Bortezomib Potentiates the Effect of Roscovitine Via DNA Damage Induced Apoptosis in A549 Lung Cancer Cells

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ABSTRACT
The adoption of new treatment modalities remains crucial as lung cancer has one of the lowest survival rates, along with liver and pancreatic cancer. Bortezomib is a proteasome inhibitor that has higher antitumor activity when combined with cyclin-dependent kinase (CDK) inhibitor-roscovitine in vitro. Apoptosis related gene expression levels of p53, Noxa, Puma, Bcl-xL, Bak, Casp-3 and Casp-7 were measured by quantitative PCR (qPCR) upon treatment with 10-20μM roscovitine and in combination with 30nM bortezomib for 24 hours. Synergistic effect on apoptosis was also investigated at protein levels by analyzing p53, Cleaved Casp-3 and Cleaved Parp expressions. Induction of autophagy was determined by western blotting of B-catenin and LC3B I-II. Roscovitine combined bortezomib treatment induced apoptosis by upregulating p53 pathway and its downstream mediators. Bortezomib increased Parp and Caspase3 cleavage significantly at 24h. Bortezomib inhibited B-catenin and triggered autophagy induction at 24 and 48hours. As cancer cells evade programmed cell death, CDK inhibitors might be used to direct cancer cells into apoptosis. This study concludes that bortezomib potentiates the effect of roscovitine via DNA damage induced apoptosis in A549 lung cancer cells.

Keywords: CDK inhibitor, Lung cancer, Bortezomib, Apoptosis, Roscovitine.

Introduction
Lung cancer remains to be the leading cause of deaths worldwide and it has one of the lowest survival rates, along with liver and pancreatic cancer [1,2]. Survival rates are low due to diagnosis at an advanced stage of the disease [3]. Therefore the adoption of new treatment modalities is indispensable.

Roscovitine is a small molecule that targets various CDKs (CDK2, CDK7 and CDK9) in cell cycle [4]. Roscovitine is an investigational drug that is currently in phase trials for the treatment of solid cancers, cshusings syndrome and active ulcerative colitis [5]. Roscovitine derivatives are also being used in order to increase the efficacy of the drug [6]. As cancer cells reprogramme cell death by evading apoptosis, cyclin-dependent kinase inhibitors might be used in treatment to direct cells into apoptosis.

Bortezomib is a proteasome inhibitor that is used in the treatment of multiple myeloma and mantle cell lymphoma [7]. It inhibits 26S proteasome where ubiquitinated proteins are degraded [8]. The ubiquitin-proteasome pathway maintains homeostatic intracellular concentrations of proteins which is a crucial mechanism that promotes protein degradation [9]. As ubiquitin-proteasome pathway is dysregulated during cancer progression, targeting this pathway is crucial in treatment [10]. Bortezomib became the first FDA approved proteasome inhibitor and clinical trials are ongoing since to investigate its anticancer activity in solid tumors [11]. Bortezomib is also found to increase the effect of traditional anticancer agents [doxorubicin, cisplatin, paclitaxel, irinotecan, and radiation etc] [12]. Therefore, the aim of this study was to investigate whether bortezomib could potentiate the anticancer effect of cyclin-dependent kinase [CDK] inhibitor-roscovitine in vitro. Bortezomib and roscovitines’ anti-proliferative effect on A549 lung cancer cells were previously identified as single treatment agent and IC50 values were identified as 30nM for bortezomib and 10μM for roscovitine in A549 cells [13,14] However their synergistic effect in lung cancer cells has not been studied before. To investigate the synergic effect, A549 cells were treated with 10 and 20μM roscovitine +30 nM bortezomib for 24 and 48 hours. Previous researches identified that roscovitine requires higher IC50 concentration (10-20μM) than bortezomib [30nM]. Therefore lower (10μM) and higher (20μM) roscovitine alone and their combination with the constant concentration of bortezomib (30nM) was used in order to leverage roscovitine’s effect.

Apoptosis related gene expressions (p53, Noxa, Puma, Bcl-xL, Bak, Casp-3 and Casp7) were measured by qPCR at 24 hours. In order to shed more light into the molecular mechanism of these drugs, P53, Parp and Casp-3 cleavage were detected by western blot. Induction of autophagy was also determined by measuring LC3BI, LC3B-II and B-Catenin protein expression profiles by western blot.

