Chapter 3

Protein nanomedicine targeted to aberrant AML epigenome
3.1. Introduction

Emerging data support the perception that recruitment of aberrant HDAC activity by oncogenic fusion proteins, resulting from chromosomal translocations, contributes to silencing of vital genes in hematopoiesis and promotion of acute myeloid leukemia.\(^1\)\(^-\)\(^4\) 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 histone (H3) acetylation levels in AML compared with progenitor cells, which suggested that a large number of genes are epigenetically silenced in AML.\(^5\) Pharmacologic reversal of atypical epigenetic silencing in AML is assumed to restore normal bone marrow function leading to enhanced clinical response.

Vorinostat (suberoylanilide hydroxamic acid; SAHA, Merck), is a potent pan-HDACi shown to inhibit the enzymatic activity of Class I (HDAC1, HDAC2 and HDAC3) and Class II (HDAC6) HDACs at nanomolar concentrations (IC\(_{50}\) < 86 nm) and induce cytotoxicity at concentrations ranging to 10 µM.\(^6\)-\(^8\) Vorinostat induces cell cycle arrest and apoptosis in cancer cell lines, improved survival and/or produced antitumor effects in rodent models of leukemia and has demonstrated activity against AML patients.\(^8\)-\(^\text{12}\) Pooled data from vorinostat clinical trial programs have emphasized on its anti-leukemic effect with acceptable safety and tolerability profiles.\(^\text{13},\text{14}\) An important attribute of HDACi is that they induce cancer cell death at concentrations to which normal cells are relatively resistant, possibly due to potentially large differences in the acetylome of normal versus tumour cells, making them well suited for leukemia therapy.\(^\text{15,16}\)

Although the drug presents excellent therapeutic potential, vorinostat faces shortcomings of 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, thus hindering development of i.v. formulations of the same.\(^\text{17}\) Vorinostat also possess sub-optimal pharmacokinetics
including low bioavailability (43% for humans), extensive serum clearance and a short elimination half-life of approximately 2 h in both animal and human studies\textsuperscript{17-20}. Owing to these characteristics, high doses of vorinostat (500 mg thrice daily) is required to be administered to achieve desirable anti-leukemic effects.\textsuperscript{13} Therefore, it is of remedial importance to develop novel formulations of vorinostat for parenteral administrations that improve solubility and the overall disposition profile of vorinostat.

Mostly, hydrophobic anti-cancer agents rely on solvent-based (e.g., Cremophor EL) delivery vehicles for i.v. administration, which are associated with serious and dose-limiting toxicities.\textsuperscript{21} Improvised drug delivery platforms utilizing human serum protein, albumin, have facilitated delivery of significant amount of drug to target site while avoiding toxicities of solvent-based formulations.\textsuperscript{22-27} Albumin-bound paclitaxel (130 nm nab\textsuperscript{TM}-paclitaxel; Abraxane\textsuperscript{®}) exemplified the first FDA approved, clinically successful albumin based nanomedicine intended for IV administration, for treatment of metastatic breast cancer, currently under clinical trials for numerous other solid tumors.\textsuperscript{23} Endogenous proteins like albumin possess advantages of offering stealth aiding evasion from RES to prolong systemic circulation times.\textsuperscript{22} In addition, hydrophobicity, neutral charge and significant protein binding efficiency (\textasciitilde71\%) of vorinostat is assumed to aid reversible and non-covalent binding of the drugs to distinct hydrophobic pockets, formed by the lipophilic amino acid side chains of the polypeptide backbone, for efficient cellular uptake and release.

Considering the above points, we report the development of a human serum albumin bound vorinostat nanomedicine and its excellent anti-leukemic activity towards primary leukemic cells derived from a heterogeneous set of nine AML patients, including refractory and relapsed cases and three AML cell lines representing different FAB classes. Interestingly, irrespective of clinical characteristics, all patient samples showed promising sensitivity towards nano-vorinostat.
3.2. Research questions and Hypotheses

1. **Qn:** Can we improve drug efficacy by introducing a nanoformulation of vorinostat?

2. **Qn:** Which material system may provide high loading efficiency for vorinostat?

**Hypothesis:** An albumin bound vorinostat nanoformulation may increase its anti-leukemic efficacy by improving its aqueous solubility and overall disposition.

