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Reversal of platinum drug resistance by the histone deacetylase inhibitor belinostat

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ABSTRACT

Objectives: To investigate and elucidate the mechanism for the potentiation of cisplatin anticancer activity by belinostat in platinum (Pt)-resistant lung cancer cells.
Materials and methods: Combination of cisplatin and belinostat was investigated in two pairs of parental and cisplatin-resistant non-small cell lung cancer (NSCLC) cell lines. The Pt-resistant cell models exhibited overexpression of the efflux transporter ABCC2 and enhanced DNA repair capacity. Cellular accumulation of cisplatin and extent of DNA platination were measured by inductively coupled plasma optical emission spectrometer. Expression of Pt transporters and DNA repair gene were determined by quantitative real-time PCR. Inhibition of ABCC2 transport activity was examined by flow cytometric assay. Regulation of ABCC2 at the promoter level was studied by chromatin immunoprecipitation assay.
Results and conclusion: In Pt-resistant lung cancer cells, belinostat apparently circumvent the resistance through inhibition of both ABCC2 and DNA repair-mediated mechanisms. The combination of belinostat and cisplatin were found to display synergistic cytotoxic effect in cisplatin-resistant lung cancer cell lines when the two drugs were added concomitantly or when belinostat was given before cisplatin. Upon the concomitant administration of belinostat, cellular accumulation of cisplatin and formation of DNA-Pt adducts were found to be increased whereas expression levels of the efflux transporter ABCC2 and the DNA repair gene ERCC1 were inhibited in Pt-resistant cells. Belinostat-mediated downregulation of ABCC2 was associated with an increase association of a transcriptional repressor (negative cofactor 2) but reduced association of a transcriptional activator (TFIIB) to the ABCC2 promoter. The data advocates the use of belinostat as a novel drug resistance reversal agent for use in combination cancer chemotherapeutic regimens.

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1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide [1]. Non-small cell lung cancer (NSCLC) is a major histologic subtype constituting over 80% of all lung cancers. While surgical resection is curative for early stage lung cancer, most NSCLC patients present with locally advanced or metastatic disease at the time of diagnosis. Although significant progress has been made in the management of NSCLC in recent years with the use of molecular-targeted drugs, only a subpopulation of patients carrying specific genetic abnormality respond to these new agents [2]. The mainstay treatment for patients with advanced NSCLC is the conventional platinum (Pt)-based doublet regimens [3], usually composed of cisplatin plus gemcitabine/pemetrexed/vinorelbine or carboplatin plus paclitaxel. However, resistance to Pt drugs develops rapidly, which can be caused by decreased influx/increased efflux of drug, glutathione or metallothionein conjugation, activated DNA repair, or skipping lesions during DNA replication [4]. New approaches to reverse drug resistance are urgently needed.

Belinostat, a hydroxamate-type inhibitor of class I, II and IV histone deacetylases (HDACs) [5], is approved for the treatment of refractory peripheral T-cell lymphoma [6]. It has also been reported to inhibit the growth of solid cancers including lung and ovarian cancer [7,8]. HDAC inhibitors have been shown to potentiate cyto-

Abbreviations: ABC, ATP-binding cassette; CFDA, carboxy-2',7'-dichlorofluorescein diacetate; MDR, multidrug resistance; NC2, negative cofactor 2; NSCLC, non-small cell lung cancer; Pt, platinum; SRB, sulforhodamine B.

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toxic therapy and radiation but the precise mechanisms have not been fully elucidated [9,10].

The aim of this study was to investigate the potentiation effect of belinostat on the anticancer activity of cisplatin in Pt-resistant NSCLC.

2. Materials and methods

2.1. Chemicals and reagents

Belinostat was purchased from Selleckchem (Houston, TX, USA). Benzbromarone and carboxy-2′,7′-dichlorofluorescein diacetate (CFDA) were obtained from Sigma Chemical (St Louis, MO, USA). Cisplatin was purchased from Acros Organics (Thermo Fisher Scientific, New Jersey, USA).

2.2. Cell culture

Human NSCLC cell lines H460 and A549 are generous gift from Dr. Susan Bates (National Cancer Institute, USA). Cisplatin resistance was induced in H460 and A549 cells by prolonged incubation in increasing concentration of cisplatin to generate cisplatin-resistant sublines, H460 cisR and A549 cisR, respectively. Human NSCLC H1299 cells were purchased from American Type Culture Collection (Manassas, VA, USA). The human embryonic kidney cell line HEK293 and its stable pcDNA3-, or ABCC2-transfected sublines were used to demonstrate the effect of belinostat on ABCC2. A549 and A549 cisR were maintained in DMEM medium whereas H460 and H460 cisR were grown in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 units/mL streptomycin sulfate, and 100 units/mL penicillin G sulfate, and incubated at 37 °C in 5% CO_2. The transfected HEK293 cells were cultured in DMEM medium supplemented with 2 mg/mL G418.

