Table 1.4).

### Table 1.4. Classification of HDACs

<table>
<thead>
<tr>
<th>Class</th>
<th>Enzyme</th>
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<tbody>
<tr>
<td>I</td>
<td>HDAC1, HDAC2, HDAC3 and HDAC8</td>
</tr>
<tr>
<td>II</td>
<td>IIA: HDAC4, HDAC5, HDAC7 and HDAC9</td>
</tr>
<tr>
<td>III</td>
<td>Sirtuins – SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7</td>
</tr>
<tr>
<td>IV</td>
<td>HDAC11</td>
</tr>
</tbody>
</table>

A disrupted equilibrium between HDACs and HATs, with preponderance of deacetylase activity, leads to transcriptional repression of a diverse set of genes involved in the regulation of cell proliferation, differentiation and apoptosis. Aberrant gene transcription caused by abnormal activity of HDACs is commonly observed in AML, frequently as a direct result of chromosomal translocations (Table 1.5).
**Table 1.5.** Recurring cytogenetic rearrangements in AML associated with aberrant HDAC recruitment

<table>
<thead>
<tr>
<th>Translocation</th>
<th>Fusion protein</th>
<th>Recruited HDAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(8;21)</td>
<td>AML1-ETO</td>
<td>HDAC3</td>
</tr>
<tr>
<td>t(15;17)</td>
<td>PML-RAR</td>
<td>HDAC3</td>
</tr>
<tr>
<td>t(11;17)</td>
<td>PLZF-RAR</td>
<td>HDAC4</td>
</tr>
<tr>
<td>11q23 rearrangements</td>
<td>many</td>
<td>many</td>
</tr>
<tr>
<td>t(8;16)</td>
<td>MOZ/CBP</td>
<td>HDAC6</td>
</tr>
<tr>
<td>inv(8)</td>
<td>MOZ-TIF2</td>
<td>HDAC6</td>
</tr>
</tbody>
</table>

ChIP on microarray studies in primary leukemia samples, have demonstrated that AML blasts exhibit significant alterations in histone H3 acetylation (H3Ac) levels at > 1000 genomic loci compared with CD34+ progenitor cells. Importantly, core promoter regions tended to have lower H3Ac levels in AML compared with progenitor cells, which suggested that a large number of genes are epigenetically silenced in AML.\(^97\)

**1.3.6.1. Inhibition of HDAC activity**

Modulation of protein lysine acetylation through inhibition of histone deacetylases is currently being considered as an attractive new therapeutic strategy.\(^98,99\) Histone deacteylase inhibitors (HDACi) have a diverse array of mechanisms of action, and the complexity of their effects on malignant cells has only begun to be unraveled. As a well-defined model system, acute promyelocytic leukemia (APL) offers an interesting paradigm for studying HDACi action.\(^98\) HDACi have little or no effect on short-term cultures of normal haematopoietic progenitors *in vitro* or on haematopoiesis in normal mice. HDACi are not effective on cells at the pre-leukemic stage, which behave similarly to normal cells. Following transformation, HDACi are able to induce members of the tumour-necrosis factor-related apoptosis inducing ligand (TRAIL) or FAS pathways, leading to tumour-specific cell death. It is noteworthy
that normal cells are relatively resistant to HDACi-induced cell death, whereas a broad variety of transformed cells are sensitive to inhibitor-induced cell death.\textsuperscript{100-103}

1.3.6.1.1. Vorinostat

Vorinostat (suberoylanilide hydroxamic acid; SAHA, Merck), a hydroxamic acid inhibitor of class I and class II HDACs,\textsuperscript{104} was approved by the US FDA for the treatment of recurrent cutaneous T-cell lymphoma.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{vorinostat.png}
\caption{Mode of action of histone deacetylase inhibitor, vorinostat (Image courtesy: http://nuit-blanche.blogspot.in)}
\end{figure}

Vorinostat induces cell cycle arrest and apoptosis in cancer cell lines, improved survival and/or produced anti-tumor effects in rodent models of leukemia and has
demonstrated activity against AML patients.\textsuperscript{104-107} Vorinostat has been shown to induce reactive oxygen species, growth arrest and apoptosis in leukemia cells and leukemia mouse models.\textsuperscript{108} It was shown to downregulate WT1 expression and modify Wnt, FLT3 or NF-κB pathways. The drug is also found to modify the turnover of key oncogenes such as EGFR, RAF1 or ABL through the modulation of HSP90 by HDAC6 and HDAC10.\textsuperscript{102-108} These diverse actions may collaborate to promote cell death in AML cells.