3.3. Materials and Methods

3.3.1. In silico docking and synthesis of nano-vorinostat

Vorinostat was obtained as from Selleck chemicals (USA). 10 mg/mL vorinostat stock (37.83 mM) was prepared in dimethyl sulfoxide and aliquots were stored at −80 °C. Prior to wet-chemical synthesis, ligand protein docking simulations were performed using Auto Dock 4.2 to understand the possible chemical interactions and associated binding energy between vorinostat and human serum albumin. Nano-vorinostat was prepared employing a modified coacervation method previously reported by our group. In 39.6 µL of vorinostat is added into 3 mL of 1 mg/mL HSA in order to get a final concentration of 500 µM. This mixture is kept for overnight stirring at 37°C to aid interaction between the drug and HSA. 3.2 mL of absolute ethanol was added to the above mixture until a colloidal solution of HSA-vorinostat nano-coacervate is obtained. The solution was kept for overnight evaporation of ethanol. Thereafter, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was added to the solution in order to cross-link the protein matrix, thereby stabilizing nano-vorinostat. The suspension was kept under stirring in dark at room temperature for about 2-4 h. The nanoformulation was then subjected to dialysis using 3 mL Slide-A-Lyzer Dialysis Cassette (MWCO: 10 kDa; Thermo Scientific, Rockford, IL, USA), to remove unbound drug and EDC. The cassette containing nano-vorinostat was immersed in a beaker containing PBS (0.1 M, pH 7.4) containing 0.2% Tween 20, for 1 h at 37°C. The buffer in the beaker was maintained under stirring. The final suspension was stored at 4°C or used for cell culture studies.
3.3.2. Physico-chemical characterization of nano-vorinostat
Particle size analysis of nano-vorinostat was determined using Dynamic Light Scattering technique (DLS; Particle Sizing Systems – NICOMP 380 ZLS Particle Size Analyzer, Port Richey, Florida, USA) and Scanning Electron Microscope (SEM; JEOL JSM-6490LA, Akishima, Tokyo, Japan). Drug loading efficiency and drug content was determined by UV-Visible spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Kyoto, Japan). The percentage entrapment of the drug was determined from standard graph of vorinostat prepared in DMSO (λ_{max} vorinostat = 241 nm). Calibration curves were linear in the ranges of standard concentrations measured. Drug content and percentage encapsulation efficiency (% EE) was calculated using the formula:

\[
\text{Drug content} = \left( \frac{\text{Amount of drug in nanoparticles}}{\text{Weight of the nanoparticles}} \right) \times 100
\]

\[
\text{Encapsulation efficiency} = \left( \frac{\text{Residual amount of drug in nanoparticles}}{\text{Feeding amount of drug}} \right) \times 100
\]

3.3.3. Drug release profile of nano-vorinostat
Drug release experiment was performed using dialysis membrane technique. 5 mg of freeze dried nano-vorinostat resuspended in 5 ml of phosphate buffer saline (PBS 0.1 M, pH 7.4) was taken in a dialysis tubing cellulose membrane (MWCO: 10 kDa, Sigma-Aldrich, USA) which was then introduced into a beaker containing 50 mL PBS. At specific time points, 2 mL of PBS was withdrawn for analysis which was replaced with fresh PBS. The concentration of the released vorinostat was quantified using UV-Visible spectrophotometer at absorbance of 241 nm.

3.3.3. In vitro studies
Human primitive AML cell line, KG-1a and human myeloblastic cell line, HL-60 were purchased from National Centre for Cell Sciences (NCCS), Pune, India. MV4-11 was
procured from American Type Culture Collection (ATCC), Manassas, USA. All cell lines were cultured and maintained in Iscove’s Modified Dulbecco’s Medium supplemented with 20% Fetal Bovine Serum, 2 mM L-glutamine, 50 IU/mL penicillin and 50 µg/mL streptomycin. Peripheral blood/bone marrow samples were obtained from AML patients and healthy donors and mononuclear cells were isolated from the samples. AML patient samples and healthy human bone marrow samples were obtained from volunteers after informed, written consent and approval by the Institutional Ethical Committee (IEC), at Amrita Institute of Medical Sciences and Research Centre, Kochi, Kerala, India. Mononuclear cells were isolated from the above samples using density gradient centrifugation technique and were cultured in serum free StemSpan™ H3000 medium supplemented with StemSpan™ CC100 cytokine cocktail and used for subsequent experiments. All cells were maintained at 37°C, at 95% relative humidity and 5% CO₂.

3.3.4. Characterization of AML cell lines
3.3.4.1. Immunophenotyping of KG-1a
For phenotyping, 1×10⁵ KG-1a cells were incubated with 5 µL of FITC conjugated CD34 and APC conjugated CD38 monoclonal antibodies in a single tube along with the respective isotype controls for 30 minutes at room temperature in dark. Later, cells were washed and resuspended in PBS for flow cytometry analysis wherein fluorescence was detected using 488 nm and 633 nm excitation filters for FITC and APC, respectively. Cells were imaged using confocal microscope (Leica TCS SP5 II, Leica Microsystems CMS GmbH, Mannheim, Germany) with 543 nm He–Ne laser.

3.3.4.2. Detection of FLT3 ITD mutation in MV4-11 by polymerase chain eaction (PCR)
High molecular weight genomic DNA was isolated from 5×10⁶ MV4-11 cells, using a DNA extraction kit (QIAamp DNA Mini Kit, QIAGEN, Netherlands) according to manufacturer’s protocol. DNA yield and purity was determined by measuring absorbance at 260/280 nm using NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Quality of genomic DNA was checked with 1%
Agarose Gel Electrophoresis. MV4-11 DNA was analyzed for mutations in exons 14 and 15 of Flt3 gene using relevant primers which were designed using PRIMER 3 software and procured from BioServe primers (Invitrogen BioServices India Pvt. Ltd, Bangalore, India). Briefly, PCR conditions performed in a 60 μL reaction set-up contained genomic DNA (50 to 100 ng), PCR Master Mix (Taq PCR Master Mix Kit, QIAGEN, Limburg, Netherlands) and 30 pmol of each oligonucleotides. Reaction was carried out in a thermal cycler (G-Storm GS4, Somerset, TA117JH, UK) programmed at initial denaturation at 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, 58°C for 45 seconds, 72°C for 30 seconds, and a final step for extension at 72°C for 10 minutes. Amplified products were visualized on 2% agarose gel with 100 bp DNA ladder (GeneRuler 100 bp DNA Ladder, Thermo Scientific, Illinois, USA) to view the 297 bp product (FLT3 wild-type) using gel documentation system (Universal Hood II Gel Imager, Bio-Rad, California, USA).

