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HDAC inhibitor Vorinostat and BET inhibitor Plx51107 epigenetic agents’ combined treatments exert a therapeutic approach upon acute myeloid leukemia cell model

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Abstract
The process of cancer initiation and development is regulated via the transcriptional expression of cells going under genomic and epigenetic changes. Targeting epigenetic “readers”, i.e., bromodomains (BRD) and post-translational modifications of nucleosomal histone proteins regulate gene expression in both cancerous and healthy cells. In this study, the new epigenetic agent BRD inhibitor PLX51107 and histone deacetylase (HDAC) inhibitor SAHA’s (Vorinostat) single/combined applications’ reflections were analyzed in case of cell proliferation, cytotoxicity, apoptosis, cell cycle arrest, and finally target gene expression regulation upon both AML and healthy B-lymphocyte cells; HL60 and NCIBL2171, respectively; in vitro. Since mono treatments of either Vorinostat or Plx51107 regulated cellular responses such as growth, proliferation, apoptosis, and cell cycle arrest of tumor cells; their combination treatments exerted accelerated results. We detected that combined treatment of Plx51107 and Vorinostat strengthened effects detected upon leukemic cells for gaining more sensitization to the agents, decreasing cell proliferation, dramatically inducing apoptosis, and cell cycle arrest; thus regulating target gene expressions. We have shown for the first time that the newly analyzed BRD inhibitor Plx51107 could be a promising therapeutic approach for hematological malignancies and its mono or combined usage might support a rapid transition to clinical trials.

Keywords AML · Epigenetic · Bromodomain · PLX51107 · HDAC · Combined therapy

Introduction
Hematopoiesis originates from the Greek word that stands for “blood production”, and provides the controlled production of blood cells, covering the processes of proliferation, differentiation, and maturation of cells, developing from pluripotent hematopoietic stem cells (HSCs; [8, 27]).

Acute myeloid leukemia (AML) is a malignant disease of the bone marrow characterized by low and variable production of healthy hematopoietic cells, differentiation of myeloid progenitor cells to immature myeloid cells with abnormal proliferation, and clonal expansion; called blasts [33]. The diagnosis of acute leukemia is determined by the presence of 20% or more blasts found in the bone marrow or peripheral blood [9].

Epigenetics is defined as the changes in gene function that can be inherited through cellular divisions and are not associated with any change in the primary sequence of nucleic acids [16]. Although there are multiple mechanisms, DNA methylation, and histone modifications are the best characterized and most studied ones [37]. Enzymes that detect, recognize, and remove DNA methylation are divided into three classes. While writers catalyze the addition of methyl groups to cytosine residues, erasers modify and remove the methyl group, and the readers recognize and bind to methyl groups to regulate gene expression [25]. Histone modifications regulate transcriptional repression, gene activation, and DNA repair, and are divided into three classes depending on their functions. Writers are functional in histone changes [histone acetyltransferases (HATs)] that catalyze the addition of epigenetic marks to histone/DNA complex. Erasers are histone deacetylases (HDACs) that catalyze the separation of epigenetic marks from histones or DNA. Readers such as bromodomain (BRD) proteins take

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up secondary chromatin modifications or transcriptional mechanism elements of epigenetic marks [18].

BRDs regulate gene transcription by binding acetylated lysines to histone tails, targeting chromatin-modifying enzymes and other protein mechanisms to specific regions in chromatin [28]. BRDs’ extra-terminal domain “BET” performs regulatory function by recruiting specific effector proteins, and as a result of the binding of two acetyl groups with a single bromodomain; BRD2, BRD3, BRD4, and BRDT have been identified [28, 40]. Since BET proteins exert critical functions in transcription and malignancy, the development of BET inhibitors has been deeply investigated [1]. BET BRD inhibitors disrupt BET protein binding to acetylated lysine residues of chromatin and thus, suppress the transcription of various genes, including the oncogenic transcription factors [31] and downregulate the transcription of genes regulated by “super-enhancers-activators” such as MYC and other genes essential for neoplastic cells [3].

Both HDACs and BRD inhibitors are functional in many biological processes, including transcription, chromatin remodeling, cell cycle, signal transduction, and regulation of gene expression in various malignancies [20, 30]. Vorinostat [suberoylanilide hydroxamic acid (SAHA)] is one of the first HDAC inhibitors that supports protein acetylation, modulates gene expression, and inhibits differentiation and growth of tumor cells which in turn induces apoptosis; thus, its preclinical and clinical studies are progressing rapidly. The oral non-selective, second-generation polar compound Vorinostat [4] has been approved by the US FDA for the treatment of relapsed/refractory cutaneous T-cell lymphoma under the name “Zolinza” [13]. Besides, Vorinostat has promising clinical reflections against different hematological tumors, both as monotherapy and combined therapies [17].

The initially defined BET inhibitors were two small molecules namely JQ1 and IBET762; have been deeply investigated in various hematological malignancies, and especially early clinical studies have been performed particularly in AML [1, 11]. But PLX51107, a new selective BRD4 BET inhibitor is a promising pharmacological agent for clinical cancer research for disrupting chromatin remodeling and regulating gene expression.

In this study, the new epigenetic agent BRD inhibitor PLX51107 and HDAC inhibitor SAHA’s single/combined treatment responses were analyzed in case of cell proliferation, cytotoxicity, apoptosis, cell cycle arrest, and target gene expression regulation in AML and healthy B-lymphocyte cells; HL60 and NCIBL2171, respectively; in vitro.

