Chapter 4

Effect of nano-vorinostat on leukemic stem/progenitor cell population
4.1. Introduction
Persistence of a resilient, residual population of LSPC is clinically implicated in the inability of current conventional chemotherapy to eradicate AML, causing therapeutic failure and relapse.\textsuperscript{1} Established line of evidences have conferred LSPC with CD34\textsuperscript{+} CD38\textsuperscript{−} CD123\textsuperscript{+} immunophenotype, owing to preferential overexpression of IL-3 receptor α, CD123 (IL3-Rα) within CD34\textsuperscript{+} CD38\textsuperscript{−} subset of AML cells, over healthy CD34\textsuperscript{+} CD38\textsuperscript{−} hematopoietic stem cells (HSC).\textsuperscript{2,3} LSPC possessing low mitotic index are missed even by the most aggressive chemoregimens, which target rapidly dividing leukemic blasts alone. Considering AML as an elderly malignancy with majority of affected population above 60 years, intense chemotherapy leads to acute myelosuppression and subsequently infections and related comorbidities, resulting in higher mortality rates (> 40%). Hence clinical interventions in AML should be suitably modified to address and ablate both, leukemic blasts as well as LSPC compartments, without disrupting normal hematopoiesis. We recently reported development of protein-vorinostat nanomedicine and demonstrated its exceptional anti-leukemic activity in AML patient samples (n=9) and AML cell lines (n=3).\textsuperscript{4} Notably nano-vorinstat selectively impaired clonogenic proliferation of leukemic progenitors, while largely sparing healthy bone marrow cells indicating that this formulation may potentially destabilize LSPC fraction.

In the present chapter, we have analyzed the effect of nano-vorinostat on CD34\textsuperscript{+} CD38\textsuperscript{−} CD123\textsuperscript{+} LSPC and inspected for myelosuppressive or hematotoxic potential. Besides, we also probed for possible synergism of lower concentrations of nano-vorinostat with DNA methyl transferase inhibitor, decitabine (Dacogen®, Astex/Eisai) for a prospective, more tolerable and effective, dual epigenetic targeted therapeutic regimen for elderly AML patients

4.2. Research questions and Hypotheses
1. \textbf{Qn:} What would be the differential activity effect of nano-vorinostat on CD34\textsuperscript{+} CD38\textsuperscript{−} CD123\textsuperscript{+} leukemic stem/progenitor cell population versus healthy bone marrow cells?
Hypothesis: Nano-vorinostat may ablate both leukemic blasts and LSPC fractions, without affecting healthy bone marrow cells.

2. Qn: What would be the level of synergism of lower concentrations of nano-vorinostat with DNA methyl transferase inhibitor, decitabine?

Hypothesis: Owing to well preserved chemical stability and molecular activity of encapsulated vorinostat within the nanoformulation, lower concentrations of nano-vorinostat may synergize with another epigenetic targeted drug, decitabine, to exert simultaneous assault on AML cells.

4.3. Materials and Methods

4.3.1. Patient bone marrow cell isolation and MACS enrichment

Blood or bone marrow samples were obtained after written informed consent from AML patients and healthy stem cell donors upon approval from Institutional Ethics Committee, Amrita Institute of Medical Sciences and Research Centre, Kochi, Kerala, India. Mononuclear cells were isolated using a standard density gradient/centrifugation method with Histopaque and red blood cells depleted from the sample using ammonium chloride. Isolated cells were enriched using magnetic separation of cells incubated with EasySep® Human CD34 Positive Selection Kit (STEMCELL Technologies, Canada). MACS enriched cells 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.

4.3.2. Flow cytometric enumeration of leukemic stem/progenitor cell (LSPC) population

For this, we looked at the viable LSPC population (7-AAD⁻ CD34⁺ CD38⁻ CD123⁺) at the start of the assay (t=0 h) and after in vitro treatment with 1 µM nano-vorinostat (t=72 h). For quantifying viability of LSCP, cells were incubated with 10 µg/mL 7-amino-actinomycin D (7-AAD; Sigma-Aldrich, USA). Cells treated with 1 µM nano-vorinostat were washed once with PBS and incubated with 5 µL each of FITC CD34,
4.3.3. Clonogenic proliferation assays
5x10^6 cells were seeded and treated with 1 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 ≥50 cells were enumerated.