3.3.4.3. Cytomorphological staining of HL-60

Narrow film of HL-60 cell suspension was prepared by smearing it on a clean glass slide, which was then air dried. Smear fixing was performed in absolute methanol for 10 minutes after which it was immersed in coupling jar containing May-Grunwald's stain (SD Fine Chemicals, Mumbai, India) in phosphate buffer (pH 6.8). The slides were then transferred to another jar containing Geimsa stain (NICE Chemicals Pvt Ltd Kochi, Kerala, India) freshly diluted in phosphate buffer and kept for 30 minutes, washed thrice with distilled water and air-dried.

3.3.5. Intracellular uptake studies

To study intracellular uptake using confocal microscope, albumin component of the nanomedicine was made fluorescent by doping it with atomic clusters of gold (Au$_{28}$) which emits intense red fluorescence. Typically fluorescent Au clusters are stabilized by albumin and hence it is easy to form albumin nanoparticles with Au clusters in it. For uptake study, 5 × 10$^5$ KG1a cells were incubated with 0.5 mg/mL of gold doped nanomedicine for 2 h. Later, cells were washed, resuspended in PBS and uptake was analyzed using confocal microscopy (543 nm He–Ne laser).
3.3.6. Cytotoxicity studies
2×10^4 KG-1a, HL-60, MV4-11, BMMC or patient sample derived cells were treated with 0.1, 0.5, 1, 2.5 and 5 µM free and nano-vorinostat for 72 h. Untreated cells were used as control. All concentrations were used in triplicates. Cell viability was assessed after 72 h using MTT assay. All experiments were performed in triplicates.

3.3.7. Isolation of nuclear extract and estimation of HDAC activity
5×10^5 MV4-11 or BMMC were treated with 1, 2.5 and 5 µM free and nano-vorinostat for 72 h. Untreated cells served as the control. Cells were washed in PBS and nuclear extract was isolated. Inhibition of HDAC activity was analyzed using colorimetric HDAC Activity Assay Kit (BioVision Inc. Milpitas, California, USA) according to manufacturers’ protocol.

3.3.8. Detection of intracellular ROS and mitochondrial superoxide
In order to determine the role of ROS generation in cytotoxicity, intracellular ROS generation was measured using an oxidation sensitive dye 2,7-dichlorofluorescein diacetate (DCFH-DA; Invitrogen, CA, USA). The oxidation product of DCFH-DA has excitation/emission maxima of 495 nm/529 nm enabling detection using flow cytometry and confocal laser scanning microscopy. Typically 5×10^5 HUVECs and KB cells after 24 h of exposure to 2.5 µM free and nano-vorinostat were re-suspended in phenol red-free culture medium containing 5 mM of DCFH-DA for 30 min and intracellular ROS generation was evaluated using flow cytometry.

3.3.9. Cell cycle analysis
MV4-11 cells at 5×10^5 cells/mL were treated with 2.5 µM free and nano-vorinostat for 36 and 72 h. Following drug exposure, samples were rinsed with PBS and fixed in 70% ethanol and kept overnight at −20°C. Cells were subsequently prepared for analysis by further washes with 1% FBS containing cold PBS followed by staining with 50 µg/mL propidium iodide (PI; Molecular Probes, Invitrogen, USA) and 100 µg/mL RNase A solution (Sigma-Aldrich, USA) for 15 minutes at 37°C in the dark followed by incubation at 4°C until analysis. Samples were then analyzed using flow cytometry.
3.3.10. Apoptosis assay

$5 \times 10^5$ cells were treated with 2.5 µM free and nano-vorinostat for 72 h. Untreated cells served as control. Apoptosis assay was performed using Annexin V/PI Apoptosis Detection Kit (BD Pharmingen, San Jose, California, USA) according to manufacturers’ protocol. Cells were analyzed by flow cytometry, measuring the fluorescence at 530 and 575 nm. Apoptotic cells were imaged using confocal microscopy (He–Ne and Ar lasers).

3.3.11. Patient peripheral blood/bone marrow cell isolation and immunophenotyping

Peripheral blood/bone marrow derived mononuclear cells were isolated by Ficoll gradient centrifugation and aliquots were cryopreserved for later use. Single-cell suspensions were washed with PBS containing 2% FBS and stained with APC CD3, FITC CD7, PE CD13, APC CD19, APC CD33, PE CD34, PerCP CD45, APC CD117, APC HLA-DR and FITC MPO monoclonal antibodies (BD Biosciences, USA) in different tubes, considering appropriate fluorochrome combinations, for 30 minutes. Cells were then washed and resuspended in 300 µL of PBS and analyzed using flow cytometry.

3.3.12. Clonogenic proliferation assays

$5 \times 10^6$ patient sample derived cells were seeded and treated with 2.5 µM free and nano-vorinostat for 72 h. After incubation, treated cells were rinsed in PBS, resuspended in 1 mL StemSpan H3000 and required microlitres of cell suspension (to obtain $10^4$ cells) was diluted into a final volume of 400 µL StemSpan H3000, and added to 4 mL of MethoCult H4035 Optimum without EPO (STEMCELL Technologies, Canada), plated in triplicate, and incubated at 37°C and 5% CO$_2$ in a humidified environment for 14 days prior to scoring colonies. Colonies with $\geq 50$ cells were enumerated.