### Materials and method

#### Culturing conditions of cell lines

Acute promyelocytic myeloid leukemia cell line HL60 (CCL-240™) and non-leukemia cell line NCIBL2171 (CRL-5969™) were obtained from ATCC (American Type Culture Collection). Culturing of the cells was performed in RPMI 1640 medium (Biological industries, Cat#52400-025), 10% fetal bovine serum (Gibco, Cat# 11503-056), 2 mM l-glutamine (Biological industries, Cat#03-020-1B), 100 U/mL penicillin, 0.1 mg/mL streptomycin (Biological industries, Cat#03-031-1B) were used for cell growth and proliferation. The cells were maintained in a humidified incubator with 95% air and a 5% CO₂ atmosphere at 37 °C and followed up under an inverted and light microscope. All experimental setup of the cells was carried out in a laminar flow cabinet.

#### PLX51107 and Vorinostat agents’ stock solution preparation

HDAC inhibitor Vorinostat (MW 264.3 g/mol) was purchased (Sigma, Cat#S1047) and diluted with 4.5 mL of the serum-free medium for the main stock of 0.75 mM and the lower concentrations were prepared as a serial dilution from this main stock. The serial dilution concentrations were prepared as 10–7.5–5–2.5–1 μM/mL, respectively.

BET inhibitor PLX51107 (MW 438.48 g/mol) was purchased (Sigma, Cat#sc-518021) and diluted with 1.5 mL of the serum-free medium for the main stock of 7.602 mM and the lower concentrations were prepared as a serial dilution from this main stock. The serial dilution concentrations were prepared as 10–7.5–5–2.5–1 μM/mL, respectively.

#### Cell proliferation and cell cytotoxicity assays

A cell proliferation assay was set up to determine the number of cells to be used previously for cytotoxicity analysis spectrophotometrically in a microplate reader at 450 nm absorbance at 620 nm reference range (Thermo Scientific Multiskan FC instrument using “Scanning for multiscan F.C.2.5.1 software; Vantaa, Finland) by XTT analyses (Cell Proliferation Kit XTT, Biological industries, Cat# 20-300-1000) in triplicates for 96 h.

To determine the cytotoxic IC₅₀ dose of Vorinostat and PLX51107 specific for both non-leukemic healthy control and leukemic cells; a cell viability and proliferation assay was carried out by the same XTT method for 72 h. The cells were seeded in 96 well plates at a density of 10×10³ in 0.1 mL medium/well and increasing doses of agents were applied as 1 to 10 μM for HL60 cells and 1 to 7.5 μM for
NCIBL2171 cells and their untreated control counterparts. Quantitative measurement of cell viability and cytotoxic IC$_{50}$ values were determined in the same microplate reader. The obtained data were assessed, cell proliferation curves were generated and IC$_{50}$ values were calculated with the CalcuSyn 2.0 (Biosoft) for each agent specific for HL60 and NCIBL2171 cells for both 48th, and 72nd hours.

**Combined treatment of Vorinostat and PLX51107 upon cells**

Based on the determined mono IC$_{50}$ doses of each agent upon cells, another cell viability XTT assay was carried out to designate the combination IC$_{50}$ doses as $\times$0.75, $\times$1/2, $\times$1.5, and $\times$2 of the mono doses for 48th and 72nd hours. Synergistic, additive, or antagonistic reflections of the combination doses were determined in CalcuSyn 2.0 (Biosoft) software with cytotoxicity percentage values. The dose-effect curves were drawn and the combination indexes (CI) and dose reduction indexes (DRI) of the doses were also determined by the software.

**Apoptosis analysis**

Apoptotic cases of HL60 and NCIBL2171 cells following treatment of the determined mono and combination IC$_{50}$ doses of Vorinostat and PLX51107 were assessed through the “FITC Annexin V-PI apoptosis kit” (Sigma-Aldrich Chemie, Cat#SK00482-06) for 48th and 72nd hours and the obtained data were evaluated in BD Accuri™ C6 Flow Cytometer (New York NY, USA). For calculating apoptotic cell rates in each group, early and late apoptotic cell rates were combined.

**Cell cycle analysis**

For the leukemic HL60 cell line, the cells were treated with the mono and combined IC$_{50}$ doses of Vorinostat and PLX51107 for 72 h; whereas non-leukemic control cells were treated for 48 h and “BD Cycletest Plus DNA Reagent Kit (Becton Dickinson, Cat#340242)” was used for cell cycle arrest analysis. The effects of the treatments on the cell cycle were evaluated in the flow cytometry (BD Accuri™ C6 Plus flow cytometer) device.

**Determining gene expression profiles by qRT-PCR**

Another experimental setup was carried out to determine the expressional regulations of selected 19 target genes (the primer pairs are given in Table 1) following Plx51107, Vorinostat mono, and combination doses at 48th and 72nd hours by real-time RT-qPCR in triplicates. For this purpose, at the end of incubation time, total RNA was isolated from each cell via the “Magna Pure Compact RNA Isolation” kit manual in Magna Pure Compact System (Roche). The concentration and purity of the RNA samples were determined in the “NanoDrop™ 2000/2000c Spectrophotometer” (Thermo Scientific™, Catalog number: ND-2000). cDNA synthesis reaction was performed with the “OneScript® Plus cDNA Synthesis Kit” (Abm, Cat# G236) and used in the qPCR reactions with the “FastStart Essential DNA Green Master” (Roche, Cat#06402712001) for quantification of the target genes in LightCycler® 480 Instrument (Roche Diagnostic, Product No. D 100 03) in triplicates. The expression profiles of the target genes within each cell group were determined by $2^{-\Delta\Delta Ct}$ method. Statistical analyzes were performed using the Student T-test via the “GraphPad Prism ver:8.0.2” program.