4.3.4. Hemocompatibility tests
4.3.4.1. Hemolysis analysis
Haemolytic potential of nanoparticles was analysed spectrophotometrically by means of the soret band based absorption of free haemoglobin in blood plasma at 415 nm. Whole blood was collected into 3.8% trisodium citrate anticoagulant (anticoagulant: blood = 1:9). 450 mL of whole blood was treated with 50 mL of samples for 3 h at 37°C under mild mixing condition. PBS and 1% Triton X-100 were used as negative and positive controls, respectively. The treated blood was then centrifuged at 4000 rpm for 15 min. The plasma was collected and diluted with 0.01% sodium carbonate and absorbance was measured spectrophotometrically at 380, 415 and 450 nm. Amount of plasma haemoglobin was calculated using below equation.

\[
\text{Amount of plasma hemoglobin (mg/dL)} = \frac{2 \times A_{415} - (A_{380} + A_{450}) \times 1000 \times \text{dilution factor}}{(E \times 1.655)}
\]

where \(A_{415}, A_{380}\) and \(A_{450}\) are the absorbance values at 415, 380 and 450 nm. \(A_{415}\) is the soret band based absorption of haemoglobin. \(A_{380}\) and \(A_{450}\) are correction factors applied for uroporphyrin absorption falling in the same wavelength range. \(E\) is molar absorptivity value of oxyhaemoglobin at 415 nm which is 79.46. 1.655 is the correction.
factor applied due to turbidity of plasma sample. The hemolytic property of nanoparticles was plotted as % hemolysis versus different sample concentrations. Experiment was repeated with 3 independent donor samples.

\[
\% \text{ Hemolysis} = \frac{\text{Plasma Hb value of sample}}{\text{Total Hb of blood}} \times 100
\]

4.3.4.2. Plasma coagulation study
Interaction of the test material with the plasma coagulation factors was analysed by coagulation time measurements. Platelet poor plasma (PPP) was separated from peripheral blood by centrifugation at 4000 rpm for 15 min at 19C. 450 µL of PPP was treated with 50 µL of sample at 37°C for 30 min. 100 µL of prothrombin reagent (Diagnostica Stago, France) was added to 50 µL of treated plasma and the time taken for the plasma to coagulate, i.e., prothrombin time (PT) was measured. In case of activated partial thromboplastin time (aPTT) measurement, 50 µL of aPTT activator (Diagnostica stago, France) was added to 50 µL of plasma and incubated for 180 sec before the addition of 50 µL of 0.025 M CaCl₂. After CaCl₂ treatment, the time taken by plasma to coagulate was measured as aPTT.

4.3.4.3. Platelet aggregation study
For platelet aggregation analysis, PRP was collected from whole blood by centrifugation at 150 g for 10 min. 800 µL of PRP was treated with 200 µL of sample to be tested at 37°C for 15 min. Platelet count was done in a haematology analyser (Abbott CELL-DYN 3700). PBS and 50 mM ADP served as negative and positive controls, respectively. To study the interference nanoparticles with platelet count analysis, a control of nanoparticles alone without platelets was run and no count was obtained which proved that the particles do not interfere with the platelet count.

