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Original article

Romidepsin induces cell cycle arrest, apoptosis, histone hyperacetylation and reduces matrix metalloproteinases 2 and 9 expression in bortezomib sensitized non-small cell lung cancer cells

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ABSTRACT

Histone deacetylase (HDAC) inhibitors have been proven to be effective therapeutic agents to kill cancer cells through inhibiting HDAC activity or altering the structure of chromatin. We recently reported that chemotherapy by the HDAC inhibitor, romidepsin activates the anti- apoptotic transcription factor NF-κB in A549 non-small cell lung cancer (NSCLC) cells and fails to induce significant levels of apoptosis. We also demonstrated that NF-κB inhibition with proteasome inhibitor bortezomib enhanced HDAC inhibitor induced mitochondrial injury and sensitize A549 NSCLC cells to apoptosis through the generation of reactive oxygen species. In this study, we investigate whether combined treatment with romidepsin and bortezomib would induce apoptosis in A549 NSCLC cells by activating cell cycle arrest, enhanced generation of p21 and p53, down-regulation of matrix metalloproteinases (MMPs) 2,9 also altering the acetylation status of histone proteins. Our data show that combination of romidepsin and bortezomib caused cell cycle arrest at Sub G0-G1 transition, up-regulation of cell cycle protein p21 and tumour suppressor protein p53. In addition, romidepsin down-regulated the expression of MMP-2,9 and hyperacetylation of histone H3 and H4 in bortezomib sensitised A549 NSCLC cells. From this study we concluded that romidepsin and bortezomib cooperatively inhibit A549 NSCLC cell proliferation by altering the histone acetylation status, expression of cell cycle regulators and MMPs. Romidepsin along with bortezomib might be an effective treatment approach for A549 NSCLC cells.

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1. Introduction

Lung cancer is the most common invasive cancer and cause of cancer death worldwide [1]. Lung cancer accounts for nearly one third of all cancer-related death in men and one quarter of all cancer death in women. Lung cancer deaths for men and women are decreasing; conversely, more people still die of lung cancer every year than of breast, colon, prostate and pancreas cancer combined. As per the 5-year existence for lung cancer is presently 16%, the destruction due to this single cancer type alone is disturbing [2]. Between lung cancers, 80% are categorized as non-small cell lung cancer (NSCLC) and 20% are small cell lung cancer (SCLC) [3]. NSCLC has a poor diagnosis and remains the prominent cause of death associated to cancer worldwide [4]. New approaches are required in the treatment of NSCLC in order to have any impact in this disease.

Histone deacetylases (HDACs) and histone acetylases (HATs) are enzymes that are answerable for deacetylating and acetylating, respectively, lysine residues located at the amino-terminal tails of histones [5]. The mechanism is deregulated in many neoplasms, in which genes regulating the cell cycle and apoptosis are commonly mutated or abnormally expressed [6]. It is commonly recognised that acetylation of histones, but also of other proteins bound to chromatin, are associated with an increase of transcriptional activity, while in contrast, deacetylation of these proteins is associated to transcriptional suppression which is expected to be mediated by chromatin condensation. Therefore, in order to retain a physiological regulation of transcriptional activity the balance between acetylation and deacetylation of chromatin-related proteins must be accurately regulated [7].

An increasing number of signs indicate an abnormal gene expression in cancer cells that in particular cases has been related with modifications in the expression, the activity and the enrolment of HATs and HDACs [8]. Therefore, HDACs are measured today as an essential cancer targets and HDAC inhibitors (HDACi) are becoming an encouraging new class of anticancer drugs [7]. HDACi induce, to a variable extent, growth arrest, variation or apoptosis in vitro and in vivo [9]. In this circumstance romidepsin, a class I and II HDAC inhibitor has been recently approved by the Food and Drug Administration for the treatment of cutaneous

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T-cell lymphoma (CTCL) and peripheral T-cell lymphoma (PTCL) [10,11].

Despite their promising activity in preclinical models, histone deacetylase inhibitors, which induce differentiation, cell cycle arrest and apoptosis in some malignancies, are only moderately efficacious in NSCLC, and this relates in part to the activation of the antiapoptotic transcription nuclear factor-kappaB (NF-κB) [12–14]. NF-κB is generally sequestered in the cytoplasm by the inhibitory protein IκB. NF-κB stimulation arises by both cytokines and intracellular stress signals that cause in Akt, IKK, or together kinase-mediated phosphorylation of IκB. Precisely, phosphorylation of IκB on serines 32 and 36 marks IκB for ubiquitination and degradation by the 26S proteasome [7]. IκB degradation help nuclear translocation of the transcriptionally active NF-κB isoform p50/p65, prominent to transcription of NF-κB dependent anti-apoptotic genes. More recently, our previous report shows direct evidence that the insensitivity of A549 NSCLC cells to apoptosis following the inhibition of HDAC activity by romidepsin is due to NF-κB activation and indicating that NF-κB activation is responsible for the differential responses of cells to romidepsin. We also found that inhibition of NF-κB with proteasome inhibitor bortezomib enhanced the sensitization of A549 NSCLC cells to romidepsin-induced apoptosis.

