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Research paper

Epigenetic drug combination overcomes osteoblast-induced chemoprotection in pediatric acute lymphoid leukemia

Anthony Quagliano[a,b], Anilkumar Gopalakrishnapillai[a,*], Sonali P. Barwe[a,b,*]

[a] Nemours Center for Childhood Cancer Research, Alfred I. duPont Hospital for Children, Wilmington, DE 19803, United States
[b] Department of Biological Sciences, University of Delaware, Wilmington, DE 19716, United States

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ABSTRACT

Although there has been much progress in the treatment of acute lymphoblastic leukemia (ALL), decreased sensitivity to chemotherapy remains a significant issue. Recent studies have shown how interactions with the bone marrow microenvironment can protect ALL cells from chemotherapy and allow for the persistence of the disease. Epigenetic drugs have been used for the treatment of ALL, but there are no reports on whether these drugs can overcome bone marrow-induced chemoprotection. Our study investigates the ability of the DNA methyltransferase inhibitor azacitidine and the histone deacetylase inhibitor panobinostat to overcome chemoprotective effects mediated by osteoblasts. We show that the combination of azacitidine and panobinostat has a synergistic killing effect and that this combination is more effective than cytarabine in inducing ALL cell death in co-culture with osteoblasts. We also show that this combination can be used to sensitize ALL cells to chemotherapeutics in the presence of osteoblasts. Finally, we demonstrate that these effects can be replicated *ex vivo* in a number of mouse passaged xenograft lines from both B-ALL and T-ALL patients with varying cytogenetics. Thus, our data provides evidence that azacitidine and panobinostat can successfully overcome osteoblast-induced chemoprotection *in vitro* and *ex vivo* in both B-ALL and T-ALL cells.

1. Introduction

Acute lymphoblastic leukemia (ALL) is the most commonly diagnosed pediatric malignancy. While 90% of patients will undergo remission during treatment, around 20% of those patients will have a recurrence of the disease, which is often much more aggressive and difficult to treat [1,2]. Moreover, T-ALL patients have a worse prognosis [3]. The higher rate of relapse and treatment challenges in ALL can be partially attributed to the role of the bone marrow microenvironment in leukemogenesis and leukemia progression [4–6]. The bone marrow allows leukemic cells to interact with different cell types such as: osteoblasts, stromal cells, endothelial cells, and mesenchymal stem cells, as well as extracellular matrix (ECM) proteins like fibronectin and osteopontin. These interactions can activate intracellular signaling pathways that protect leukemic cells from chemotherapy [4,7].

In order to decrease the high relapse rate in ALL and improve patient outcomes, treatment options that either diminish or circumvent the effects of bone marrow-induced chemoprotection must be explored. Our lab has previously identified that disrupting the interaction between p11 protein (S100A10) and annexin II was able to chemosensitize ALL cells in co-culture with osteoblasts [8]. Natalizumab, which specifically targets integrin alpha4 and disrupts stromal adhesion of ALL cells, is currently being tested in clinical trials to block integrin alpha4 chemoprotective activity [9].

Aberrant epigenetic modulation is a crucial mediator of leukemogenesis [10]. Epigenetic alterations may also play a role in the development of chemotherapy resistance in ALL [11]. These epigenetic alterations offer new therapeutic targets as they are easily amenable to change unlike genetic mutations. While there has been some success in the clinical use of epigenetic drugs, their role in overcoming microenvironment-induced chemoprotection in ALL has not been characterized.

In this study, we investigate the ability of the epigenetic drugs azacitidine (DNA methyltransferase inhibitor) and panobinostat (histone deacetylase inhibitor) to kill ALL cells and overcome

* Corresponding authors at: Nemours Center for Childhood Cancer Research, Alfred I. duPont Hospital for Children, 1701, Rockland Road, Wilmington, DE ,19803, United States.
E-mail addresses: anil.g@nemours.org (A. Gopalakrishnapillai), barwe@medsci.udel.edu (S.P. Barwe).