4.3.4.4. Lymphocyte proliferation and immunosuppression analysis
PBMCs were isolated from peripheral blood by density gradient separation. 100 µL of 10⁵ cells were seeded to each well in 96-well plates. 100 µL of samples and controls were added to the cells. After incubation for 3 days, MTT assay was done to
study lymphocyte proliferation. PBS and 100 mg/mL PHA-M (Phytohaemagglutinin) served as negative and positive controls, respectively. Lymphocyte proliferation was calculated according to equation given below.

\[
\text{% Cell proliferation} = \left( \frac{OD_S - OD_N}{OD_N} \right) \times 100
\]

where OD\(_S\) and OD\(_N\) are the optical density values at 570 nm for sample and negative controls, respectively. Immunosuppression analysis was done by studying the interference of nanoparticles with normal lymphocyte proliferation activated by lectins. PBMCs were isolated and treated with a combination of samples and 100 mg/mL of PHA-M. Incubation was done for 3 days followed by MTT assay. OD value obtained at 570 nm was plotted against different sample concentrations tested.

4.3.5. Evaluation of synergism with decitabine

Typically 2×10\(^4\) patient sample derived cells were treated with 0.1µM nano-vorinostat and varying concentrations of decitabine (0-1000 nM) alone and in combination, 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.

4.4. Results and Discussion

4.4.1. Effect of nano-vorinostat on CD34\(^+\) CD38\(^-\) CD123\(^+\) LSPC population

Our preliminary set of experiments with nano-vorinostat (NV) in primary AML cells from patient samples (n=9) and cell lines (n=3) has indicated its potential for striking the LSPC compartment without affecting normal bone marrow cells. The clinical validity of observed NV induced toxicity was evaluated in CD34\(^+\) CD38\(^-\) CD123\(^+\) leukemic cells and in normal bone marrow cells derived from healthy stem cell donor. Clinical characteristics of the patients at the time of sampling are summarized in Table 4.1. Both elderly patients showed high blast percentage (> 80%), normal karyotype and exhibited refractoriness to conventional induction regimen, which convey poor prognostic outcome.
Table 4.1. Clinical characteristics of the patients at the time of sampling

<table>
<thead>
<tr>
<th>Patient sample #</th>
<th>Age/Gender</th>
<th>Sample</th>
<th>Diagnosis with  subgroup 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>68/M</td>
<td>BM</td>
<td>M4 AML</td>
<td>81.2</td>
<td>None detected/46, XY</td>
<td>Refractory to Ara-C / Daunorubicin regime</td>
</tr>
<tr>
<td>2</td>
<td>72/F</td>
<td>BM</td>
<td>M2 AML</td>
<td>92</td>
<td>None detected/46, XX</td>
<td>Refractory to Ara-C / Daunorubicin regime</td>
</tr>
</tbody>
</table>

* at the time of sampling

We analyzed the anti-LSPC effect by monitoring absolute numbers of viable (7-AAD negative) CD34\(^+\) CD38\(^-\) CD123\(^+\) cells before (t=0 h) and after (t=72 h) treatment with 1 µM nano-vorinostat. Figure 4.1 A and B shows the effect of NV on patient sample 1 (PS1) and 2 (PS2), respectively. Interestingly, NV was shown to completely eradicate the LSPC population in PS1, since there were practically no viable cells in the P4 quadrant representing 7-AAD\(^-\) CD34\(^+\) CD38\(^-\) CD123\(^+\) fraction (Figure 4.1 A, bottom panel). In PS2, after 72 h of NV treatment, only 2 viable events were registered in the P4 quadrant (Figure 4.1 B, bottom panel). Since both PS1 and PS2 showed high blast percentage, and an absolute wipe-off of cells were observed post NV treatment, it could be appropriately deciphered that NV exerted its anti-leukemic activity on both blast and LSPC fractions, effectively ablating both bulk and stem/progenitor compartments.

To confirm the flow cytometry data, we have evaluated the colony forming capability of LSPC after nano-vorinostat treatment. Figure 4.2 shows that 1 µM NV completely disrupted the colony formation in leukemic BMC as evident from virtual absence of colonies in nano-vorinostat treated PS1 and PS2 samples (Figure 4.2. B and D). In contrast clonogenic growth pattern of healthy BMC was not hindered (Figure 4.2. F) upto 5 µM NV. Healthy BMCs treated with 5 µM NV were shown to produce CFU-G and CFU-GM colonies.
**Figure 4.1.** Effect of nano-vorinostat on CD34<sup>+</sup> CD38<sup>−</sup> CD123<sup>+</sup> cells from (A) patient sample 1 and (B) patient sample 2. In figures, upper panel: isotype controls; middle panel: absolute number of cells before nano-vorinostat treatment; bottom panel: absolute number of cells after nano-vorinostat treatment.
Figure 4.2. Effect of nano-vorinostat on the clonogenic proliferation of (A & B) healthy bone marrow cells and (C-F) two refractory patient samples, PS1 and PS2.

