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Research paper

Potent induction of apoptosis by givinostat in BCR-ABL1-positive and BCR-ABL1-negative precursor B-cell acute lymphoblastic leukemia cell lines

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ABSTRACT

We have previously shown that givinostat can induce potent apoptosis in the *BCR-ABL1*-positive, *TP53*-wild type B-cell acute lymphoblastic leukemia (B-ALL) cell line SUP-B15. We extend our studies here to two additional B-ALL cell lines, *BCR-ABL1*-negative CCRF-SB and p210 *BCR-ABL1*-positive NAML1. Givinostat induced significant cell growth inhibition in both cell lines, with an IC50 of 0.65 ± 0.052 μM and 0.25 ± 0.028 μM in CCRF-SB and NAML1, respectively. The key signal protein of the BCR-ABL1, Crk-L1, was significantly reduced by givinostat treatment in NAML1. As in SUP-B15, givinostat induced apoptosis in both cell lines but showed different levels of cleavage of the procaspase proteins Casp-3, Casp-7 and PARP. Levels of cell cycle-DNA repair regulator p21, CHK1 and FANCD2 levels were markedly affected by givinostat treatment. These data further enrich our understanding of the mechanisms of the antineoplastic effects of givinostat in B-ALL and provide a preclinical rationale for the inclusion of givinostat or similar agent in leukemia therapy.

1. Introduction

Given the key role epigenetic dysregulation in mediating growth and maturation arrest in hematopoiesis, histone deacetylases (HDACs) are potential targets in the treatment of leukemia. Consequently, inhibitors of HDACs (HDIs) are being studied for therapeutic purposes [1,2]. HDIs promote or enhance several different anticancer mechanisms, such as induction of apoptosis, cell-cycle arrest, and cellular differentiation [3,4]. Recently, three HDACi (vorinostat, belinostat and romidepsin) received FDA approval for treatment of cutaneous or peripheral T-cell lymphoma, and the HDACi panobinostat for the treatment of multiple myeloma in combination with others medications [5,6].

There is the need for improved treatment of acute lymphoblastic leukemia (ALL), which particularly in adults has a low cure rate and high mortality. There are a large number of previous studies on the effects of HDACi on cultured cell lines as well as xenograft tumor models, which have indicated activity of these agents in lymphoid malignancies. Givinostat exerted significant DNA damages in some responders of T-cell ALL xenografts with apoptosis induced by this agent operating at least in part through genotoxic effects and modulation of DNA repair pathway [6,7].

Our recent study showed that givinostat significantly inhibited cell proliferation of p190 *BCR-ABL1* positive SUP-B15 cell line through inhibition of BCR-ABL1 signaling pathway. In that line, givinostat also activated caspase cascades and increased expression of p21 protein [8]. This study investigates the effects of givinostat treatment in B-cell ALL cell line with the p210/major variant of *BCR-ABL1* or the absence of BCR-ABL1 fusion.

2. Materials and methods

2.1. Reagents

BAX, Bcl-xL, AIF, LC3B, BECN1, PUMA, FANCD2, and ELM4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other sources for antibodies, including phospho-CrkL, caspase-3 and −7, PARP1 and GAPDH, were described previously [8]. Sources and preparation of propidium iodide (PI) and FITC Annexin V-FITC detection kit was described previously [8].

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2.2. Cell culture and drug treatment

CCRF-SB and NAML1 cells (ATCC) were grown in IMDM or RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (ATCC). The cells were seeded at 2×10^5/ml in 5% CO^2 at 37 °C in a T-50 polypropylene flask (Becton Dickinson Labware, Lincoln Park, N.J.), with the cap loosely placed to allow for gas exchange. The cells were grown with dimethyl sulfoxide vehicle (DMSO), 0–1.0 μM Givinostat, or imatinib for 12–72 h (hrs) when intact cells for MTT test (ATCC) or cells were harvested for protein or RNA extraction. *BCR-ABL1* fusion genes were detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR) as previously described [9,10].

2.3. Cell proliferation assay (MTS assay)

Cell proliferation was assessed using CellTiter96 AQueous One solution cell proliferation assay kit (Promega, Madison, WI). Four replicates per condition were measured using 100 μl of cell suspension at a density of 5×10^4 cells/well. Givinostat at 1.0 μM or DMSO was added to the suspension and cultured at 37 °C in 5% CO2. Before the conclusion of the experiment at 24, 48, 72 and 96 h, 20 μl of the tetrazolium reagent MTS was added to each well with additional culture for 4 h. The absorbance was measured at 490 nm using a microplate spectrophotometer (PowerWave, BioTek Instruments, Winooski, VT, USA), and the results were expressed as a percentage (%) of the control (vehicle alone).

2.4. Western blot analysis

Briefly, CCRF-SB (1×10^6) or NALM1 (1×10^6) cells were harvested, washed with PBS and resuspended in 1 × Laemmli sample buffer containing proteinase and phosphatase inhibitors. Fifty (50) μg of protein was separated by sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes. Membranes were probed with antibodies against corresponding proteins. The blots were visualized with ECL reagent (Amersham, Arlington Heights, IL) and then exposed to Hyperfilm (Amersham) as described previously [8,9].