**Quantitative assessment of BRD4 protein expression by western blot analyses**

Protein extraction of both of the cells was performed using “Ripa Buffer” (Sigma Aldrich, Cat#RO278) instructions. The protein amounts were assessed by the Bradford method using bovine serum albumin standards starting from 0.25 to 2 mg/mL concentrations (Thermo Scientific, Fermentas, USA). An amount of 18 µg/mL for each protein sample was resolved at 4–12% NuPAGE Bis–Tris Gels” (Invitrogen by Thermo Fisher, Cat#NP0321BOX) and run in the “XCell SureLock Mini-Cell Electrophoresis System” (ThermoFisher, Cat#EI0001) at 200 V for 50 min; and transferred to PVDF membranes using the iBlot dry transfer system (Invitrogen Corporation, Carlsbad, CA, USA). The target BRD4 and the loading control primer antibody concentrations used were as follows: 1:1000 diluted BRD4 (Santa Cruz Biotechnology, Cat#sc-518021) and β-actin (Thermo, Cat#8457S), Cell Signaling Technology, Beverly, MA, USA). Primary antibody incubation, blotting, and secondary antibody incubation steps were performed via the Western Breeze Chromogenic Detection Kit (Invitrogen Corporation, Carlsbad, CA, USA, Cat#WB7103). Quantitative detection of proteins was evaluated with a gel view using Image J 1.46r. software and “ImageJ application” program (http://rsbweb.nih.gov/ij/).

| Table 1 | Identified target genes |
| --- | --- |
| Apoptosis | Bcl-x, Bax, Casp3, Casp8, Bcl-2 |
| Cell cycle | ATM, CCND1, CDKN2A |
| Epigenetic | DNMT3a, BRD2, BRD4 |
| AML | RUNX1, GATA1, TIF2 |
| Housekeeping gene | GAPDH |
| Other | STAT1, STAT3, IKZF1, IKZF3 |

This completes the summary of the document's content.
Statistical analyses

Vorinostat and PLX51107 IC₅₀ values were calculated with the CalcuSyn and the graphs were created with GraphPad 8.0.2 software. Target genes’ mRNA expression levels detected by qRT-PCR, apoptotic analyses, and cell cycle assay results were evaluated with Student T-test referring to the significance of p < 0.05 in GraphPad 8.0.2 software. Quantitative detection of proteins was evaluated using Image J 1.46r software and the “ImageJ application” program (http://rsbweb.nih.gov/ij/). Advanced functional analysis was performed using CalcuSyn and GraphPad software.

Results

Proliferation assay

A proliferation assay was set up to determine the number of cells to be used in the cytotoxicity analysis for NCIBL2171 and HL60 cells. The cell numbers required for the experiments were determined as 4 × 10⁴ cells/mL for NCIBL2171 cells and 5 × 10⁴ cells/mL for HL60 cells (Fig. 1).

Effects of PLX51107 and Vorinostat on the viability of leukemic cells: the agents selectively display cytotoxic effects

The cytotoxicity assays performed by the XTT analyses revealed that Plx51107 mono treatment upon NCIBL2171 cells pointed out the IC₅₀ dose as 1.24 µM (r-value = 0.97949) for 48th and 0.61 µM (r-value = 0.60) for 72nd hours; it was detected as 22.46 µM (r-value = 0.95) for 48th and 6.68 µM (r-value = 0.99520) for 72nd hours for HL60 cells (Fig. 2). As for the Vorinostat’s mono treatment results; while the IC₅₀ dose was detected as 2.01 µM (r-value = 0.87650) for 48th and 23.10 µM (r-value = 0.22) for 72nd hours for NCIBL2171 cells; it was detected as 31.72 µM (r-value = 0.76159) for 48th for HL60 cells and 4.3 µM (r-value = 0.92783) for 72nd hours for HL60 cells (Fig. 3). All data are given in Table 2.

Cytotoxicity of combination treatments of Plx51107 and Vorinostat

The effects of different combinations of Vorinostat and Plx51107 on HL60 and NCIBL2171 cell lines were evaluated. Dose-effect curve, median-effect graphs, and conservative isobologram graphs were determined as a result of the combination cytotoxicity analyses; using IC₅₀ × 0.5, IC₅₀ × 0.75, IC₅₀ dose; IC₅₀ × 1.5; IC₅₀ × 2 doses. Determined cell numbers as a result of proliferation assay were seeded in each well for treatment and varying Plx51107 and Vorinostat doses were applied to cells. At the end of the 72nd hour, the combined effect of the agents upon cells was determined.

When treatment doses were evaluated for NCIBL271 cells according to their CI values; since an antagonistic effect was detected for any differing combination dose; downstream experiments were carried out with determined IC₅₀ mono doses of the agents.

Combination analysis revealed synergistic killing effects between Plx51107 and Vorinostat against HL60 cells at different exposure times. So, Vorinostat IC₅₀ dose with Plx51107 IC₅₀ dose (combination 1) and Vorinostat IC₅₀ dose with 10 µM Plx51107 IC₅₀ dose (combination 2) was revealed for further analyses (Fig. 4).

