Title:	Multimodal nanotherapy abrogating polycomb mediated epigenetic regulation of acute myeloid leukemia
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Abstract: Acute myeloid leukemia is an aggressive hematological cancer of hemopoietic stem cells or myeloid progenitor cells. Clonal heterogeneity of myeloid precursors leading to a fatal hematologic malignancy known as acute myeloid leukemia (AML) is characterized by inadequate differentiation of myeloid blasts into mature cells that can self-renewal and undergo aberrant proliferation of myeloid lineages. The long-term survival of AML patients < 60 years old remains 40%, demonstrating that despite treatment improvements, the prognosis of AML requires better knowledge to understand the significant genetic and epigenetic changes associated with AML to address them for developing a therapeutic regime. The polycomb group, protein subunit Enhancer of Zeste Homolog 2 (EZH2), which functions as a histone methyltransferase, is highly expressed and required for leukemic cell survival. EZH2/EZH1 regulates molecular, metabolic, and proliferative processes and modulates extracellular microenvironments. The histone methyltransferases EZH2/EZH1, a critical regulator of tumor suppressor gene silencing as a potential therapeutic target in the context of AML. Immune recognition implies that targeting EZH2 may repress AML progression. This thesis demonstrates the evolution of multitargeted and multimodal anti-AML treatment regimens that include EZH2 as a critical regulator in the therapeutic intervention. The specific aims were to assess the mechanisms of EZH2/1 that may facilitate leukemia and how inhibition may abrogate the leukemic growth by protein-based nanostructures because of their structural, composition, and functional versatilities in maintaining AML in vitro and in vivo mice models. The first chapter in the present thesis describes the development of EPZ011989; an EZH2- specific inhibitor encapsulated in human serum albumin nanoformulation. The non-covalent interactions between EPZ011989 and HSANPs facilitated loading and sustained release of inhibitor molecules and enhanced internalization and nuclear localization in human AML cell lines. The nanoformulation caused marked inhibition of EZH2, BMI1, H3K27me3, and H2AK119ub, the hallmarks of epigenetic silencing of tumor suppressor genes. Pre-clinical validation results show that EPZ011989-loaded HSANPs alter the expression of CD11b+ and CD45+ positive cell populations in the peripheral blood and bone marrow of AML engrafted nude mice model the suppression of malignant clonal enrichment of undifferentiated myeloid lineages. The EZH2 and indirect targeting of BMI-1 and c- Myb were established as the basis of the collective epigenetically targeted anti-AML activity. The ubiquitination and proteasomal degradation pathways mediate the decrease of EZH2 and c-Myb proteins. In the in vivo system, 2 the current nanoformulation demonstrated better systemic compatibility. The findings provide crucial experimental evidence for targeted epigenetic therapy of AML by overcoming drug absorption and solubility issues that trigger superior anti-leukemic activity. Next, In chapter 2, we utilized the identical human serum albumin (HSA) nanoparticles for packaging small interfering RNA (siRNA) for targeted inhibition of EZH2- in AML. The EZH2 siRNA loaded in a polyethyleneimine (PEI) conjugated HSA can overcome the systemic instability limitation of siRNA and target the AML cell population for enhanced EZH2 gene silencing. These stable nano-complexes (HSANPs-PEI@EZH2siRNA), stabilized by mutual electrostatic interactions between PEI and EZH2 siRNA, have higher systemic stability, hemocompatibility, and EZH2 gene silencing activity in vitro when compared to standard transfection reagents. HSANPs- PEI@EZH2siRNA exposure depletes the EZH2 in AML cells and is also associated with a reduced level of Bmi-1 protein and H3K27me3, H2AK119ub marks. The ubiquitin-mediated proteasomal degradation mechanism was driving the downregulation of EZH2. Detailed molecular investigation confirms the binding interaction between EZH2 and c-Myb can be linked to the regulation of leukemogenesis. The systemic administration of HSANPs- PEI@EZH2siRNA to AML engrafted immunodeficient nude mice resulted in effective EZH2 gene silencing, reducing the AML cell population evident from bone marrow and peripheral blood. The