Materials and Methods

Cell Culture and Chemicals
A549 cells were grown in DMEM medium supplemented with 10% Fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO, USA). Cells were grown in an
incubator in 5% CO2 at 37°C. Roscovitine and bortezomib were supplied from Santa Cruz, Texas, USA. Roscovitine and bortezomib were dissolved in DMSO, the maximum concentration of DMSO was used as a vehicle control. A549 cells were treated with 10-20μM roscovitine and in combination with 30nM bortezomib for 24 and 48 hours.

**Protein Expression Analysis by Western Blotting**

Protein lysates were collected after 24 and 48 hours treatment with the indicated drugs and quantified by using the BCA Assay Kit (Thermo Pierce, Rockford, IL, USA). Lysates (20 μg) were denatured for 5 minutes at 95°C in LDS non-reducing sample buffer (Pierce, Rockford, IL, USA) and then loaded to the 10% Tris-glycin gels. The gels were transferred to the PVDF membrane (Merck Millipore, Darmstadt, Germany) at 300 mAmp for 90 minutes. Membranes were blocked with 5% non-fat milk powder in TBS-T for 1 hour at room temperature and incubated overnight at 4°C with the primary antibodies for P53, cleaved Parp, cleaved Casp-3, LC3B, B-Catenin and B-actin at 1:1000 dilution (Thermo Pierce, Rockford, IL, USA). Blots were washed with TBS-T subsequently. Protein bands were detected by using the secondary antibody (Thermo Pierce, Rockford, IL, USA) and the blots were visualized by BioVision ECL Western Blotting Substrate Kit (Biovision, California, USA).

**Gene Expression Analysis by RT-qPCR**

RNA was isolated by using Trizol reagent (Invitrogen, Thermo Fischer Scientific, USA) 2000 ng of total RNA was reverse transcribed by Superscript III cDNA Synthesis Kit (Invitrogen, Thermo Fischer Scientific, USA) Forward and reverse primer sequences was provided in the appendix. 10–20 cycles of specific target amplification was performed by SYBR Green qPCR master mix. RT-qPCR analysis was performed in Roche LightCycler qPCR. Gapdh was used as internal control when calculating Cq value. ΔΔCq method was used to quantify the gene expression levels. mRNA expression levels for p53, Noxa, Puma, Bcl-xL, Bak, Casp-3 and Casp-7 were measured by qPCR. Data was analyzed and plotted in Graphpad prism software.

**Results and Discussion**

**Bortezomib and Roscovitines’ Effects on Apoptosis**

P53 pathway and related downstream target genes that are involved in apoptosis were quantified by qPCR in order to assess roscovitine’s anticancer activity and its synergic effect with bortezomib. A549 cells were treated with 10 and 20μM roscovitine and in combination with 30nM bortezomib for 24 hours. mRNA expression levels for p53, Noxa, Puma, Bcl-xL, Bak, Casp-3 and Casp-7 were quantified by qPCR. mRNA expression were normalized to the Gapdh housekeeping control for each gene.

Roscovitine treatment decreased p53 levels but when bortezomib was added p53 gene expression levels were upregulated (Figure 1a). Noxa mRNA expression levels were downregulated upon 10, 20μM roscovitine and 10 μM + 30nM bortezomib treatment, however it increased with 20μM + 30nM bortezomib treatment (Figure 1b). Puma is known as p53 upregulated modulator of apoptosis, consistent with the p53 upregulation, Puma levels increased at all treatment conditions (Figure 1c). Bortezomib induced Bcl-xL upregulation at 24 hours (Figure 1d). Bak is also a member of the BCL2 protein family. Bak mRNA levels increased upon 20μM roscovitine, 10 and 20μM roscovitine + 30nM bortezomib treatment (Figure 1e). Caspases are crucial mediators of apoptosis. Caspase 3 mRNA expression levels upregulated with all treatment conditions (Figure 1f), whereas Caspase 7 remained unchanged or decreased (Figure 1g).

To further investigate the induction of apoptosis upon roscovitine and bortezomib treatment; p53, cleaved parp and cleaved caspase 3 protein expression levels were determined by western blotting at 24 hours. Consistent with the mRNA expression, p53 protein expression levels were upregulated upon treatment. Cleaved caspase 3 is an indicator of apoptosis. Cleaved caspase 3 levels increased in a dose-dependent manner. It was found that bortezomib potentiated the effect of roscovitine via Parp cleavage (Figure 2).
Figure 1. P53 pathway and related downstream target genes that are involved in apoptosis were quantified by qPCR. A549 cells were treated with 10 and 20μM Roscovitine and plus 30nM Bortezomib for 24 h. mRNA expression levels for p53, Noxa, Puma, Bcl-xL, Bak, Casp-3 and Casp7 were measured by qPCR. mRNA expression values were normalized to the Gapdh housekeeping control.