Apoptotic effects of Plx51107, Vorinostat, and combined treatments upon cells

Apoptotic changes were evaluated using the Annexin V method, in the scope of the determined IC₅₀ doses for cells. For NCIBL2171 cells, Plx51107 and Vorinostat agents were applied at 1.24 µM and 2.01 µM, respectively, for 48 h; and the apoptosis rates were detected. Due to the antagonistic effect of the combination of the agents observed in the

![Fig. 1 Proliferation analysis for a HL60 cells as 5 × 10⁴ cells/mL and b NCIBL2171 cells 4 × 10⁴ cells/mL.](image)
Instead of combined treatment, single IC_{50} doses were applied, while 0.7% apoptotic rate was observed with Vorinostat, it was 1.5% for Plx51107 when compared to untreated control cells (Fig. 5).

As for HL60 cells, the determined IC_{50} doses were 6.68 μM for Plx51107, and 4.3 μM for Vorinostat; as a result of the combination analysis, the observed synergistic effect dose was 10 μM indicating the ×1.5 fold of detected IC_{50} for Plx51107. Compared to the untreated control group, 94.8% apoptosis at the IC_{50} dose of Plx51107, and 97.4% apoptotic rates for 10 μM Plx51107 dose were detected. Vorinostat IC_{50} dose treatment resulted in 97.4% of apoptosis induction. Afterward, apoptotic effects of combination doses were revealed and, while 57.4% apoptosis was observed

Table 2 Plx51107 and Vorinostat IC_{50} dose for NCIBL2171 and HL60 cell lines

|          | NCIBL2171 |          | HL60 |          |
|----------|-----------|----------|------|----------|
| IC_{50} (μM) | r-value | IC_{50} (μM) | r-value |
| 48 h     |           |             |     |          |
| Plx51107 | 1.24  | 0.97 | 22.46 | 0.95 |
| Vorinostat | 2.01 | 0.87 | 31.72 | 0.76 |
| 72 h     |           |             |     |          |
| Plx51107 | 0.61  | 0.60 | 6.68  | 0.99 |
| Vorinostat | 23.10 | 0.22 | 4.3   | 0.92 |

Fig. 2 Plx51107 cytotoxicity analysis in a NCIBL2171 cell line as 1.24 μM (r-value = 0.97949) for 48th and 0.61 μM (r-value = 0.60) for 72nd hours and b HL60 cell line as 22.46 μM (r-value = 0.95) for 48th and 6.68 μM (r-value = 0.99520) for 72nd hours

Fig. 3 Vorinostat cytotoxicity assay in a NCIBL2171 cells as 2.01 μM (r-value = 0.87650) for 48th and 23.10 μM (r-value = 0.22) for 72nd hours and b HL60 cells as 31.72 μM (r-value = 0.76159) for 48th for HL60 cells and 4.3 μM (r-value = 0.92783) for 72nd hours
for combination 1 (6.68 μM Plx51107 + 4.3 μM Vorinostat), apoptosis rate was 88.8% for combination 2 (10 μM Plx51107 + 4.3 μM Vorinostat). In the light of these results, more apoptotic induction was detected in single treatments of Plx51107 and Vorinostat IC50 doses for leukemic cells; but also combination 2 indicated higher rates of apoptotic induction. All data are given in Fig. 6.

Effects of Plx51107, Vorinostat, and combination treatments on cell cycle

After treating NCIBL2171 and HL60 cells with the previously determined IC50 doses either with Plx51107 and Vorinostat or their combinations, cell cycle arrest analyzes were performed. Untreated NCIBL2171 cells appeared to be in 43.1% of the G0/G1 phase. Plx51107 and Vorinostat IC50 dose treatment showed an increase of up to 50% and 69.9% of G0/G1 arrest, respectively. (Fig. 7; Table 3). Based on this, it was concluded that NCIBL2171 cells caused G0/G1 arrest. For HL60 cells, it was observed that 52.3% of the untreated control cells were in the G0/G1 phase and 20.7% were in G2/M. The percentage of cells in the G0/G1 phase was increased to 59.0% following Plx51107 IC50 dose treatment and to 66.9% with 10 μM Plx51107 treatment. Vorinostat IC50 treatment led to an increase of 33.6% of G2/M arrests. Combination 1 increased G0/G1 arrest to 59.4%, and combination 2 increased it to 57.8% (Fig. 8; Table 3). The combination doses were observed in the G0/G1 arrest. In general, it is detected that Plx51107 single treatments and combined treatments accelerated the leukemic cell arrest in G0/G1 phase. But it is detected that Vorinostat single-dose treatments accelerated the leukemic cell arrest in the G2/M phase.

Effects of Plx51107, Vorinostat, and combination doses upon gene expression profiles

NCIBL2171 cells were treated with the IC50 dose determined for Plx51107 and Vorinostat; as 1.24 μM and 2.01 μM, respectively, and expressional regulations of selected target genes were evaluated by comparing the results referring to as untreated control cells. The target genes Bcl-x, Bax, Casp3, Casp8, Bcl-2, ATM, CCND1, CDKN2A, STAT1, STAT3, BRD2, TIF2, IKZF3 expressions exhibited a significant > ×2 or more increases by Vorinostat IC50 dose treatment. Besides, DNMT3A (14-fold), GATA1 (2.50-fold), Runx1 (1.10-fold), and BRD4 (1.22-fold) expressions were down-regulated. As for Plx51107 treatment, DNMT3A, GATA1, RUNX1, and
BRD4 genes expressions were < × 2-fold decreased; a 2-fold or more increase was observed in the expression of other genes (Table 4).