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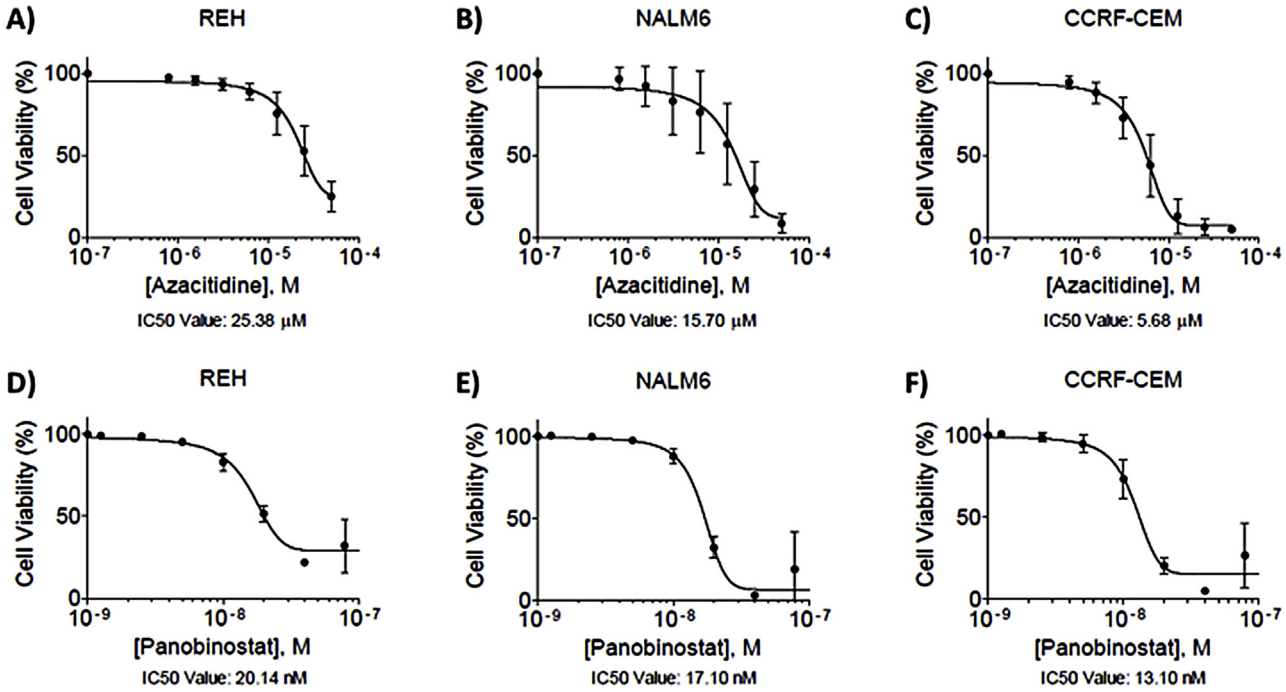

Fig. 1. Determination of IC50 concentrations of azacitidine and panobinostat. REH, Nalm6, and CCRF-CEM were treated with different concentrations of azacitidine and panobinostat. Cell viabilities were determined using flow cytometry. Azacitidine IC50s were determined to be A) 25.38 μM B) 15.70 μM and C) 5.68 μM, where as panobinostat IC50s were D) 20.14 nM E) 17.10 nM and F) 13.10 nM for REH, Nalm6 and CCRF-CEM respectively. IC50 values were calculated from 3 to 4 independent experiments performed in duplicates.

osteoblast-induced chemoprotection in a variety of ALL samples. We identify that azacitidine and panobinostat show synergy in inducing ALL cell death *in vitro* and *ex vivo* and that the combination is more successful in overcoming osteoblast-induced protection than other DNA-damaging chemotherapeutics. Additionally, we show that azacitidine and panobinostat can sensitize ALL cells to chemotherapy treatments and these effects are replicated *ex vivo* in a number of patient samples with varying cytogenetics.

2. Materials and methods

2.1. Cell lines, patient samples, and reagents

REH (CRL-8286), CCRF-CEM (CCL-119), and Saos-2 (HTB-85) cells were obtained from American Type Culture Collection (ATCC), Manassas, VA. Nalm6 cells were purchased from DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. Leukemic cell lines were cultured in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS), 2 mM/L L-glutamine, 25 U/mL penicillin, and 25 μg/mL streptomycin. Saos-2 cells were cultured in DMEM/F12 (1:1) with supplements described above.

Primary B-ALL and T-ALL cells isolated from bone marrow aspirates or peripheral blood of patients treated at Nemours/Alfred I. duPont Hospital for Children are banked by the Nemours BioBank. Samples were collected under a Nemours Delaware Institutional Review Board (IRB) protocol approved by the Nemours Office of Human Subjects Protection. Patient samples were passaged in mice following the guidelines of the Nemours Institutional Animal Care and Use Committee (IACUC) as described previously [12]. Mouse passaged primary ALL cells were used for *ex vivo* studies.

Azacitidine (S1782), panobinostat (S1030), cytarabine (S1648), and daunorubicin (S3035) were obtained from Selleckchem. Powder was dissolved in DMSO to appropriate concentrations.

2.2. Determination of IC50 concentrations

Leukemic cells (30,000) were plated in 96-well plates and treated with varying concentrations of azacitidine and panobinostat. Drugs were diluted in RPMI-1640 media to the highest concentration used then serial diluted to lowest concentration and added to the corresponding wells. Viability was determined using a NovoCyte flow cytometer (ACEA Biosciences, Inc.) using a forward scatter by side scatter plot to draw independent gates demarking live and dead populations. These populations were confirmed with propidium iodide staining. GraphPad Prism was used to determine IC50 with a log(agonist) vs. response – variable slope curve analysis using the 95% confidence interval.

2.3. Synergy assay

Leukemic cells (30,000) were plated in 96-well plates. IC50 concentrations of azacitidine and panobinostat were diluted in RPMI-1640 both singularly and in combination and added to the cells for 48 h. The percentage of viable cells at the end of treatment was determined using flow cytometry as described above. Drug synergy was estimated by calculating relative risk ratio (RRR). RRR is calculated as the ratio between the actual value and expected value of the percentage of surviving cells following treatment. RRR = (Percentage of viable cells in sample treated with azacitidine-panobinostat combination)/[(Percentage of viable cells in azacitidine-treated sample x Percentage of viable cells in panobinostat-treated sample)/100], as described previously and shown to correlate with combination index [13,14].

For patient samples, 1×10^6 cells were plated on 24-well plates in the presence of 20,000 Saos-2 cells and treated with specified concentrations of azacitidine and panobinostat alone and in combination for 48 h. Synergy was determined as mentioned above.