2.5. Flow cytometry analysis

Cells treated or untreated cells for 48 h were fixed with ice-cold 70% ethanol for 1 h and/or stored at −20 °C until use. The cells were then washed with cold PBS and treated with RNase for 15 min at 37C, stained with 50 μg/ml PI and analyzed in flow cytometric analysis (FACSCalibur, Becton Dickinson, CA, USA). DNA content was calculated in the untreated cells with intact G1, S, and G2/M phases using Mod-Fit software (Verity Software House). The percentage of sub-G0/G1 were averaged based on triplicate measurements.

Flow cytometric analysis measures necrosis/apoptosis using an Annexin V-FITC/PI detection kit [11]. After treatment with 0.5 μM of Givinostat or vehicle for 12–48 h, cells were harvested by centrifugation and resuspended in 0.5% FBS-PBS. The cell suspension was double-stained with FITC annexin-V (50 μg/ml) and PI (50 μg/ml) and kept in the dark for 15 min. The fluorescence of each cell was analyzed by a using a nitrogen argon laser operating at 488/535 nm excitation/emission for annexin-V, with the fluorescence captured on the FL-1H channel, and at 488/620 nm excitation/emission for PI, with the fluorescence captured on FL-2H channel with logarithmic amplification. For each determination, 10,000 cells were counted. Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

2.6. PCR and RT-PCR assays

Total RNA was isolated from CCRF-SB and NALM1 cells using Trizol (QIAzol) and miRNeasy Mini Kit (Qiagen) followed by cDNA reverse transcription by using random hexamer, dNTP, and SuperScript™ II Reverse Transcriptase according to the manufacturer's procedure (Invitrogen, USA). PCR volume was 25 μl, including 5 μl of cDNA (or DNA), primers, and master mix with Hotstar Taq polymerase. The primers for amplifications of p190 and p210 of *BCR-ABL1* were published as previous [10]. Conventional PCR was performed as described previously [8]. The amplicon sizes were analyzed by capillary electrophoresis on ABI 3130 (Life Technology) with appropriate size controls. DNA sequencing of *TP53* was performed using the dideoxy chain-termination (Sanger) method.

2.7. Statistics

All results were expressed as means ± SD unless stated otherwise. The unpaired Student's *t*-test was used to evaluate the significance of differences between groups, accepting $p < 0.05$ as level of significance.

3. Results

3.1. Inhibition of proliferation induced by treatment with givinostat but not by imatinib in CCRF-SB and NALM1

In CCRF and NAML1, imatinib treatment at a dose range of 0.01–0.5 μM showed minimal toxicities or growth inhibition (by MTS) with 24–72 h of treatment (data not shown), which is similar to relative resistance noted for SUP-B15 [8]. In contrast, treatment with 0.1 μM Givinostat inhibited 66% of NALM1 growth and 0.5 μM givinostat inhibited 39% of CCRF-SB growth at 48 h (Fig. 1A). The IC_{50} values at 48 h for a single givinostat treatment were determined from cell survival plots (SigmaPlot) and were 0.65 ± 0.3 μM and 0.25 ± 0.3 μM in CCRF-SB and NALM1, respectively. The treatment of the cells by Givinostat at 1 μM resulted in time-dependent decrease of pCrk-L1 by Western blot (Fig. 1C). As expected, qRT-PCR showed an absence of *BCR-ABL1* fusion gene in CCRF-SB but p210 (b2a2) transcripts detected in NALM1 (Fig. 1B).

3.2. Givinostat treatment induced cell cycle arrest at G1-phase and DNA content changes

We analyzed the effect of givinostat on the cell cycle of the treated cells by flow cytometry. As shown in Fig. 2 and summarized in Table 1, there were significant increases in the G1 fraction in CCRF-SB and NALM1 with 24 and 48 h of givinostat treatment. Consistent with MTS result above, there was a significant decrease in the S phase in 24 h-treated NALM1 cells. Therefore, our data suggests that givinostat can arrest G1-phase on ALL cells, similar to its effect on SUP-B15 [8].

Treated cells at 24 h also showed slight increases in the sub-G0/G1 fractions, likely representing fragmented or subdiploid DNA content that were significantly increased by 48 h compared to control ($P < 0.001$) (Table 1). Givinostat induced 3-fold and 6.6-fold increases in sub-G0/G1 signal in CCRF-SB and 7.6- and 22.8-fold increases in NALM1 at 24 and 48 h, respectively.

3.3. Givinostat treatment induced apoptosis

The increased signal in the sub-G0/G1 fractions could be due to apoptotic or necrotic cells due to direct genotoxic effects of high-dose givinostat. To distinguish these possibilities, we treated the cells with 0.5 μM givinostat and analyzed fractions for apoptotic cells and necrotic cells by flow cytometric detection of FITC-Annexin V/PI staining. As shown in Fig. 3, apoptosis was already apparent after 24 h of treatment, with more than 50% of the total counts/cells at 48 h in both cell lines. As summarized in Table 2, the apoptotic fraction in treated CCRF-SB at 24 h was 18.4% as compared to 9% in the control, with a