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Research paper

Combination of cabazitaxel and plicamycin induces cell death in drug resistant B-cell acute lymphoblastic leukemia

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ABSTRACT

Bone marrow microenvironment mediated downregulation of BCL6 is critical for maintaining cell quiescence and modulating therapeutic response in B-cell acute lymphoblastic leukemia (ALL). In the present study, we have performed a high throughput cell death assay using BCL6 knockdown REH ALL cell line to screen a library of FDA-approved oncology drugs. In the process, we have identified a microtubule inhibitor, cabazitaxel (CAB), and a RNA synthesis inhibitor, plicamycin (PLI) as potential anti-leukemic agents. CAB and PLI inhibited cell proliferation in not only the BCL6 knockdown REH cell line, but also six other ALL cell lines. Furthermore, combination of CAB and PLI had a synergistic effect in inhibiting proliferation in a cytarabine-resistant (REH/Ara-C) ALL cell line. Use of nanoparticles for delivery of CAB and PLI demonstrated that the combination was very effective when tested in a co-culture model that mimics the *in vivo* bone marrow microenvironment that typically supports ALL cell survival and migration into protective niches. Furthermore, exposure to PLI inhibited SOX2 transcription and exposure to CAB inhibited not only Mcl-1 expression but also chemotaxis in ALL cells. Taken together, our study demonstrates the utility of concomitantly targeting different critical regulatory pathways to induce cell death in drug resistant ALL cells.

1. Introduction

B-cell acute lymphoblastic leukemia (ALL) manifests itself as an accumulation of poorly differentiated malignant lymphoid cells within the bone marrow (BM), resulting in the disruption of normal hematopoiesis [1]. BM is also the most common site of disease relapse, contributed by minimal residual disease (MRD), a major factor associated with poor prognosis and mortality [2]. Unlike T-cell lymphoblastic leukemia, MRD in ALL has been less completely studied and its etiology still remains to be more clearly delineated [3]. However, there is sufficient evidence that the maintenance of MRD is due, in part, to the ability of the BM microenvironment to provide sanctuary to the ALL cells allowing malignant cells to survive, even in the presence of chemotherapy [4,5]. Interestingly, MRD cells became sensitive following release from their BM niche, emphasizing the crucial role of the BM microenvironment in contributing to therapeutic resistance [6]. Within the BM niche, in addition to often being non-responsive to chemotherapy, ALL cells have been described as quiescent with 'stem cell like' characteristics [6–8]. Hence, in order to consider strategies to eradicate MRD, the ability of ALL cells to migrate to the BM niche has to be considered in addition to the quiescent and drug resistant phenotype acquired when resident within the niche.

We previously developed an *in vitro* co-culture model of ALL cells with either primary human-derived BM stromal cells or osteoblasts (components of the BM niche) [9]. From this co-culture we characterized a drug resistant sub-population of leukemic cells referred to as "phase dim" (PD), based on their lack of light refraction coincident with their migration beneath adherent layers of stroma or osteoblasts. The PD tumor cells are used to model cells that contribute to MRD *in vivo* based on phenotypic similarities [9]. Using this niche-based co-culture model, we have reported that primary ALL samples, or ALL cells in co-culture with the BM cellular components, have reduced BCL6 expression in the PD cell population [10]. Furthermore, reduction in BCL6 resulted in disruption of cell cycle progression, with cyclin D3-

Abbreviations: ALL, acute lymphoblastic leukemia; BM, bone marrow; MRD, minimal residual disease; CAB, cabazitaxel; PLI, plicamycin; NP, nanoparticles; PD, phase dim

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dependent accumulation of cells in the G0/G1 phase. The importance of BCL6 in maintaining cell quiescence, drug resistance and the resulting MDR phenotype was further validated *in vivo* by demonstrating significant event free survival in mice treated with a combination of caffeine (stabilizer of BCL6) and cyatarabine (Ara-C) when compared to mice treated with Ara-C alone [10]. BCL6 has also been shown to be a master regulator of glycolysis by directly repressing the overall gene program of the glycolytic pathway [11]. Not surprisingly, we have shown that drug resistant PD ALL cells, characterized by reduced expression of BCL6, demonstrate increased glycolysis coincident with upregulation of several molecules that modulate the metabolic pathway, including hexokinase II [9,10]. Based on these observations we screened for drugs that induce death in leukemic cells with diminished BCL6, with the intent to identify agents that could be tested for efficacy in targeting MRD in ALL.

In the present study, we have successfully screened a library of FDA-approved oncology drugs in a BCL6 knockdown ALL cell line and identified cabazitaxel (CAB) and plicamycin (PLI) as potential candidates that could target and eliminate drug resistant leukemic cells. We further validated the anti-leukemic activity of CAB and PLI in six ALL cell lines and demonstrated that part of the anti-leukemic activity was attributed to cell cycle arrest. Furthermore, to show activity in low expressing BCL6 cells, we demonstrated synergism of the CAB/PLI combination in a cytarabine resistant REH cell line (REH/Ara-C) and our co-culture model. Collectively our observations suggest this combination therapy, with inhibition of chemotaxis and downstream modulation of SOX2 and Mcl-1, warrants further evaluation in settings that are refractory to traditional chemotherapy.