3.4. Results and Discussion
3.4.1. *In silico* docking, synthesis and physico-chemical characterization of nano-vorinostat

With the aim of developing a nanoformulation for vorinostat, we have employed *in silico* docking simulations prior to wet chemical synthesis of human serum albumin (HSA) bound vorinostat nanomedicine, hereafter termed as nano-vorinostat. Ligand-protein docking simulations (Auto Dock 4.2) were performed to investigate possible chemical interactions and associated binding energy between vorinostat and HSA.

*Figure 3.1. In silico molecular modeling of interactions between human serum albumin (HSA) and vorinostat*

Docking vorinostat with Pocket II A of A-chain of HSA, one of the most promising and experimentally determined drug binding pockets\(^\text{28}\) indicated that the drug could make strong hydrophobic interactions with Ser 192, Tyr 150, Lys 199, Leu 238 and Leu 219 residues in albumin (Figure 3.1). A single molecule of albumin was found to hold at least 5 molecules of vorinostat with an average binding energy of -6 kcal/mol. The interaction was further strengthened by the formation of hydrogen bonds by vorinostat with Ser 192, Ala 291 and Arg 222 residues of HSA. In addition, the presence of Vander Waal’s forces and intermolecular interactions also strongly contribute towards the stable interaction of vorinostat with albumin residues. Figure 3.2. illustrates the synthesis steps involved in the preparation of nano-vorinostat.
Coacervation technique was implemented for nano-vorinostat synthesis. Ethanol served as the desolvating agent, which when introduced into human serum albumin containing vorinostat, yielded protein-vorinostat coacervates. These coacervates were subsequently subjected to EDC mediated crosslinking to further reinforce drug entrapment and avoid premature drug release.

**Figure 3.2. In silico molecular modeling of interactions between human serum albumin (HSA) and vorinostat**

**Figure 3.3.** (A) SEM image showing spherical particles of ~100 nm. Inset: DLS data showing hydrodynamic diameter of ~94±8 nm (B) Photograph of nano-vorinostat exhibiting excellent colloidal stability (C) Encapsulation efficiency, drug content, and zeta potential of nano-vorinostat.
SEM image of nano-vorinostat (Figure 3.3. A) indicated formation of well dispersed spherical particles measuring ~ 100 nm in size, which was in line with the DLS measurement showing mean hydrodynamic diameter of ~91 ± 14 nm (Figure 3.3. A, inset). Figure 3.3. B shows the photograph of nano-vorinostat colloidal solution. Unbound free drug was removed from nano-vorinostat suspension by dialysis, and UV–Vis absorption studies of the washed precipitate indicated relatively high loading efficiency of 72.4% and drug content of 10.77 w/w. The nanoformulation recorded an average zeta potential of -31.12 mV, suggesting colloidal stability in aqueous medium (Figure 3.3. C). Drug release studies performed at physiological pH showed that ~85% of encapsulated vorinostat was released from protein nanoparticles within 72 h, which reached almost 100% by 168 h (Figure 3.4).

![Graph](image1.png)

**Figure 3.4.** *UV-VIS data showing drug release profile of nano-vorinostat at pH 7.4*

It was observed that the drug was released in a sustained fashion, without any significant burst release.

**3.4.2. In vitro cytotoxicity studies and functional assays in AML cell lines**

With the aim of investigating single agent anti-leukemic activity of nano-vorinostat in patient samples, initially, we have tested the same in three AML cell lines, KG-1a, HL-60 and MV4-11, representing three different FAB classes, namely FAB M0, M3 and
M5. Prior to the experiments, the cell lines were characterized for their molecular or morphological characteristics (Figure 3.5. A-C). KG-1a cells derived from the parent KG1 cell line (established from erythroleukemia patient evolving to AML, between passages 15 and 35) are morphologically, cytochemically, immunologically, and functionally less mature than the latter and belongs to the AML FAB M0 class\(^{29}\). KG-1a, owing to its primitive disposition, by virtue of expression of hematopoietic stem cell markers (CD34\(^+\) CD38\(^-\)), is actively pursued as an appropriate cell model for leukemic stem cell research\(^{29}\).

**Figure 3.5.** Molecular characterization of AML cell lines. (A) Confocal image of CD34\(^+\) CD38\(^-\) fraction of KG-1a (C) Optical micrograph of HL-60 cells exhibiting cytological staining pattern of promyelocytes (D) Agarose gel electrophoresis of MV4-11 PCR product showing band specific to FLT3-ITD

Immunophenotyping previously done by us had revealed that ~76% cells exhibited CD34\(^+\) CD38\(^-\) stem cell phenotype\(^{27}\). Figure 3.5. A shows the confocal microscopic image wherein most of KG-1a cells are stained positive for CD34 alone (CD38\(-\)), indicating immature, stem cell-like population and a few cells dual stained for CD34 and CD38, representing relatively differentiated population. HL-60 cell line originally established from an acute promyelocytic leukemic patient represents AML FAB M2 with maturation\(^{30}\). Consistent to its origin, our cytological studies showed HL-60 to possess myeloblastic or promyelocytic nature. Figure 3.5. B shows optical micrograph of HL-60 cells, exhibiting cytological staining pattern indicative of promyelocytic nature, with pale staining areas in the nucleus, basophilic cytoplasm and primary granules. MV4-11 cell line derived from patient with acute myelomonocytic leukemia
(AML FAB M5) express a typical FLT3-ITD (internal tandem duplication) mutation consisting of a 30-bp insertion at nucleotide 1857 and further harbours a t(4;11)(q21;q23) MLL translocation, representing mixed lineage or biphenotypic leukemia having poor prognosis.\textsuperscript{31,32} The presence of FLT3–ITD mutation in AML patients significantly correlates with an aberrant proliferative capability resulting from constitutive phosphorylation of anti-apoptotic proteins, increased risk of relapse and dismal overall survival (< 6 months).\textsuperscript{33} Figure 3.5. C shows agarose gel electrophoresis of MV4-11 PCR product showing band specific to FLT3-ITD mutation, which represents worse prognosis scenario.