present study demonstrates a non-viral siRNA delivery system targeting polycomb EZH2 and confers superior molecular anti-leukemic therapy. The third chapter delves into the Leukemic stem cell targeting via protein immune active complex for AML cure. Leukemic stem cells play key roles in leukemogenesis, development, and recurrence must be eliminated to obtain a clinical cure. To better understand the fundamental physiological and molecular pathways involved in stem cell maintenance, we employed novel immune-active proteinnanoconjugate- based immunotherapy of these leukemic stem cells in AML. The immune-active IgG protein gives a uniform nanosized IgG nanoparticle (IgGNPs) conjugated with human IFN - γ (IgGNPs@IFN-γ) for precise LSCs targeting. The non-covalent interactions between the IFN- γ and IgGNPs nanoparticles are major forces that improve the IFN- γ release profile, human LSCs (CD34+/CD38-), and AML cell uptake. In-vitro cell conjugation assays stimulate human NK cells toward LSCs and AML cells. The therapy suppresses the expression of OCT3/4, which is linked with reduced levels of EZH1, implying chromatin compaction in LSC. The ubiquitination and proteasomal degradation pathways suppress the EZH1, and OCT3/4 proteins as EZH1 directly interacts with OCT3/4. Pre-clinical evaluation of IgGNPs@IFN-γ in patient-derived CD34+/CD38- xenografted mice model reveals decreased CD34+/CD38- LSCs population, which correlates with reduced C-Kit+ and proliferation Gr-1 marker. After adaptive NK cell 3 implantation in a xenografted mouse model constructed with an EZH1 knockout CD34+/CD38- cell population, fewer LSCs seen in the peripheral and bone marrow fractions. The quiescent LSCs expressed the highest levels of OCT3/4 and polycomb subunit EZH1 in the AML hierarchy and inactivation of EZH1/OCT3/4 with IgGNPs@IFN-γ + NK eradicated quiescent LSCs to cure AML initiation and progression. Thus, the results demonstrated that immune-active nanocomplex activates NK cells for specific targeting of leukemic stem cells owing to EZH1 in sensitizing the AML immunotherapy. Chapter 4 demonstrates the approach of targeted stimulation of the immune cells for selective immune targeting of the AML. The innate immune system activation is required to generate antigen-specific immune responses in immunotherapy. Natural killer T (NKT) cells are innate immune cells that release interferon and activate dendritic cells to present tumor antigens to T cells, activated by adjuvant alpha galactosylceramide (Galcer). Galcer is a safe and effective immune booster in human cancer, with the disadvantage of increasing NKT cell anergy. We hypothesize that the preparation of Galcer loaded protein nanoparticles preferentially delivers it to dendritic cells and prevents anergy induction in NKT cells. Immunoglobulin protein nanoparticles (IgGNPs) tagged with Galcer and decorated with peptide/tumor membrane components activate NKT cells in vitro and in vivo. The nano-complex inhibits the overexpressed PcG subunit EZH2/1 and targets Nuclear factor-kappa enhancer-binding protein (NFkB). After nanoformulation exposure, the ubiquitin-proteasome pathway maintains the cellular levels of EZH2/1, Bmi1, and NFkB in AML cells. NFKB has a direct interaction binding at the EZH2/1 promoter and regulates its expression in AML pathogenesis. Xenograft AML model established in immunodeficient mice using naïve U937 and EzH2knockdown U937 shows a lower level of CD45+/C kit positive cells and elicits CD11b expression in peripheral blood after systemic administration of IgGNPs@Galcer immune active complex. The proliferation of CD161+ NKT cells in the EZH1/EZH2 knockdown group, along with a decrease in the leukemic population, indicated the efficacy of the active immune complex in AML therapy. The results show that utilizing immune-inspired nanoformulation for selective activation of NKT cells with targeted depletion of the epigenetic EZH1/2 induces an anti-AML immune response and may be translated into potent cancer immunotherapy. Finally, the current thesis elaborates on the comprehensive approach to preparing bioinspired protein nanostructures and utilizing their functional versatility in overcoming pharmacologic barriers in drug delivery, non-viral delivery of siRNA, selective NK cells used immune targeting the Leukemic stem cells, and modulating the NKT cell response for anti-AML.

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