Roscovitine-treated HeLa cells finalize autophagy later than apoptosis by downregulating Bcl-2

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Received December 4, 2013; Accepted May 30, 2014

DOI: 10.3892/mmr.2014.2902

Abstract. The cell cycle is tightly regulated by the family of cyclin-dependent kinases (CDKs). CDKs act as regulatory factors on serine and threonine residues by phosphorylating their substrates and cyclins. CDK-targeting drugs have previously demonstrated promising effects as cancer therapeutics both in vitro and in vivo. Roscovitine, a purine-derivative and specific CDK inhibitor, has been demonstrated to arrest the cell cycle and induce apoptosis in a number of different cancer cell lines, including HeLa cervical cancer cells. In the present study, roscovitine was able to decrease both the cell viability and cell survival as well as induce apoptosis in a dose-dependent manner in HeLa cells by modulating the mitochondrial membrane potential. The decrease of anti-apoptotic B-cell lymphoma 2 (Bcl-2) and Bcl-2 extra large protein expression was accompanied by the increase in pro-apoptotic Bcl-2-associated X protein and P53-upregulated modulator of apoptosis expression. The marked decrease in Bcl-2 following exposure to roscovitine (20 µM) for 48 h prompted us to determine the autophagic regulation. The outcome revealed that roscovitine triggered Beclin-1 downregulation and microtubule-associated light chain 3 cleavage starting from 12 h of incubation. Another biomarker of autophagy, p62, a crucial protein for autophagic vacuole formation, was diminished following 48 h. In addition, monodansyl cadaverin staining of autophagosomes also confirmed the autophagic regulation by roscovitine treatment. The expression levels of different Bcl-2 family members determined whether apoptosis or autophagy were induced following incubation with roscovitine for different time periods. Downregulation of pro-apoptotic Bcl-2 family members indicated induction of apoptosis, while the downregulation of anti-apoptotic Bcl-2 family members rapidly induced autophagosome formation in HeLa cells.

Introduction

Cyclin-dependent kinases (CDKs) strictly orchestrate the cell cycle machinery through the binding to their specific cyclin counterparts at cell cycle checkpoints. The CDK-cyclin complex formation ensures the appropriate and accurate transition between cell cycle phases, which is essential for cell proliferation (1-3). Cancer cells frequently demonstrate deregulated CDK activity that causes uncontrolled cell proliferation, which is a hallmark of malignant cell turnover. Therefore, recent approaches targeting CDKs with specific small molecule inhibitors have a crucial role both in developmental biology and cancer therapy. Roscovitine (CYC202 or seliciclib), a purine derivative, is a novel promising candidate for CDK inhibition and is able to induce apoptosis in a variety of cancer cell types; including lung, prostate, breast and colon cancers as well as multiple myeloma (4-5). Roscovitine mainly acts as a competitive inhibitor of CDKs while showing a broad affinity for specific CDK ends, and blocks the ATP-binding catalytic site of kinases (1,3). Recent studies demonstrated that roscovitine induces cell cycle arrest at the G1/S or G2/M phase transition in MCF-7 breast (1), lung cancer (2), HeLa cervical (3) and HCT116 colon cancer (4) cells. In addition, roscovitine treatment may synergize the anti-tumor effects of other chemotherapeutics by inducing further apoptotic cell death (5).

Although previous studies have established the apoptotic potential of roscovitine in HeLa cells (3), the autophagic regulation following roscovitine treatment has not been fully elucidated. Autophagy is a regulated process of degradation and recycling of cellular components in vacuoles inside the cell, allowing organelle and energy turnover. Autophagy has a crucial role in tissue dynamics, homeostasis, development and disorders. Autophagy is responsible for the degradation of cytoplasmic macromolecular and protein structures

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Abbreviations: ABTS, 2,2'-azino-di-3-ethylbenzthiazoline sulfonate 6 diammonium salt; AO, acridine orange; DiOC6, 3,3'-dihexyloxacarbocyanine iodide; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylosulfoxide; MDC, monodansylcadaverine; PBS, phosphate-buffered saline; PI, propidium iodide; POD, peroxidase