\textbf{Figure 3.6.} Confocal image of gold doped nano-vorinostat treated KG-1a showing internalized nanoparticles (6 h).

We have studied intracellular uptake of nano-vorinostat in these cell lines and a representative confocal image (Figure 3.6) shows > 90% KG-1a cells showing internalized nanoparticles. For this imaging, nanoparticles were doped with fluorescent Au clusters in albumin matrix as reported earlier.\textsuperscript{24} Further, cytotoxicity caused by free and nano-vorinostat was estimated in all the above cell lines (Figure 3.7. A-C) and also in bone marrow derived mononuclear cells (BMMC) from healthy individuals (Figure 3.8 A).
The tested concentrations of free and nano-vorinostat showed dose dependant progressive loss of cell viability in all of these cell lines. However, nano-vorinostat showed enhanced cytotoxicity over the free drug with 2.5 µM nano-vorinostat invariably inducing > 80% cytotoxicity towards all three cell lines, which was increased to ~100% at 5 µM (Figure 3.7. A-C). This indicates the efficacy of nano-vorinostat towards different FAB classes of AML, irrespective of their primitiveness, differentiation or mutational status.

Interestingly, both free drug and nano-vorinostat hardly induced any cytotoxicity towards bone marrow derived mononuclear cells (BMMC) from healthy...
individuals. This was further substantiated by employing methylcellulose based clonal progenitor assay capable of supporting myeloid colony formation.

**Figure 3.8.** (A) Nano-vorinostat sensitivity towards healthy BMMC. CFU assay showing formation of colonies in (B) control and (C) 5 µM nano-vorinostat treated healthy bone marrow cells.

Treatment with maximum test concentration of 5 µM nano-vorinostat was found to have no adverse effects on clonogenic growth pattern of healthy BMMC (Figure 3.8. C), suggesting less toxicity of vorinostat to healthy stem/progenitor cells. We also quantified level of HDAC activity in the three AML cell lines and BMMC, which indicated that cell lines showed ~4-5 fold increase in HDAC activity compared to normal bone marrow cells (Figure 3.9). The differential toxicity of the drug towards the AML cell lines could be due to their inherent high HDAC activity (Figure 3.9). It is believed that differences in the acetylome and expression levels of thioredoxin (TXN; natural ROS scavenger) in tumor and normal cells may also contribute to the relative
resistance of the latter to HDACi induced cytotoxicity. Normal cells accumulate thioredoxin after treatment with HDACi. Therefore, ROS generated in normal cells gets neutralized by TXN, whereas transformed cells lacking TXN, succumbs to oxidative stress. Moreover, normal cells, possessing intact survival mechanisms, are able to recover from HDAC inhibition. However, cancer cells having multiple, collective defects in proliferation, differentiation and senescence cascades, may succumb to HDAC inhibition.

**Figure 3.9.** *Data showing high HDAC activity in AML cells compared to healthy BMMC*

Anti-cancer effects of HDAC inhibition by vorinostat have been associated with a multitude of mechanisms including intracellular generation of ROS and cell-cycle arrest, which commits neoplastic cells to apoptosis. We next proceeded to evaluate the mechanism of action of nano-vorinostat by studying HDAC inhibition, intracellular ROS generation, cell cycle arrest and apoptosis in MV4-11, cell line harbouring MLL translocation and FLT3-ITD mutation, representing FAB M5 class and showing dismal prognosis. Our HDAC inhibition assay revealed enhanced activity of nano-vorinostat over its free drug counterpart. More than 90% HDAC inhibition was registered by 1 µM nano-vorinostat compared to ~ 65% by free drug. Concentrations above 1 µM registered almost complete inhibition as seen from Figure 3.10.
Previous studies have reported that intracellular ROS and oxidative injury is one of the prime mechanisms of HDACi-induced apoptosis. Our results also showed that the nanoformulation succeeded in producing high levels of intracellular ROS than free vorinostat. Nano-vorinostat has shown to inhibit HDAC more effectively than free drug, probably due to the enhanced cellular uptake of albumin-bound drug, which retained its chemical stability as well as activity at the molecular level. Inhibition of HDACs by vorinostat occurs through direct interaction of drug with the catalytic site of enzyme, leading to a state of histone hyperacetylation, which assists in chromatin remodelling to permit expression of repressed genes.\textsuperscript{8}

Earlier studies have reported that intracellular ROS and oxidative injury is one of the prime mechanisms of HDACi-induced apoptosis.\textsuperscript{6,8,11} ROS generation capability of free and nano-vorinostat was assessed in MV4-11 as shown in Figure 3.11. Flow cytogram showed that ~ 93\% of cells treated with 2.5\,\mu M nano-vorinostat showed positive staining for ROS compared to ~78\% cells treated with free vorinostat, indicating enhanced activity of nanoformulation. This was confirmed by confocal microscopic image where MV4-11 cells treated with 2.5 \,\mu M nano-vorinostat showed

**Figure 3.10.** Data showing high HDAC activity in AML cells compared to healthy BMMC

![Graph showing HDAC activity](chart.png)
bright green fluorescence of oxidized DCF, corresponding to high levels of ROS in the cytosol.