Thus NV even at a concentration of 1 µM was found to exert cytotoxicity specifically against LSPC without exhibiting any myelosuppressive property up to 5 µM, a highly desirable trait in contrast to myeloablative chemoregimens. Neutropenia, characterized by an abnormally low number of neutrophils, is the most common side effect of chemotherapy resulting in higher risk of bacterial and fungal infections. Colony forming unit of granulocyte-macrophage (CFU-GM) assay screens probability of anti-cancer agents to induce neutropenia. These results are in agreement with our previously observed data, where NV treatment led to loss of colony formation of leukemic BMC largely sparing the normal BMC giving rise to regular colonies, albeit at a lower concentration of 1 µM.

Although complete remissions with vorinostat have been reported in clinical trials, on account of lack of sustained anti-leukemic effect and/or considerable dose-dependent side effects, HDACi have not been approved for AML. We believe that albumin-bound vorinostat might aid enhanced intracellular uptake and sustained drug release, which might help overcome challenges faced by free vorinostat (FV). With well preserved chemical stability and molecular activity, lower concentrations of the
drug could have rendered its established pleiotropic effect on the LSPC, effectively killing them. Moreover sustained release profile of NV may enable prolonged anti-leukemic effect compared to transient effect of FV.

4.4.2. Hemocompatibility profile of nano-vorinostat

4.4.2.1. Hemolysis analysis

Initially, the hemolytic potential of the nanoformulation was assessed where Figure 4.3. A revealed that nano-vorinostat did not induce any hemolysis up to 10 µM.

![Hemolysis data of free and nano-vorinostat treated whole blood showing non-hemolytic action of the tested samples. Inset: Photograph of the whole blood treated with Triton, 1 µM, 5 µM and 10 µM nano-vorinostat. Scanning electron micrograph of RBC treated with (B) PBS and (C) 10 µM nano-vorinostat.](image)

This is evident from Figure 4.3, A inset, where supernatants of NV treated whole blood samples remained clear in contrast to red coloured supernatant of triton (positive control) treated sample. Erythrocytes treated with 10 µM NV showed intact morphology (Figure 4.3. C) similar to those treated with PBS (negative control; Figure 4.3. B).
4.4.2.2. **Platelet aggregation studies**

NV did not induce any platelet aggregation upto 10 µM (Figure 4.4, A). SEM image of 10 µM NV treated platelets were seen to spread on the coverslip, without any significant aggregation, similar to PBS treated platelets (Figure 4.4, B and C).

![Platelet aggregation studies](image)

**Figure 4.4.** (A) Platelet count data of free and nano-vorinostat treated platelet rich plasma showing no significant change in count after the treatment. Scanning electron micrograph of platelets treated with (B) PBS (C) 10 µM nano-vorinostat

4.4.2.3. **Effect on plasma coagulation factors**

The effect of NV on intrinsic and extrinsic plasma coagulation factors was studied using prothrombin time (PT), international normalized ratio (INR), and activated partial thromboplastin time (aPTT) measurements. PT, INR and aPTT values of NV treated platelet poor plasma (PPP) fell within respective normal ranges (shaded regions in Figure 4.5, A-C) suggesting that the nanoparticles did not interfere with any of the coagulation factors.
Figure 4.5 (A) PT (B) INR ratio and (c) aPTT measurements of free and nano-vorinostat treated blood plasma samples. Normal range is shown as shaded region in graphs.