Key words: roscovitine, cervical cancer, autophagy, apoptosis, Bcl-2

and damaged or aged organelles in the cells. The most significant marker of autophagy is the appearance of double membrane-enclosed vesicles, which engulf portions of cytoplasm and/or organelles in the cytoplasm (6-8). Due to different stress factors, including starvation, autophagy can result in cellular adaptation as an alternative to cell death or survival. Although autophagy has been considered to be a stress adaptation mechanism in response to certain conditions, including starvation, apoptosis and autophagy is now considered to have a specific function. In variable cellular settings and in the presence of different external factors, autophagy is regarded as an alternative cell death pathway. Depending on the type of the stimulus, the two types of cell death may be triggered at the same time, corresponding to each other by common upstream signals, or occasionally the cell switches in between these death pathways in a successive manner. Thus, the clarification of the joint molecular targets in both apoptosis and autophagy regulation processes is critical to evaluate the therapeutic potential of novel chemotherapeutic drugs. One of the links between apoptosis and autophagy is B-cell lymphoma 2 (Bcl-2), which is an anti-apoptotic member of the Bcl-2 family. Bcl-2 exerts a dual role to inhibit apoptosis and eliminate pro-apoptotic Bcl-2 family members or autophagy by binding Beclin-1, which has a similar structure to that of pro-apoptotic Bcl-2 proteins. Beclin-1 is a Bcl-2 homology (BH) 3 only protein which binds to anti-apoptotic Bcl-2 family members; myeloid cell leukemia-1 (Mcl-1), Bcl-2 or Bcl-2 extra large (Bcl-xL) (9,10). It has been suggested that anti-apoptotic Bcl-2 family members exert a dual role in inhibiting both apoptotic and autophagic pathways in the cell. Beclin-1 is an initiator factor for autophagy and recruits Vps34 and Vps15 to form pre-autophagosomal structures (11). Therefore, the expression profile of Beclin-1 akin to Bcl-2 may be a crucial switch molecule between apoptosis and autophagy (12).

The present study aimed to examine the potential therapeutic role of roscovitine in HeLa cervical cancer cells by investigating apoptotic and autophagic molecular signaling molecules in a time-dependent manner.

Materials and methods

Drugs, chemicals and antibodies. Roscovitine (seliciclib, 2-(1-ethyl-2-hydroxy-ethylandino)-6-benzylamino-9-isopropylpurine) was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) to establish a 10 mM stock solution, and the aliquots were maintained at -20°C. Propidium iodide (PI), DAPI, 3,3′-dihexyloxacarbocyanine iodide (DiOC6) and acridine orange (AO) were purchased from Invitrogen Corporation, Tokyo, Japan. The number of viable cells (y-axis) was plotted against the time (x-axis).

Fluorescence microscopy. The HeLa cells were seeded at a density of 5x10⁴ cells per well in 12-well plates and treated with roscovitine for 24, 48 and 72 h. The cells were then counted by staining with 0.4% (w/v) trypan blue dye. Viable and dead cells were counted under light microscopy (IX71; Olympus Corporation, Tokyo, Japan). The number of viable cells (y-axis) was plotted against the time (x-axis).