As for the expressional regulation of HL60 cells upon the different type and combination doses of the agents, significant changes were detected for Bcl-x, Bax, Casp3, Casp8, Bcl-2, ATM, CCND1, CDKN2A, STAT1, STAT3, DNMT3A, GATA1, RUNX1, BRD2, TIF2, IKZF3, genes compared to their untreated control counterparts (Table 5). For the Plx51107 IC_{50} dose, decreases were observed in the expression of other genes except for the GATA1 (2.46-fold increase). For the dose of 10 μM Plx51107, except for the IKZF1 (0.28-fold increase) gene, decreased expression of all other genes was observed. For the dose of Vorinostat IC_{50}, the expression of Bcl-x (1.07-fold), Bax (1.91-fold), CCND1 (1.87-fold), DNMT3A (1.05-fold), GATA1 (5.8-fold) and IKZF1 (1.01-fold) genes’ expressions were increased, whereas other genes’ expressions were down-regulated. The decreased expressional regulation for most of the genes was detected for combination doses. Representative heatmaps for up-regulated and down-regulated genes in NCIBL2171 and HL60 cells are given in Fig. 9.

**Evaluating BRD4 protein expression following treatments**

To further investigate the translational regulation of the BRD4 protein of the cells following Plx51107, Vorinostat, and the combination doses treatment; western blot analyses were performed. The results showed that treatment of NCIBL2171 cells with Plx51107 and Vorinostat led to a decrease in BRD4 protein expression. As for HL60 cells, either mono or combined treatment of the agents indicated expressional down-regulation of BRD4 protein. Plx51107, Vorinostat, and combination 1 and 2 doses caused a decrease in the BRD4 expression (Fig. 10).
Fig. 6 Mechanism of cell death induced by Plx51107, Vorinostat and their combination in HL60 cells (a), 6.68 μM Plx51107 dose (b), 10 μM Plx51107 dose (c), 4.3 μM Vorinostat dose (d), combination 1 (6.68 μM Plx51107 + 4.3 μM Vorinostat) and combination 2 (10 μM Plx51107 + 4.3 μM Vorinostat) doses (e, f) and fold changes of the apoptotic effect at 72 h (g) (****p < 0.0001)
Discussion

AML treatment focuses on targeted treatment strategies with a higher degree of affinity and reduced toxicity to gain increases in improved survival and recovery rates [19], thus epigenetic therapeutics particularly seems to be an efficient candidate in this area [43]. This study aimed to evaluate the cellular effects of the newly discovered BRD4 inhibitor Plx51107 and the HDAC inhibitor Vorinostat, on the AML model HL60 cells and non-leukemic control cells, in vitro. Also, the hypothesis that a combination of Plx51107 and Vorinostat might have resulted in more remarkable outstanding and anticancer effects in AML when compared to either agent single usage for HL60 leukemic cells were tested. This seems to be the first study revealing the cellular effects of the Bet inhibitor Plx51107 and HDAC inhibitor Vorinostat combination treatment in AML to the best of our knowledge and literature research.

The Bet inhibitor Plx51107 was reported to be effectively combined with an anti-PD-L1 inhibitor to delay tumor growth in melanoma cells [26], whereas, we evaluated the cytotoxic effects of it upon AML cell line HL60 for the first time. As the determined IC50 value of Plx51107 was 6.68 μM at 72nd hours for HL60 cells; the widely studied BET inhibitor JQ1 caused a dose-dependent

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Table 3  Percent values of cell cycle changes and fold change results of Plx51107, Vorinostat, and combination doses of (a) NCIBL2171 cells and (b) HL60 cells

|                          | Go-G1 | S      | G2-M   |
|--------------------------|-------|--------|--------|
| NCIBL2171 Kontrol (%)    | 43.1  | 39.3   | 18.5   |
| NCIBL2171 Plx. (%)       | 50    | 45.4   | 5.6    |
| NCIBL2171 Plx Fold change| 1.16  | 1.15   | 0.30   |
| NCIBL2171 Vor. (%)       | 69.9  | 28     | 2.8    |
| NCIBL2171 Vor Fold change| 1.62  | 0.71   | 0.15   |

(b)

|                          | Go-G1 | S      | G2-M   |
|--------------------------|-------|--------|--------|
| HL60 control (%)         | 52.3  | 25.2   | 20.7   |
| HL60 IC50 Plx. (%)       | 59    | 23.9   | 15.2   |
| HL60 10 μm Plx. (%)      | 66.9  | 20.5   | 10.1   |
| HL60 Plx. IC50 fold change| 1.12  | 0.94   | 0.73   |
| HL60 Plx. 10 μm plx. Fold change| 1.27 | 0.81 | 0.49 |
| HL60 Vor. (%)            | 44.5  | 22.6   | 33.6   |
| HL60 Vor. Fold change    | 0.85  | 0.89   | 1.62   |
| HL60 combination 1 (%)   | 59.4  | 23.2   | 12.2   |
| HL60 combination 2 (%)   | 57.8  | 24.6   | 12.2   |
| HL60 combination 1-fold change| 1.13 | 0.92 | 0.58 |
| HL60 combination 2-fold change| 1.10 | 0.97 | 0.58 |
decrease in cell viability with an IC$_{50}$ of ∼ 500 nmol/L for OCI-AML3, RAGI, and K562 cells [35]. So, it can be concluded that Plx51107 exhibited a higher dose of IC$_{50}$ for AML cells compared to the JQ1 inhibitor. As for Vorinostat treatment, a 4.3 µM IC$_{50}$ dose was detected in HL60 cells at the 48th hour; and similar results were reported as 1.52 ± 0.07 µmol/L in HL60 cells [12] and less than 10 µM in both HL60 and MCF-7 cells [41]. The IC$_{50}$ values determined for Plx51107 and Vorinostat in NCIBL2171 healthy control cells were 1.24 µM and 2.01 µM at 72nd hours, respectively, and lower than HL60 leukemic cells as expected. The combination treatments of Plx51107 and Vorinostat in HL60 cells seemed to be the first one in the literature, and we detected a synergistic effect only for HL60 cells since the combination IC$_{50}$ dose was reduced to 3.26 µM; that led us to conclude that sensitization to the agents was accelerated in leukemic cells. It was reported that mono and combination treatments of JQ1 with Nutlin increased apoptosis in OCI-AML3 cell lines, and the combination was more effective [35]. In another study, JQ1 inhibitor was reported to increase the apoptotic cell rate from 6 to 48% in leukemia and lymphoma cell lines [24]. Vorinostat increased apoptosis in mouse bone marrow [2], also while causing an apoptotic rate of 5% in HL60 cells, it was increased to 20% in combination with decitabine [42]. So, we detected higher apoptosis rates reaching ∼98% rather than in the literature. Based on the results, it is possible to say that single doses and combination doses induce apoptosis at similar rates.