2. Materials and methods

2.1. Cell culture and chemicals

The development of doxycycline-inducible REH BCL6 knockdown cells and its comparative REH scrambled stable cells has been previously published [10]. SUPB15 (ATCC #CRL-1929) and JM1 (ATCC #CRL-10423) were purchased and maintained in RPMI 1640 containing 10% FBS, 0.05 mM β-mercapto-ethanol and 1X streptomycin/penicillin antibiotics. REH (ATCC #CRL-8286), NALM1 (ATCC #CRL-1567), NALM6 (DSMZ ACC #128), BV173 (DSMZ ACC#20), RS4 (ATCC #CRL-1873) and SD1 (DSMZ ACC#366) were purchased and maintained in RPMI 1640 containing 10% FBS and 1X streptomycin/penicillin antibiotics. Human osteoblasts (HOB) was purchased from PromoCell (Cat No: C-12720, Hiedelberg, Germany) and cultured according to the vendor's recommendations. All the ALL cell lines were authenticated by short tandem repeat (STR) analysis (University of Arizona Genetic Core, Tuscon, Az) and maintained in 6% CO_2 in normoxia at 37 °C. Primary immune cells $CD3^+$ T cells, $CD19^+$ B cells, peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells (BMMC) were all purchased from ALLCELLS and maintained in Lymphocyte growth medium −3 (Lonza, Cat No: CC-3211) containing 10% FBS and 1X streptomycin/penicillin antibiotics. De-identified primary human ALL cells were obtained from the West Virginia University Health Sciences Center and West Virginia University Cancer Institute Biospecimen Processing Core. CAB and PLI were purchased from Fisher Scientific (Cat No: 501014444 and 50488911, respectively) and stored at −80 °C as a 10 mM stock.

2.2. High throughput screening assay

FDA-approved oncology drug set VII was obtained from NCI/NIH in 96 well plate format with each well containing 20 µl of 10 mM drug stock. REH BCL6 knockdown and scrambled cells were treated with 1 µg/ml of doxycycline for 24 h (to induce shRNA expression) and then cultured in a 96 wells plate at 50,000 cells per well. Drugs were added in triplicate at log concentrations with the lowest concentration of 0.1 µM and the highest concentration of 100 µM. The cells were incubated for 72 h in a humidified atmosphere under 5% CO_2 at 37 °C following which the cell viability and the IC_{50} was measured and analyzed as described below.

2.3. Cell viability assay

ALL cells were grown in 96-well plates at 50,000 cells per well and treated with CAB or PLI at indicated concentrations. Cell viability was measured 72 h post-treatment using a cell counting kit according to the manufacturer's instructions (Dojindo Molecular Technologies Inc., Cat No: CK04). Briefly, 10 µl of the assay reagent was added to each well and incubated for 2 h at 37 °C in normoxia, after which the plates were read on a BioTek Cynergy 5 plate reader at 450 nm absorbance. Untreated cells were used as controls. IC_{50} and combination index (CI) was analyzed using compusyn (www.combosyn.com).

2.4. Cell cycle analysis

ALL cells at 10^6 cells/ml were treated with either 3 nM of CAB or 15 nM of PLI for 24 h. Post-treatment the cells were washed with phosphate buffered saline (PBS) and fixed using 70% ethanol for 30 min at 4 °C. After incubation, the cells were washed with PBS and re-suspended in a staining buffer containing 50 µg/ml of propidium iodide (PI) and 100 µg/ml of RNase A. Cells were incubated for 30 min in the staining buffer, followed by wash and analysis of the DNA content using flow cytometry.

2.5. Long-term co-culture and isolation of leukemic cell population

Long-term co-culture conditions have been previously described [9]. Briefly, 1 million REH cells were seeded on 85% confluent HOB layer and maintained in 5% O_2. The co-culture was fed every 4 days and on the 12th day in culture, REH cells were isolated for western blotting. The leukemic cell population that were in suspension and not interacting with the osteoblasts were removed and spun down and designated as suspended cells (S). The REH cells which are buried under the HOB were separated by size exclusion with G10 sephadex after vigorous washing. These buried REH cells were designated as phase dim cells (PD) and have been previously described to be representative of MRD [10,12].

2.6. Nanoparticle drug delivery system

The nanoparticles were prepared as described earlier [13]. Briefly, 2 mg of CAB or PLI were dissolved in 500 microliters of acetone containing 20 mg PEG-PLGA-COOH and added dropwise into a constantly stirring 25 mM of MES (pH 5). The organic solvent was allowed to evaporate in a fume hood and the nanoparticles (NP) were activated for 1 h using a 25 mM solution of MES containing 20 mg NHS and 20 mg EDC (pH 5). The quality of the resultant NP was analyzed using NanoSight NS300 (Malvern Instruments Ltd, UK).

2.7. Cell death analysis

12-day co-culture experiments were carried out as described above in a 24-well plate. On day 10, the cells were treated with either CAB, PLI, combination of CAB and PLI (C + P) or Ara-C. After 48 h (day 12), the live ALL cells were counted using trypan blue dye exclusion method and the % viability was calculated.

2.8. Hemosphere assay

SD1 cells were plated at 100,000 cells/well and allowed to form spheroids in a 96 well plate. The formation of spheroids was visualized and confirmed using light microscope and then treated with 15 nM of