**Figure 3.11.** Flow cytogram showing enhanced intracellular ROS generation in nano-vorinostat treated cells. Confocal image showing bright green fluorescence of oxidized DCF in cytosol of nano-vorinostat treated cells

An almost universal effect of HDACi is cell cycle arrest, due to the upregulation of cell cycle genes like CDKN1A (encodes the production of p21 cyclin-dependent kinase inhibitor). Cell cycle phase distributions induced by 2.5 µM free and nano-vorinostat is shown in Figure 3.12. The results revealed that MV4-11 cells after 36 h showed significant decrease in G0/G1 phase content (24.6%) and increase in G2/M (35.6%), compared to untreated control and free vorinostat, suggestive of cell cycle arrest at G2/M phase. Similarly, free vorinostat also registered decreased G0/G1 content (32%) and G2/M arrest (35.6%) with cells trailing to sub-G1 phase. However, considering cell count into account; nanoformulation showed more pronounced effects on cell cycle progression. At 72 h, nano-vorinostat treatment increased sub-G1 fraction to 69.1% compared to its free drug counterpart showing 55.6% cells.
Figure 3.12. Flow cytogram showing induction of G2/M arrest by nano-vorinostat treatment within 36 h, followed by accumulation of cells in the sub-G1 phase by 72 h.

Significant accumulation of cells in sub-G1 phase is indicative of apoptotic mode of cell death, which was verified using Annexin V/PI based apoptosis assay. After 72 h, 2.5 µM free vorinostat treatment showed 72.3% cells in early stage apoptosis, 13.2% in late stage apoptosis and 0.4% in necrosis stage, leaving 14.1% cells in the live quadrant. In contrast, same concentration of nano-vorinostat showed 88.5% cells in early apoptosis stage, 9.2% in late apoptosis, 0.2% in necrosis, leaving 2.1% cells alone in the live quadrant indicating higher apoptosis rates compared to free vorinostat (Figure 3.13). This was further confirmed using confocal microscopy which clearly showed both early and late apoptotic cell fractions in nano-vorinostat treated cells. We speculate that the combined effect of enhanced HDAC inhibition, generation of high intracellular levels of ROS and cell cycle arrest, inflicted by nano-vorinostat treatment, proved traumatic enough to trigger apoptosis in AML cell lines.
Collectively, all functional assays pointed towards the efficacy of nano-vorinostat, which exhibited enhanced cytotoxicity towards the representative cell line, compared to its free drug counterpart which, probably could be due to the enhanced cellular uptake of albumin-bound drug, which retained its chemical stability as well as activity at the molecular level to exert its effects more efficiently.

3.4.3. Nano-vorinostat sensitivity in patient sample derived leukemic cells
The clinical validity and relevance of the observed anti-leukemic effect of nano-vorinostat was further evaluated in primary leukemic cells isolated from peripheral blood/bone marrow samples of AML patients (n=9). FAB class, blast percentage, expression levels of CD33 and CD34 and mutation/cytogenetic abnormalities were taken into account for analyzing their respective roles in nano-vorinostat sensitivity.
Clinical characteristics of the patients at the time of sampling are summarized in Table 3.1.

<table>
<thead>
<tr>
<th>Patient sample #</th>
<th>Age/Gender</th>
<th>Sample</th>
<th>Diagnosis with FAB class</th>
<th>Blasts* (%)</th>
<th>Translocations/Molecular markers/Cytogenetics*</th>
<th>Clinical status at the time of sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57/M</td>
<td>PB</td>
<td>AML with antecedent polycythemia vera</td>
<td>88.2</td>
<td>JAK2-Val1177F/6, XY</td>
<td>Induction chemotherapy</td>
</tr>
<tr>
<td>2</td>
<td>66/F</td>
<td>PB</td>
<td>M2 AML</td>
<td>82</td>
<td>None detected/45, XX, del(9)(p)</td>
<td>On low dose cytarabine due to co-morbidities</td>
</tr>
<tr>
<td>3</td>
<td>27/M</td>
<td>PB</td>
<td>Biphenotypic AML (AML with T-lymphoid markers)</td>
<td>58.9</td>
<td>None detected/46, XY</td>
<td>Untreated</td>
</tr>
<tr>
<td>4</td>
<td>31/M</td>
<td>PB</td>
<td>M2 AML showing aberrant CD19 expression</td>
<td>09.1</td>
<td>None detected/46, XY</td>
<td>Refractory to induction</td>
</tr>
<tr>
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<td>11/M</td>
<td>PB</td>
<td>M2 AML</td>
<td>81.1</td>
<td>None detected/na*</td>
<td>First relapse</td>
</tr>
<tr>
<td>6</td>
<td>49/F</td>
<td>PB</td>
<td>M1 AML</td>
<td>96.7</td>
<td>None detected/46, XX</td>
<td>Refractory to induction</td>
</tr>
<tr>
<td>7</td>
<td>60/F</td>
<td>BM</td>
<td>M4 AML</td>
<td>50</td>
<td>t(4;11)/(46, XX</td>
<td>On chemotherapy with decitabine</td>
</tr>
<tr>
<td>8</td>
<td>61/F</td>
<td>BM</td>
<td>M1 AML</td>
<td>33.1</td>
<td>None detected/46,XX</td>
<td>On chemotherapy with decitabine</td>
</tr>
<tr>
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<td>54/M</td>
<td>DM</td>
<td>M5 AML</td>
<td>80.4</td>
<td>t(4;11)/(46, XY</td>
<td>Refractory to induction</td>
</tr>
</tbody>
</table>