Following cytotoxicity assays, apoptotic analyses were performed and detected that control NCIBL2171 cells showed very low apoptosis rates about ∼ 1.5% for Plx51107 and Vorinostat IC$_{50}$ doses. But leukemic cells exhibited very high rates of apoptosis either with mono or combination doses of the agents; but superior for mono dose treatments. It was reported that mono and combination treatments of JQ1 with Nutlin increased apoptosis in OCI-AML3 cell lines, and the combination was more effective [35]. In another study, JQ1 inhibitor was reported to increase the apoptotic cell rate from 6 to 48% in leukemia and lymphoma cell lines [24]. Vorinostat increased apoptosis in mouse bone marrow [2], also while causing an apoptotic rate of 5% in HL60 cells, it was increased to 20% in combination with decitabine [42]. So, we detected higher apoptosis rates reaching ∼98% rather than in the literature. Based on the results, it is possible to say that single doses and combination doses induce apoptosis at similar rates.

Then, the effects of treatments on the cell cycle were analyzed, and detected that either Vorinostat or Plx51107 caused G$_{0}$/G$_{1}$ arrest in NCIBL2171 cells. Both Plx51107 and Vorinostat mono treatment doses induced G$_{0}$/G$_{1}$ phase arrest. It is observed that Plx51107 caused an increase in G$_{0}$/G$_{1}$ arrest and Vorinostat causes arrest in G$_{2}$/M. When two agents were used together, this caused G$_{0}$/G$_{1}$ arrest. Based
on these results, when our data were compared with the literature, it was observed that treatment of HL60 cells with Vorinostat caused G2/M arrest and a dose-dependent reduction of cells in the G1 and S phases. This suggested that our results were consistent with the Vorinostat dose [32]. In another study, it was concluded that JQ1 treatment, which is a BET inhibitor, decreased cell viability with G0/G1 cell cycle arrest in myeloid leukemia cell lines, and it was consistent with our results [29].

Anti-tumor activities of BET BRD inhibitors have been demonstrated in different preclinical models from hematological or solid tumors, and BET BRD inhibitors reduce the transcription of genes regulated by “super-enhancers-activators” such as MYC and other genes essential for neoplastic cells. BET BRD inhibitors are in ongoing oncology/hematology phase I clinical trials [3]. Since these agents prevent the expression of some growth-promoting genes in leukemic cells, induce apoptosis, and inhibit proliferation in tumor cells by overexpressing BRD proteins, next we checked some target genes’ differing expression profiles. As for the apoptotic genes, Plx51107 down-regulated anti-apoptotic Bcl-x and pro-apoptotic Bax genes in HL60 cells, whereas Vorinostat caused down-regulation of other apoptotic genes except for Casp8; and combination treatments caused down-regulation of all apoptotic genes.

### Table 4 Calculation of fold change for NCIBL2171 cells in genes normalized to GAPDH

| Genes  | NCIBL2171 1.24 μM Plx | NCIBL2171 2.01 μM Vor |
|--------|----------------------|----------------------|
| BCL-X  | 43.41 Log2 5.44     | 36.50 Log2 5.19      |
| BAX    | 144.01 Log2 7.17    | 158.68 Log2 7.31     |
| CASP3  | 178.53 Log2 7.48    | 335.46 Log2 8.39     |
| CASP8  | 114.5632 Log2 6.84  | 145.01 Log2 7.18     |
| BCL2   | 410.15 Log2 8.68    | 968.76 Log2 9.92     |
| ATM    | 160.9 Log2 7.33     | 116.16 Log2 6.86     |
| CCND1  | 73.01 Log2 6.19     | 467.88 Log2 8.87     |
| CDKN2A | 413.00 Log2 8.69    | 1951.00 Log2 10.93   |
| STAT1  | 137.19 Log2 7.10    | 176.07 Log2 7.46     |
| STAT3  | 117.78 Log2 6.88    | 164.28 Log2 7.36     |
| DNMT3A | 0.15 Log2 −2.76     | 0.00 Log2 −14.01     |
| GATA1  | 0.12 Log2 −3.11     | 0.17 Log2 −2.52      |
| TIF2   | 9.78 Log2 3.29      | 5.74 Log2 2.52       |
| IKZF1  | 6.59 Log2 2.72      | 3.03 Log2 1.60       |
| RUNX1  | 0.45 Log2 −1.16     | 0.47 Log2 −1.10      |
| IKZF3  | 0.04 Log2 −4.74     | 5.82 Log2 2.54       |
| BRD2   | 20.53 Log2 4.36     | 38.59 Log2 5.27      |
| BRD4   | 0.85 Log2 −0.24     | 0.43 Log2 −1.22      |