Proteasome inhibitors belong to this type of agents and some of them are being widely explored for cancer therapy [15]. These inhibitors target the 26S proteasome, a component of the ubiquitin-proteasomal system, the major nonlysosomal pathway involved in proteolysis of intracellular proteins. Degradation of proteins via proteasome is an important mechanism for the regulation of key cellular processes as cell cycle progression, signaling and gene transcription [16]. Proteasome inhibition blocks the degradation of IκB and as a consequence prevents the activation of NF-κB [17]. Therefore, inhibition of proteasome-mediated degradation of IκB would inhibit consequent translocation of NF-κB to the nucleus and would be one approach to prevent stimulus induced NF-κB activation [13].

Bortezomib is the leading proteasome inhibitor approved by the US FDA for the treatment of myeloma and mantle cell lymphoma [18,19]. Bortezomib has recently been described to be a potent inhibitor of NF-κB activation. This compound is a dipeptidyl boronic acid analogue that selectively inhibits the chymotryptic activity within the 26S proteasome [20].

The purpose of this study was to conclude whether bortezomib sensitization will mediate enhanced potential of romidepsin induced apoptosis in A549 NSCLC through elucidating its molecular mechanism on proliferation, cell cycle progression, metastasis and epigenetic modifications.

2. Materials and methods

2.1. Reagents and antibodies

The Romidepsin was purchased from carbosynth Ltd, UK. Bortezomib (PS-341) was kindly provided as a gift sample from Janssen Pharmaceuticals, Mumbai, India and Dr. Sophia Karabela (Laboratory of Inflammation and Autoimmunity, Center of Immunology and Transplantation Athens, Greece). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, DMSO (cell culture grade), MTT (dimethyl thiazolyltetrazolium bromide), Acridine orange and Ethidium bromide was purchased from Hi Media Laboratories, Mumbai, India. Antibodies to p21, p53, acetyl histone 3 and 4 and matrix metalloproteinases 2,9, β-tubulin were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody to β-actin was from Santa Cruz (CA, USA).

2.2. Cell cultures

A549 non-small-cell lung cancer (NSCLC) cell line obtained from the National Centre for Cell Science, Pune, India. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum (10% FBS) and 1% penicillin/streptomycin in a 5% CO_2 humidified atmosphere at 37 °C.

2.3. MTT assay

A549 NSCLC cells in monolayer containing approximately 1×10^4 were added to each well of a 96-well culture plate and incubated for overnight at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After overnight incubation, cells were exposed to increasing concentrations of romidepsin (10–100 nM), bortezomib (10–100 nM), or both romidepsin and bortezomib (50:40–70:60 nM). After treatment hours (24, 36 and 48) of incubation 20 μL of MTT was added then the cultures were further incubated for 4 h, MTT was aspirated and then 200 μL of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using microplate reader (Bio-rad, USA).

2.4. Acridine orange/ethidium bromide (AO/EB) staining

For measuring apoptotic level in the cells, 5×10^5 cells were seeded on cover slip in a 6-well plate and allowed to attach overnight. The next day, medium was replaced by fresh medium containing left alone or were treated with romidepsin, bortezomib or both and allowed to incubate for 36 h. At the end of incubation cover slip was removed from the culture plate and stained with 50 μL/mL of AO/EB was added and incubated at 37 °C with 5% CO_2 for 30 mins. The stained cover slip was washing with 1 × PBS for removing extra dye. Cover slip was fixed on glass slide and images of the cells were captured using 40 × objectives under fluorescence microscope (Carl Zeiss, Jena, Germany) (510–590 nm).

2.5. Cell cycle analysis

In order to assess the effect of romidepsin and bortezomib on cell cycle, A549 NSCLC cells were plated at 5×10^5 cells. Allowed to attach overnight incubation, cells were treated with nothing, romidepsin, bortezomib, or both allowed to incubate for 36 h. After prescribed treatment hours, cells were harvested, washed with cold 1xPBS, fixed with cold 75% ethanol at 4 °C overnight, The cells were then resuspended in PBS containing 0.5% Triton X-100 (Sigma-Aldrich 93443), 0.1 mg/mL RNase (Sigma-Aldrich R4642) and incubated for 1 hour. After 1 hour, add propidium iodide (40 μg/mL) (Sigma-Aldrich P4170) and kept in a dark room for 45 mins. After 45 mins of incubation with propidium iodide at room temperature, the cells were analyzed on a flow cytometer (FACS Calibur, Becton Dickinson), equipped with an air-cooled argon laser providing 15 mW at 488 nm (Blue laser) with standard filter setup. Ten thousand events were collected and the percentages of each cell cycle phases were analysed using Cellquest Pro software (Becton Dickinson, USA).

2.6. Western blot analysis

A549 NSCLC cells were either left alone or were treated with romidepsin, bortezomib, or both for 36 h. Cells were harvested and whole-cell lysates were prepared with Dignam buffer. Cells were lysed by incubation for 30 mins at −20 °C in 100 μL of Dignam buffer (10 mmol/L HEPES, 1.5 mmol/L $Mgcl_2$, 10 mmol/L Kcl, 0.1% NP-40, 0.5% DTT and 0.5 mmol/L PMSF). Lysates were centrifuged for 12 mins at 12,000 × g (4 °C), and 50 μg of the supernatants were mixed with equal volumes of 2xSDS-PAGE sample buffer

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