Figure 2. a) A549 cells were treated with indicated concentrations of Roscovitine and Bortezomib for 24 h and 48h. Roscovitine and Bortezomib combination increased p53 protein expression in A549 cells. Parp and Casp3 cleavages were detected by western blot analysis upon Roscovitine and Bortezomib treatment for 24 h. b) Relative protein expression were represented as fold change for p53 normalized to β-Actin. c) Relative protein expression were represented as fold change of the PARP cleavage normalized to β-Actin d) Relative protein expression were represented as fold change of the Casp-3 cleavage normalized to β-Actin

**Roscovitine and Bortezomib Induce Autolysosomal β-Catenin Degradation in A549 Cells**

A549 cells were treated with indicated concentrations of roscovitine and bortezomib for 24 h and 48h, the induction of autophagy was determined by western blotting of LC3B protein expression. B-catenin protein expression levels were also investigated to question its role in autophagy. 10 μM roscovitine+30nM bortezomib and and 20μM roscovitine+30nM bortezomib concentrations inhibited B-catenin protein expression significantly at 24h. 20μM roscovitine+30nM bortezomib treatment decreased B-catenin levels at 48h. LC3-II is an autophagy indicator. Bortezomib increased the induction of autophagy at both 24 and 48 h (Figure3).
Figure 3. a) A549 cells were treated with indicated concentrations of Roscovitine and Bortezomib for 24 h and 48h. Roscovitine and Bortezomib combination decreased B-catenin protein expression in A549 cells. The induction of autophagy was determined by LC3B protein expression. B-actin was used as loading control. b) Relative protein expressions were represented as fold change for B-catenin normalized to B-Actin. c) Relative protein expression were represented as fold change for LC3B-I normalized to B-Actin. d) Relative protein expression were represented as fold change for LC3B-II normalized to B-Actin.

Discussion

The CDKs are critical regulators of cell cycle control and have other important cellular functions such as transcription [15]. As cancer cells have uncontrolled proliferation and CDKs are a central regulator of the cell cycle control; developing CDK inhibitors as anticancer agents gained interest [16]. However, targeting human kinome is a challenging task as human genome encodes 538 different protein kinases and many of them are associated with cancer progression [17]. Bortezomib is a proteasome inhibitor that has synergic effect when used in combination with the other anticancer agents [18]. Therefore, Bortezomib was evaluated in combination with roscovitine to assess their potential for the development of new treatment modalities in lung cancer treatment.

Although the number of ongoing clinical trials with roscovitine remained limited, recently it was found that roscovitine enhanced antitumor activity of temozolomide in vitro and in vivo by regulating autophagy and Caspase-3 dependent apoptosis in glioblastoma [19]. p53 acts as a tumor suppressor and reacts to stress signals with diverse responses. One of the most important p53 functions is to induce apoptosis [20]. In this study, bortezomib potentiated the effect of roscovitine via p53 dependent pathway of apoptosis.

Bcl-xL is a Bcl-2 protein family member that modulates apoptosis by controlling mitochondrial membrane permeability [21]. It was found that bortezomib treatment increased the gene expression of the Bcl-2 protein family members involved in apoptosis (Puma, Bcl-xL, Bak). Bortezomib also induced Caspase 3 cleavage both at mRNA and protein levels. Caspase-3 catalyzes the cleavage of many key cellular proteins during apoptosis [22]. PARP cleavage is observed during programmed cell death induced by a variety of apoptotic stimuli [23]. The protein expression profiles of p53, cleaved Casp-3 and cleaved Parp were consistent with the mRNA expression data. The results revealed that using bortezomib in combination with the CDK inhibitor roscovitine promoted apoptosis both at the mRNA and protein levels. Increasing cleaved Parp protein expression levels upon combination treatment suggests that more DNA damage accumulated when cells were treated with the proteasome inhibitor. Bortezomib treatment induced more autophagy via B-catenin-LC3B axis when compared to the control treatment. Although this study is limited with a single cell line in vitro, a large body of evidence demonstrates that roscovitine and bortezomib have synergic anticancer effect via DNA damage induced apoptosis.

Conflicts of Interest

The author declares no conflicts of interest. No competing financial interests exist.
References