2.3. Reverse transcription and quantitative real-time PCR

Quantitative real-time PCR was performed as described previously [11] to evaluate the expression of ABCC2, ATP7A, ATP7B, CTR1 and ERCC1 in cells treated with belinostat. The specific primer sequences are shown in Supplementary Table 1.

2.4. Growth inhibition assay and analysis of drug combination

The growth inhibitory effect of individual anticancer drugs was evaluated by the sulforhodamine B (SRB) assay as described previously [12]. The median-drug effect analysis method was used to evaluate the nature of the drug combination [13]. Cells grown in 96-well plates were treated with either cisplatin or belinostat alone or their combination in a fixed ratio of each drug in increasing concentrations.

Combination index (CI) was then calculated to assess the outcome of the drug combination at different fraction of cells affected (Fa) as described previously [13].

2.5. Flow cytometric analysis of ABCC2 transporter activity

A flow cytometric assay was employed to study the inhibition of ABCC2 transport activity by belinostat in ABCC2-stably transfected HEK293 cells as described previously [11] by measuring the cellular retention of a fluorescent ABCC2 probe substrate (0.2 μM carboxy-2′,7′-dichlorofluorescein diacetate (CFDA)). Benzbromarone was used as a specific ABCC2 inhibitor for comparison. CFDA fluorescence was detected with a 488-nm argon laser and a 530-nm bandpass filter on LSRFortessa Cell Analyzer (BD Biosciences, San Jose, CA, USA).

2.6. Analysis of ABCC2 inhibition kinetics

The inhibition kinetic of ABCC2-mediated efflux of CFDA by belinostat was evaluated as described previously in ABCC2-stably transfected HEK293 cells [11]. The quantity of ABCC2-mediated CFDA efflux was measured by flow cytometric assay as above and calculated by subtracting values obtained at 37 °C from that at 0 °C. The inhibitory effect of belinostat on ABCC2 was analyzed by the Dixon plot.

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously [14]. ChIP was carried out overnight at 4 °C with monoclonal antibody against TFIIB, NC2, or normal IgG. The amount of immunoprecipitated DNA was assessed by quantitative real-time PCR, using primers spanning the *ABCC2* promoter (Supplementary Table 1), and compared with the amount of input DNA before immunoprecipitation. Fold enrichment in each immunoprecipitation was determined by comparing the Ct value for the immunoprecipitated DNA versus the input DNA from real-time PCR. Only 10% of the total input was used in PCR reactions.

2.8. Cellular Pt accumulation and DNA platination

Cellular Pt accumulation and DNA platination were measured as described previously [15]. Pt content in the samples was analyzed by inductively coupled plasma optical emission spectrometer (ICP-OES) (Optima 4300DV, PerkinElmer, MA, USA).

2.9. Transporter ATPase assay

The effect of belinostat on the vanadate-sensitive ATPase activity of ABCC2 was measured by using the BD Gentest ATPase assay kit (BD Biosciences) according to the manufacturer's instructions.

2.10. Apoptosis assay

After treatment with 4 μM cisplatin in the presence or absence of 0.5 μM belinostat for 48 h, parental H460 or cisplatin-resistant H460 cisR cells were collected. The proportion of apoptotic cells was determined by using the APC Annexin V Apoptosis Kit (BD Bioscience) according to the manufacturer's instruction.

2.11. Statistical analysis

All experiments were repeated at least three times. The statistical software SPSS16.0 (IBM, Armonk, NY, USA) was used for data analysis. Statistical significance was determined at $p < 0.05$ by the Student's *t*-test.

3. Results

3.1. Combination of cisplatin and belinostat was synergistic in NSCLC cells

The growth inhibitory effect of cisplatin and belinostat on two pairs of parental (H460 and A549) and cisplatin-resistant NSCLC (H460 cisR and A549 cisR) cell lines were examined by SRB assay. The IC_{50} of the two drugs in the cell lines are summarized in Supplementary Table 2. Both H460 cisR and A549 cisR were remarkably less responsive to cisplatin (relative resistance = 36-fold and 35-fold, respectively) than the parental cells. On the other hand, belinostat exhibited similar growth inhibitory effect in the parental and cisplatin-resistant cells.

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