Immunoblot analysis. The cells were lysed on ice using ProteoJET mammalian cell lysis reagent (Fermentas, Glen Burnie, MD, USA) containing protease inhibitor (Roche, Mannheim, Germany). The cell lysates were centrifuged at 16,100 x g for 20 min at 4°C and the protein concentration was determined at 570 nm (A570) using a microplate reader (Model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following separation of denatured proteins according to size by SDS-PAGE, the proteins were transferred onto a polyvinylidene fluoride membrane. Following incubation of the PVDF membrane with blocking buffer for 1 h, the membrane was incubated with a specific primary antibody for 2 h followed by a horseradish peroxidase (HRP)-conjugated secondary anti-rabbit (1:5,000) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).
fluoride membrane (Thermo Scientific) where they were labeled using antibodies specific to the target protein. The gels obtained by SDS-PAGE were placed in the Trans-Blot Turbo transfer system (Bio-Rad Laboratories) and protein transfer was conducted at 25 V for 5 min. The membrane was blocked in 5% bovine serum albumin/TBS containing 0.1% Tween-20 (TBST) for 2 h at room temperature, and then incubated with primary antibodies for each target; Bax, Puma, Bcl-2, Beclin-1, LC3B, Atg5, Atg12, p62 and β-actin overnight at 4˚C and further incubated with anti-rabbit immunoglobulin G-HRP secondary antibody overnight at 4˚C. Following washing with TBST, the proteins were detected using Super Signal West FemtoLuminol/Enhancer Solution (Thermo Scientific) and exposed to Hyperfilm-enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Densitometric analysis of immunoblots was performed using Image J (National Institutes of Health, Bethesda, MD, USA) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) software; and all of the proteins were quantified relative to the loading control.

**Determination of mitochondrial membrane potential assay.** The HeLa cells (1x10^5) were seeded in 12-well plates, treated with the indicated concentration of roscovitine and allowed to attach overnight. The cells were washed once with 1X PBS, and stained with 0.4 mM DiOC6 (Molecular Probes Life Technologies, Carlsbad, CA, USA) fluorescent probe. Mitochondrial membrane potential (MMP) loss was measured using a Fluoroskan Ascent fluorometer (excitation/emission = 488 nm/525 nm; Thermo Labsystems, Milford, MA, USA).

**Statistical analysis.** All of the experiments were statistically analyzed using GraphPad Prism 6 software. Error bars in the graphs were generated using ± standard deviation values. A P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Roscovitine decreases cell viability and inhibits proliferation in a dose-dependent manner in HeLa cells.** The effect of roscovitine on the viability of HeLa cells was investigated by an MTT assay. Exposure of the cells to roscovitine (0-50 µM) for 24 h decreased the cell viability in a dose-dependent manner (P<0.0001, each compared with the untreated control). A moderate cytotoxic effect was triggered following 20 µM roscovitine treatment, which caused 25% cell viability loss compared with the untreated control samples. The highest concentration of 50 µM roscovitine reduced the cell viability ratio by 50% within 24 h (Fig. 1A).

To confirm the cytotoxic effect of roscovitine on cell proliferation in HeLa cells, a trypsin blue exclusion assay was also performed (Fig. 1B). Exponentially growing HeLa cells were treated with roscovitine (20 µM) for different time periods within 72 h. The doubling time of HeLa cells was determined...
to be 23 h, which was in accordance with the information provided by the supplier. Treatment of the cells with roscovitine suppressed the cell viability following 24 h. However, the cells gained proliferation capacity following long-term exposure to roscovitine for 48 and 72 h. Although roscovitine diminished the cell proliferation within 24 h, it was concluded that longer treatment with roscovitine may lead to the accumulation of resistance factors in HeLa cells (P<0.0001).

Following exposure of HeLa cells to roscovitine for 24 and 48 h, the cells were stained with PI and DAPI to identify the cell death mechanism (Fig. 2A). The percentage of PI-positive cells, which are regarded as dead cells, was increased following roscovitine treatment of HeLa cells (red stain) compared with the untreated cells in a time-dependent manner. In addition, DAPI staining was performed to identify apoptotic cells which exhibited fragmented DNA and nuclear condensation. The number of apoptotic cells (bright blue stain) was increased following 24 and 48 h roscovitine treatment compared with the untreated control cells. According to the fluorescence images, the ratio of nuclear condensation was increased following 48 h of roscovitine treatment.