### Table 5 Calculation of fold change for HL60 cells in genes normalized to GAPDH

| Genes  | HL60 6.68 μM Plx | HL60 10 μM Plx | HL60 4.3 μM Vor |
|--------|-----------------|---------------|----------------|
| BCL-X  | 0.52 Log2 −0.95 | 0.86 Log2 −0.21 | 2.10 Log2 1.07 |
| BAX    | 0.24 Log2 −2.08 | 0.97 Log2 −0.05 | 3.76 Log2 1.91 |
| CASP3  | 0.13 Log2 −2.96 | 0.22 Log2 −2.20 | 0.50 Log2 −0.99 |
| CASP8  | 0.02 Log2 −5.71 | 0.02 Log2 −5.46 | 0.01 Log2 −6.55 |
| BCL2   | 0.02 Log2 −5.99 | 0.02 Log2 −5.39 | 0.04 Log2 −4.68 |
| ATM    | 0.02 Log2 −5.93 | 0.00 Log2 −16.80 | 0.01 Log2 −6.53 |
| CCND1  | 0.77 Log2 −0.37 | 0.63 Log2 −0.67 | 3.66 Log2 1.87 |
| CDKN2A | 0.11 Log2 −3.17 | 0.15 Log2 −2.70 | 0.84 Log2 −0.26 |
| STAT1  | 0.04 Log2 −4.76 | 0.08 Log2 −3.61 | 0.21 Log2 −2.22 |
| STAT3  | 0.05 Log2 −4.46 | 0.01 Log2 −7.64 | 0.01 Log2 −7.25 |
| DNMT3A | 0.14 Log2 −2.82 | 0.88 Log2 −0.19 | 2.07 Log2 1.05 |
| GATA1  | 5.50 Log2 2.46  | 17.63 Log2 4.14  | 55.72 Log2 5.80 |
| TIF2   | 0.02 Log2 −5.75 | 0.00 Log2 −9.43 | 0.00 Log2 −8.99 |
| IKZF1  | 0.86 Log2 −0.21 | 1.21 Log2 0.28   | 2.01 Log2 1.01 |
| RUNX1  | 0.01 Log2 −7.52 | 0.00 Log2 −9.33 | 0.00 Log2 −9.78 |
| IKZF3  | 0.01 Log2 −7.21 | 0.00 Log2 −8.18 | 0.00 Log2 −8.69 |
| BRD2   | 0.01 Log2 −7.48 | 0.00 Log2 −9.66 | 0.00 Log2 −10.05 |
| BRD4   | 0.01 Log2 −7.48 | 0.00 Log2 −9.64 | 0.00 Log2 −9.76 |

### Table 6 Calculation of fold change for HL60 KOMBINASYON1 and HL60 KOMBINASYON2

| Genes  | HL60 KOMBINASYON1 (6.68 μM Plx + 4.3 μM Vor.) | HL60 KOMBINASYON2 (10 μM Plx + 4.3 μM Vor.) |
|--------|---------------------------------------------|---------------------------------------------|
| BCL-X  | 0.54 Log2 −0.89 | 0.19 Log2 −2.39 |
| BAX    | 1.01 Log2 0.02  | 0.78 Log2 −0.36 |
| CASP3  | 0.15 Log2 −2.70 | 0.05 Log2 −4.23 |
| CASP8  | 0.01 Log2 −6.93 | 0.00 Log2 −9.38 |
| BCL2   | 0.02 Log2 −5.36 | 0.01 Log2 −7.05 |
| ATM    | 3.14 Log2 1.65  | 0.00 Log2 −9.64 |
| CCND1  | 1.42 Log2 0.51  | 0.43 Log2 −1.23 |
| CDKN2A | 0.13 Log2 −2.99 | 0.07 Log2 −3.8 |
| STAT1  | 0.09 Log2 −3.40 | 0.02 Log2 −5.59 |
| STAT3  | 0.00 Log2 −9.77 | 0.04 Log2 −4.5 |
| DNMT3A | 0.09 Log2 −0.01 | 0.32 Log2 −1.64 |
| GATA1  | 17.27 Log2 4.11 | 6.41 Log2 2.68 |
| TIF2   | 0.00 Log2 −9.45 | 0.00 Log2 −9.23 |
| IKZF1  | 1.26 Log2 0.33  | 1.27 Log2 0.35 |
| RUNX1  | 2288.20 Log2 11.16 | 0.00 Log2 −9.48 |
| IKZF3  | 0.00 Log2 −9.04 | 0.00 Log2 −8.21 |
| BRD2   | 0.00 Log2 −9.77 | 0.00 Log2 −9.73 |
| BRD4   | 0.00 Log2 −10.09 | 0.00 Log2 −9.82 |
and down-regulation of Bcl-2 in AML cell lines [23]. Although the cell lines were the same, the used drugs were different, similar results were obtained. HDAC inhibitors and BET inhibitors have been shown to suppress Bcl-2 genes in leukemia cells and cause a decrease in expression [30]. In our study, it was observed that Bcl-2 was down-regulated in the AML cell line HL60, whereas we observed compatible results.