**Table 3.1. Clinical characteristics of the patients at the time of sampling**

These patients included three refractory and one relapsed cases. All obtained patient samples belonged to either granulocytic (M0-M4) or monocytic (M5) lineages. Test concentrations (0.1-5 µM) of free and nano-vorinostat were selected from initial experience with leukemic cell lines. Figure 3.14 shows nano-vorinostat sensitivity profile of primary leukemic cells from these patient samples. Inset of each graph depicts immunophenotyping data of corresponding patient samples. Interestingly, within all FAB classes, nano-vorinostat exerted augmented cytotoxicity towards all patient samples, with slight variations in the percentage viability over the range of test concentrations. Interestingly, most of the patient cells showed dose dependent loss of viability, with nano-vorinostat registering enhanced cytotoxicity at lower IC50 (0.5 µM), compared to that of free vorinostat (1 µM).
Figure 3.14. Vorinostat sensitivity towards primary leukemic cells derived from patient samples (n=9).
Evaluating closely, patient sample 6 recorded highest blast percentages of 96.7 and sample 8, the least with 33.1%. Similarly, irrespective of blast (CD45<sup>dim/-</sup> cells) percentage, all patient samples responded to nano-vorinostat in a dose dependent manner. CD33 is a myeloid specific antigen whose expression is directly associated with adverse disease features and inversely associated with low-risk disease. Increased CD33 percentage is considered as an independent predictor of inferior outcome in AML<sup>35</sup>. Similarly, high expression levels of hematopoietic stem cell marker, CD34, is directly related to enhanced resistance to apoptosis and is also observed as another independent sign of poor prognosis<sup>36</sup>. Sample 4 showed highest CD33 levels of 86.7% and sample 8 had least with 0.9%. Similarly, patient sample 9 recorded highest CD34 levels of 97.7% and sample 4 the least with 11.1% and rest of samples within this wide range. Interestingly, patient samples which showed high expression levels of both CD33 and CD34 showed excellent response towards nano-vorinostat, similar to ones which expressed low levels of these markers. Inspite of such huge variations in phenotypic expressions, all patient samples showed excellent response towards nano-vorinostat. In regard with mutations/chromosomal abnormalities, patient sample 1 showed JAK2-V617F mutation and samples 7 and 9 showed MLL re-arrangements. In another aspect, JAK2-V617F mutations leads to more aggressive growth of leukemia owing to the activation of JAK2-STAT5 cascade which substantially alters apoptotic response, self-renewal and proliferative capacity of myeloid cells.<sup>37,38</sup> MLL rearrangements at 11q23 are also considered as a poor predictor of clinical outcome in AML<sup>39,40</sup> However, both these samples showed almost similar cytotoxicity patterns with patient sample 9 registering ~100% sensitivity above 0.1 µM nano-vorinostat. Sample 5 obtained from a relapsed FAB M2 patient showed > 90% cytotoxicity starting from the lowest test concentration (0.1 µM), which increased to ~100% for concentrations above 0.1 µM. Samples 4 (FAB M2), 6 (FAB M0) and 9 (FAB M5) obtained from refractory patients also showed results similar to that observed with the relapsed patients, all responding invariably to lowest test concentrations itself.
It may be noted that there are a considerable number of patients diagnosed with AML who either fail to achieve remission or relapse after induction with cytarabine and daunorubicin. Despite the development of a variety of new investigational therapies\textsuperscript{41}, relapsed or refractory AML remains to be a complicated clinical challenge. From our results, all relapsed and refractory patient samples invariably displayed excellent response to nano-vorinostat starting at lowest concentration (0.1 µM). It is noteworthy that the nanomedicine exercised superior cytotoxic effects in otherwise hard-to-treat relapsed and refractory patient samples, compared to free drug. This pointed towards the potential of nanomedicine to evolve as a promising strategy against poor prognosis cases, either as a stand-alone drug or as an adjuvant with the existing chemo-regimen.

3.4.4. Inhibition of HDAC activity and clonogenic proliferation potential of leukemic bone marrow samples

Since, AML exemplify derailed myelopoiesis, which normally involves the growth and maturation of myeloid lineage, colony forming unit-granulocyte macrophage (CFU-GM) assay was performed to study clonogenic growth patterns of vorinostat treated leukemic BMMC from AML patients. This assay has become a yardstick, functional in vitro assay, providing an alternative to in vivo animal models, for testing the efficacy and general/lineage specific toxicity of candidate drugs on hematopoietic progenitors. The number of colonies obtained in CFU assays should be linearly proportional to the CFU content of the input cell suspension provided that a sufficiently low number of cells are plated (1×10\textsuperscript{4} cells). Besides, the relatively long incubation period (14 days) also increases the sensitivity of the assay and allows the detection of more long-term effects than are detectable in a proliferation assay.