**Challenge:** Although the therapeutic potential of vorinostat is promising, vorinostat is plagued by poor aqueous solubility (0.2 mg/mL) and low permeability (a log partition coefficient of 1.9) as indicated by its Class IV designation in Biopharmaceutics Classification System.\textsuperscript{109} Owing of this, development of a parenteral formulation of vorinostat has been hindered. For instance, in early clinical studies, the intravenous (i.v.) formulations of vorinostat were dissolved in sodium hydroxide, adjusted to pH 11.2, and administered over a 2 h infusion.\textsuperscript{110} Other attempts to develop a parenteral formulation of vorinostat are limited but include a cyclodextrin formulation.\textsuperscript{109} Vorinostat is also beset by suboptimal pharmacokinetics including low bioavailability (F) (43% for humans and 11% for rats), extensive serum clearance, and a short elimination half-life of approximately 2 h in both animal and human studies.\textsuperscript{110-112} Much of the short half-life and limited overall exposure of vorinostat is related to its rapid metabolism, which is its predominate route of elimination.\textsuperscript{111} Therefore, it is of clinical importance to develop novel formulations of vorinostat for parenteral administrations that improve solubility and its overall disposition profile.

1.3.7. **Acute Myeloid Leukemia Stem Cells (AMLSC)**

Similar to normal hematopoiesis, acute myeloid leukemia (AML) encompasses functionally diverse cells, and origination from a leukemic stem cell (LSC) was initially suspected many decades ago (Figure 1.4).\textsuperscript{2,3} The cellular origin of LSC, however, remains unclear, with ongoing controversy as to whether they arise from
transformed hematopoietic stem cells (HSCs) or emerge as a result of genetic events occurring in more mature progenitor cells.\textsuperscript{1-3,113-118}

Figure 1.4. Persistance of chemotherapy resistant, residual LSC population is considered as the main reason for relapse and therapeutic failure in AML (Image courtesy: http://nextbigfuture.com)

Although there is strong evidence to suggest that the origin of AML is the LSC compartment, further definition of the LSC phenotype is needed.
1.3.7.1. Immunophenotype of AMLSC

Studies by Bonnet et al have identified LSCs in human AML as a common immunophenotype (CD34+/CD38−) and demonstrated their self-renewal potential.119 Blair et al demonstrated that only a small number of a defined subset of cells were consistently clonogenic and identified LSCs for human AML as Thy1−.120 The LSCs for human AML were identified as Thy1−, CD34+, and CD38− cells and are the only cells capable of transferring AML from the human patient to NOD/SCID mice. These cells have been referred to as SCID leukemia-initiating cells or SL-ICs. Studies by Hope et al have shown that AML originates from a hierarchy of LSC classes that differ in self-renewal capacity.121 Indeed, early recognition that some AMLs may predominantly involve committed myeloid progenitors have led to efforts targeting underlying LSCs with antibodies recognizing the CD33 (SIGLEC-3) differentiation antigen. This has been exemplified by the development of the immunoconjugate, gentuzumab ozogamicin (GO; Mylotarg).122

The LSC appears to share many of the cell surface markers, such as CD34, CD38, HLA-DR, and CD71, that have previously been identified for hematopoietic stem cells (HSCs). Several groups have reported that surface markers are differentially expressed in the 2 cell populations. In the case of human AML stem cells, a series of studies have defined their immunophenotype as CD34+, CD38−, CD71−, HLA-DR−, CD90+, CD117−, and CD123+. The expression of the last 3 antigens in AML stem cells differs from that of normal HSCs, and this difference therefore provides the means for separating normal HSCs from AML stem cells.123 The lack of c-kit (CD117) expression is a consistent feature of LSCs but not of normal HSCs. The research of Blair et al showed that the difference between AML cells with long-term proliferation and normal progenitors lies in the lack of surface expression of c-kit.124 Perhaps the most promising unique marker of LSCs is interleukin 3 receptor, also known as CD123. This marker was present on 98% of the CD34+CD38− cells of 16 AML patients but was undetectable in normal bone marrow CD34+CD38− cells. Functional assays have confirmed the presence of SL-ICs within the CD34+CD123+ cell population.125 The existence of markers such as CD123 that
are homogeneously expressed on LSCs from a wide range of AML samples points to a potentially conserved mechanism of leukemogenesis in the stem cell compartment.