As for cell cycle regulation-related genes’ expressions, CDKN2A was up-regulated in NCI602171 cells via Vorinostat treatment; whereas it was down-regulated in Plx51107 and Vorinostat combination in HL60 cells. Since cyclin-dependent kinase inhibitors accelerate cell cycle transition by suppressing CDKN2A and CDKN2B and activated mitogen-activated protein kinase signaling (Chaturverdi et al., 2013), the combination treatment acted similarly in leukemic cells. An expression increase was observed in the ATM gene in HL60 cells following Plx51107 and Vorinostat treatment; whereas ATM expression was reported to be down-regulated in AML cell lines and the bone marrow of AML patients [38, 39]. We also detected that both agents caused an expression decrease in the CCND1 in HL60 cells. Since Chen et al. [5] had shown that high expression of CCND1 caused a poor prognosis for fusion AMLs, combination treatment still indicated a therapeutic approach. These results suggest that mono and combined usage of these two
agents for induced cell cycle arrest were in line with expression regulations of these genes.

JAK/STAT family seems to be highly correlated with leukemia pathogenesis [10], then the expressional changes were detected. Similarly, STAT1 was down-regulated due to Plx5107 and Vorinostat treatment in leukemic cells. In chronic myeloid leukemia, the tyrosine kinase inhibitor Imatinib caused inhibition and decreased expression of the STAT1 gene [6], leading to a therapeutic approach. Besides, STAT3—a potent oncogene—exhibited increased expression in AML cell lines compared to control cells, as being a predictor of AML risk [14]. There was an expressional decrease in the STAT3 gene at the mono Plx51107 IC50 dose and the combination 2 (10 μM Plx51107 + 4.3 μM Vorinostat) dose in HL60 cells; so both STAT1 and STAT3 were down-regulated in leukemic cells. When another transcription factor GATA1-regulator of erythropoiesis [15] expression was evaluated, a decrease was detected with each agent treatment in leukemic cells. GATA1 knockdown was reported to be an accelerator of leukemic stem cells to undergo functional alterations due to exposure to chemotherapeutics [34]. So, GATA1 down-regulation points out a therapeutic approach to leukemia. As RUNX1 is highly expressed in all cancer cells, but especially in the AML cells [38, 39], we analyzed its expression and detected a decrease in HL60 leukemic cells following either Plx51107 or Vorinostat treatment, indicating a novel therapeutic approach.

When epigenetics-related genes’ expressional regulations were evaluated, a significant down-regulation was detected in DNMT3a in HL60 cells by treatment with PLX51107, Vorinostat, and their combinations. Similarly, Stewart et al. have reported that DNMT3a isoforms showed selective down-regulation in AML cells [36]. Other major players of epigenetics in the case of chromatin remodeling, IKZF1 and IKZF3 genes’ expressions were highly and significantly decreased in leukemia cells. It was reported for multiple myeloma cells that their growth was inhibited by ubiquitination and degradation of the IKZF1 and IKZF3 genes [21]. Finally, we checked BRD4 expression both at mRNA and protein levels, expressional down-regulations were detected. In a study that used similar agents as ours; it was revealed that the BRD inhibitor JQ1 and the HDAC inhibitor Nutlin-3 impaired the BRD4-mediated transport of p53 to chromatin regions which caused apoptosis. In addition, suppression of BRD4 resulted in cell cycle arrest, senescence, p21 up-regulation, and cell death [35]. Also, Coudé et al. reported that another BET inhibitor OTX015 caused an

Fig. 10 Western blot analysis was performed to determine the expression change of BRD4 protein in a NCIBL2171 and c HL60 cells. After normalizing the dose bands obtained using the image program with the corresponding b-actin band, we plotted the comparison of fold change in protein expression with GraphPad software for both NCIBL2171 and d HL60 cell lines (****p<0.0001)
expression decrease in BRD2 and BRD4 in most cell lines [7]. A recent study designed by Shilei Liu et al. showed that BRD4 inhibitor and HDAC inhibitor inhibited the proliferation of gallbladder cancer synergistically in vitro and in vivo (Shilei [22]. We also detected that our BET inhibitor PLX51107, Vorinostat, and their combined treatments down-regulated BRD4 expression, achieved cell cycle arrest, and induced apoptosis in leukemic cells. Overall, our data showed for the first time that the usage of new BET inhibitor PLX51107 selectively targeted leukemic cells and it could be a potential novel therapeutic agent in cancer therapy.

Conclusion

The existence of BET inhibitors will aid in the understanding of the roles of BRD-containing proteins in cancer and BRD inhibitors will likely become novel targets for the discovery of new potent and selective agents. Wide research of BET inhibitors in the preclinical and clinical stages will accelerate the potential of epigenetic-mediated approaches; leading to the development of precise, targeted, and efficient treatments for leukemia.

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Author contributions

HS and IA performed cell culture and follow-up. HS, IA, and IK performed cytotoxicity and apoptosis analysis of the agents on leukemia and non-leukemia cells. BTK determined gene expression changes by qRT-PCR and performed statistical analysis. IA and BTK has written the paper; BTK as the supervisor.

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Data availability

The datasets generated analyzed during the current study are available from the corresponding author on reasonable request. Consent to publish Since this article does not include any studies with human participants or animals, an informed consent form is not required.

Declarations

Conflict of interest

All authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by the author IA and BTK.

Informed consent

As the study was in vitro, informed consent was not required.

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