Subsequently we have assessed the nano-vorinostat treatment effect on HDAC activity and colony-forming ability of leukemic BMMC isolated from patients 7, 8 and 9, whose bone marrow samples were available. Figure 3.15. A shows the level of HDAC inhibition after free and nano-vorinostat treatment in patient sample 7, wherein 1 µM nano-vorinostat resulted in enhanced HDAC inhibition of >90%, compared to
Figure 3.15. Level of HDAC inhibition (A, C, E) and effect on clonogenic proliferation (B, D, F) of leukemic BMMC after free and nano-vorinostat treatment in patients 7, 8 and 9.

Although both samples belonged to two different FAB classes, HDAC inhibition was almost absolute in both free and nano-vorinostat treated patient samples 8 and 9 (Figure 3.15, C and E). Figure 6, D shows the HDAC inhibitory effect of nano-
vorinostat on patient sample 8 wherein nano-vorinostat treatment nearly disrupted the colony forming capability of these leukemic cells. Only few rudimentary CFU-G (colony forming unit-granulocyte) colonies were to be seen. Figure 3.15. F clearly shows that nano-vorinostat completely derailed the colony forming capability of sample 9 showing MLL re-arrangement. Figure 3.15. G shows graphical representation of median number of colony forming units formed in untreated, 2.5 µM free and nano-vorinostat treated leukemic BMMC from patients 7, 8 and 9, after 14 days.

From our results, it was clear that nano-vorinostat has succeeded in inhibiting the clonogenic proliferation capability of leukemic BMMC isolated from all three patient samples (patients 7, 8 and 9). Nano-vorinostat could disrupt both granulocytic and macrophage lineage colony formation as evident from the virtual absence of functional CFU-G, CFU-M or CFU-GM colonies in nanomedicine treated samples. In tune with these important results and considering the presence of hematopoietic progenitor cells and stem cells in bone marrow samples, it was imperative to investigate the extent of differential toxicity that may be exerted by nano-vorinostat towards healthy and leukemic cells. Interestingly, nano-vorinostat did not affect colony forming capability of normal BMMC, isolated from healthy individuals (Figure 3.8 C). This indicated that the nanomedicine exerted its toxicity specifically to leukemic cells where HDAC activity was elevated and spared normal healthy cells. The selective ablation of clonogenic growth in leukemic bone marrow samples suggests a strong possibility of nanomedicine to strike the leukemic stem cells (LSC) population. However, this needs to be confirmed by studying the effect of the same on isolated LSCs in NOD-SCID animal models.

3.4.5. Effect of kinome Vs epigenome targeted nanomedicines on patient samples
In an effort to see the comparative effects of kinome and epigenome targeted nanomedicines on primary AML cells, we have treated patient derived cells from 3 refractory patients (4, 6 and 9) which usually represent ‘tough-to-treat’ dismal prognostic group. Two separate CD33 targeted core-shell nanomedicines encapsulating
combinations of 10 nM everolimus+1 µM sorafenib and 25 nM everolimus+5 µM sorafenib, 0.5 µM nano-vorinostat and chemodrug combination of 100 nM ara-C+50 nM daunorubicin were used in the experiment. Figure 3.16 shows the effect of core-shell nanomedicines and nano-vorinostat in refractory patient samples.

![Graphs showing cell viability](image)

**Figure 3.16.** Data showing excellent sensitivity of refractory patient derived cells towards 0.5 µM nano-vorinostat compared to core-shell nanomedicine or chemodrug combination

It is evident from Figure 3.16 that lower concentration of nano-vorinostat has exerted 100% cytotoxicity invariably in all three refractory patient samples. Neither combination of higher concentrations of everolimus and sorafenib or chemodrugs was shown to exert significant cytotoxicity towards these cells. During our experiments, we have also observed that primary AML cells from refractory patients (6 and 9) were shown to express comparatively more HDAC activity than non-refractory patients (7 and 8; Figure 3.17). We speculate that refractory patients expressing aberrant HDAC activity could thus be most sensitive to HDAC inhibitors, rather than
tyrosine kinase inhibitors or chemodrugs, which might have resulted in extreme
cytotoxicity in these set of patient samples.

![Graph showing HDAC activity levels](image1)

**Figure 3.17.** Level of HDAC activity in refractory (P6 and P9) and non-refractory
patients (P7 and P8)

We were unable to perform extensive molecular characterization of the obtained
patient samples pertaining to expression levels of mTOR, MAPK and STAT5
kinases, due to sample constraints. Therefore, authoritative comments could not be
made on why the kinase targeted core-shell nanomedicines were not found to exert
significant cytotoxicity. Nonetheless, it is noteworthy that 0.5 µM nano-vorinostat
showed excellent cytotoxicity exhibiting a high E/T ratio of 16.6, indicating its wide
therapeutic index, resulting from its almost non-toxic effect towards healthy bone
marrow cells (Figure 3.18).

![Efficacy/toxicity plot](image2)

**Figure 3.18.** Efficacy/toxicity plot depicting high E/T ratio of 0.5 µM nano-vorinostat
compared to free drug combination and chemo-combination.
Concluding from the obtained results, nano-vorinostat was found to exercise superior anti-leukemic effects on all patient samples, both fair and poor prognostic groups, compared to tyrosine kinase inhibitors loaded core-shell nanomedicine pointing to the promise of epigenetic targeted therapeutic approach against AML. Considering AML as an elderly disease and observed trend of increased occurrence of unfavorable prognosis factors with increasing age and further need of a more tolerable therapeutic approach, an epigenetic targeted strategy similar to nano-vorinostat might look more promising.

3.5. References