4.4.2.4. Studies on immunogenicity and immunosuppression

Subsequently, we probed for any undesirable immunogenic or immuosuppressive potential of NV. Lymphocytes treated with NV alone did not show any spontaneous proliferation (Figure 4.6, A) up to 10 µM, indicating that nanoformulation did not mount any immune response. Whereas, lymphocytes treated with combination of phytohemagglutinin (PHA-M; mitogen) and varying concentrations of NV showed almost similar proliferation patterns as that of cells treated with PHA-M alone, suggesting that NV did not exhibit any immunosuppressive potential, by subduing lymphocyte proliferation activated by the mitogen (Figure 4.6, B). SEM images of PBS (Figure 4.6, C) and 10 µM nano-vorinostat (Figure 4.6, D) treated lymphocytes
revealed discrete cells without significant clumping. Whereas, lymphocytes treated with PHA-M and PHA-M + 10 µM NV showed agglutination (Figure 4.6, E and F).

**Figure 4.6.** (A) Percentage cell proliferation of peripheral blood derived mononuclear cells on exposure to different concentration of free and nano-vorinostat for 72 h in comparison with phytohemagglutinin (PHA-M) treatment (B) Immunosuppression studied by cell viability analysis on mononuclear cells exposed to mixture of PHA-M + free/nano vorinostat sample. Optical microscope images of mononuclear cells treated with (C) PBS (negative control) (D) 10 µM nano-vorinostat (E) PHA-M (positive control) and (F) PHA-M + 10 µM nano-vorinostat.

Our results indicated that NV exhibited better hemocompatibility, since FV showed comparatively more platelet aggregation and slightly prolonged PT value. Collectively, NV without exhibiting any undesirable hemolysis, thrombocytopenia or any immunogenic or immunosuppressive potential presented an appreciable hematocompatibility profile.
4.4.3. Synergism with DNMT inhibitor, decitabine

Considering the successful anti-leukemic activity of nano-vorinostat against both blast and LSPC, our next objective was to investigate its potential synergistic action with another epigenetic targeted therapy which is already in clinic, for elderly patients. Decitabine (Dacogen™, Eisai) is a hypomethylating agent, which acts by inhibiting DNA methyltransferase. Based on positive indications from a randomized international phase III study, decitabine was approved by the European Commission as a frontline therapy for untreated high-risk elderly AML patients. However, decitabine resistance is widely observed and in view of this, we have probed for possible synergism of lower concentration of nano-vorinostat with decitabine, in PS1 adn PS2.

![Figure 4.7. Cytotoxicity data showing the effect of decitabine alone and combination of decitabine with 0.1 µM nano-vorinostat in (A and B) PS1 (C and D) PS2.](image-url)
From Figure 4.7 A and C we can see that free decitabine alone did not cause any significant toxicity up to 1000 nM for both PS1 and PS2. Similarly, 0.1 µM nano-vorinostat alone also did not exert any appreciable toxicity (green bars in Figure 4.7. B and D). However, combination of 100 nM decitabine and 0.1 µM nano-vorinostat registered 76% toxicity in PS1 and 96% in PS2. Combination with higher concentrations of decitabine resulted 100% cytotoxicity in both patient samples. Even though number of patient samples were limited (n=2), the observed trend point towards considerable synergism between decitabine and nano-vorinostat, which could provide promise of improved therapeutic outcomes in elderly patients, compared to the conventional cytotoxic chemoregimen of cytarabine and daunorubicin.

Recently, clinical attention has focused on evaluating more tolerable therapeutic interventions for elderly AML patients, judged unfit to receive intensive chemotherapy. Considering the functional and clinical significance of the primitive leukemic cell compartments in therapeutic failure and relapse, our reports have demonstrated specific ablation of LSPC fraction, without inducing any myelosuppression or pancytopenia. This shows promise in the setting of minimal residual disease requiring prolonged treatment. Moreover, the observed synergism between NV and decitabine provides an optimisitic choice of more tolerable and effective epigenetic targeted therapeutic approach for AML over current cytotoxic chemotherapy.

4.5. References