1.3.8. Nano therapeutic advances for AML therapy

Advances in nanotechnology for drug delivery have resulted in pharmaceutical formulations that effectively combat solid tumors. However, minimal research has been performed to develop innovative therapeutic strategies for AML, aiming to patient compliant and improve therapeutic outcomes (Figure 1.5).

![Figure 1.5. Comparison of chemo- and nano therapy in AML treatment. (a) Leukemia cells originate in the bone marrow and rapidly proliferate to reach the peripheral blood, spleen, liver, lymph nodes, testes, and CNS. Conventional chemotherapeutic agents administered during therapy indiscriminately kill malignant and normal cells, causing deleterious treatment-related side effects. Nanoparticle-mediated targeted delivery of chemotherapeutic agents for leukemia induces selective apoptosis of malignant cells without harming the normal cells. This would reduce treatment-related side effects and enhance the patient compliance during and after therapy (Image courtesy: Ref [127]).]
CPX-351, a liposomal formulation of synergistic 5:1 molar ratio of cytarabine and daunorubicin is the sole nanomedicine that have been developed against AML, which entered the clinical trials. Results from phase IIb trials revealed high response rates and reduction in 60-day mortality in adults receiving standard therapy for secondary AML and presently a Phase III trial is underway in newly diagnosed secondary AML patients.\textsuperscript{127} However, this nanofomulation is not tailored for attending to our identified leads of aberrant kinome or epigenome. Moreover, LSCs residing in the quiescent, G0/G1 would not be affected by CPX-351, since its payloads target rapidly dividing cells alone. Tackling the heterogeneity of this malignancy remains as the biggest challenge. Clinical interventions in AML should preferably be modified so as to ablate both leukemic blasts and stem cells, by addressing the critically derailed kinome and epigenome, without disrupting normal hematopoietic cascades.

1.4. Objectives of the present research work
The major objective of the present thesis work was to develop nanomedicines against aberrantly activated kinases, histone deacetylases and LSC. Our three main objectives were

I. Targeting aberrant kinome using rationally designed core-shell nanosystem carrying multiple drugs

II. Targeting atypical epigenome using protein nanomedicines

III. Addressal and ablation of leukemic stem/progenitor cell population

Accordingly our specific objectives are:

I. Simultaneous inhibition of mTOR, MAPK and STAT5 pathways using polymer-protein core-shell nanomedicine loaded with everolimus and sorafenib

1. Synthesis and characterization of polymer-protein core-shell nanomedicine loaded with multiple small molecule inhibitors to aid synergistic lethality towards AML cells

2. Evaluation of \textit{in vitro} anti-leukemic efficacy in primitive AML cell line (KG1a; CD34\textsuperscript{+} CD38\textsuperscript{−})
3. Conjugation of CD33 monoclonal antibody to nanomedicine and targeted uptake studies to demonstrate targeted functionality of the nanomedicine
4. Elucidation of mechanism of the anti-leukemic activity of the core-shell nanomedicine

II. Development and evaluation of epigenetics targeted protein-vorinostat nanomedicine inducing apoptosis in heterogeneous population of primary Acute Myeloid Leukemia cells including refractory and relapsed cases
1. *In silico* molecular modelling based development and physico-chemical characterization of nano-vorinostat
2. Assessment of nano-vorinostat sensitivity in three AML cell lines and patient samples
3. Mechanistic studies to elucidate the anti-leukemic mechanism of nanomedicine
4. Assessment of effect of nano-vorinostat on the clonogenic growth potential of leukemic bone marrow derived cells and normal hematopoietic progenitors isolated from AML patients and healthy individuals, respectively.

III. Evaluation of anti-leukemic stem cell effect of nano-vorinostat, its hemocompatibility and synergism with DNMT inhibitor, decitabine
1. Assessment of anti-LSPC effect of nano-vorinostat on primitive CD34+ CD38- CD123+ cell specific fraction
2. Analysis of hemocompatibility of nano-vorinostat by a battery of studies - hemolysis assay, coagulation tests, lymphocyte proliferation and immunosuppression studies
3. Investigating possible synergism with DNA methyltransferase inhibitor, decitabine
1.5. References


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