In order to investigate the cytotoxic effect of roscovitine on HeLa cells, a Cell Death ELISA assay was performed, which demonstrated the cleavage of DNA due to drug treatment (Fig. 2B). Exposure of cells to lower concentrations of roscovitine (10 and 15 µM) induced apoptotic cell death by 1.65 and 1.85-fold compared with untreated control samples, respectively. The appropriate concentration of roscovitine, 20 µM,
increased apoptosis by 2-fold compared with the untreated control cells (P<0.0001, each compared with the untreated control). Therefore, it was concluded that roscovitine treatment for 24 h decreased the cell viability and induced apoptosis in a dose-dependent manner.

**Roscovitine induces apoptosis and autophagy through modulating levels of Bcl-2 family members.** Since Bcl-2 family members have a decisive role in apoptotic cell death mechanisms, the effect of roscovitine on the expression levels of pro- and anti-apoptotic Bcl-2 family member proteins in HeLa cells was determined. As revealed in Fig. 3A, roscovitine treatment decreased the Bcl-2 and Bcl-x<sub>L</sub> expression levels time-dependently, whereas the activation of pro-apoptotic Bcl-2 family members, Puma and Bax, was observed as an early response. Although roscovitine treatment for 24 h downregulated Bcl-x<sub>L</sub> expression levels, no significant alteration was observed for Bcl-2 at the same time-point. It was concluded that roscovitine is an apoptosis-inducing agent via modulating the ratio between anti and pro-apoptotic Bcl-2 family members in HeLa cells.

In order to examine the potential role of roscovitine on mitochondria-mediated apoptosis, DiOC6 staining was performed (Fig. 3B and C). The number of viable cells (green stained), which have normal mitochondrial function, was gradually decreased in a time-dependent manner. The loss in mitochondrial membrane potential (MMP) was also confirmed by fluorometric analysis (excitation, 485 nm; emission, 538 nm).

To investigate the effect of roscovitine on autophagic regulation mechanisms, the expression levels of the key signaling molecules associated with autophagy were investigated following roscovitine treatment in HeLa cells. As demonstrated in Fig. 4A, Beclin-1 expression levels were progressively upregulated with increasing exposure time to the drug in HeLa cells. Treatment with roscovitine for 12 and 24 h upregulated the expression of two LC3 isoforms (LC3-I and LC3-II); however, long-term treatment for 48 h with the drug downregulated the LC3-II isoform in HeLa cells (Fig. 4A). By contrast, the expression profiles of Atg5 and Atg12 were not altered following roscovitine treatment. p62 is referred to as a significant biomarker for the autophagic vacuole formation process. In the present study, it was determined that roscovitine treatment at 48 h eliminated the p62 expression levels in HeLa cells. Therefore, it was concluded that autophagic mechanisms are finalized later than roscovitine-induced apoptosis.

To demonstrate the stages of roscovitine-induced autophagy, cells were stained with MDC or AO (Fig. 4B). MDC staining was performed to visualize acidic autophagic vesicles. As a positive control, nutrient-deprived cells were stained with MDC, displaying bright green dots. Although MDC-stained cells were identified following 12, 24 and 48 h of roscovitine treatment, maximum fluorescent staining was observed after 12 h. The lysosomotropic dye AO was used
to identify lysosomes. As a positive control, nutrient-deprived cells were stained with AO, displaying bright red dots. The accumulation of AO dye was increased following roscovitine treatment in a time-dependent manner.

**Discussion**

Cervical cancer is the second most common cancer type among females worldwide and the disease etiology is mostly linked to infection with Human Papilloma Virus (HPV) (13,14). Depending on the tumor size the preferred treatments for cervical cancer are medical surgery, radiation therapy, chemotherapy or their combination. However, the presence of an increasing number of recurrent cases demonstrates an urgent requirement for improvement of therapeutic application models. Although a number of the classical chemotherapeutic agents, including cisplatin, mitomycin C and bleomycin, are used as therapeutic agents against cervical cancer, these drugs induce intrinsic resistance factors, which renders the efficiency of therapy (15,16). Therefore, the development of novel anti-tumor agents is critical for the treatment of cervical cancer patients.

Since the loss of functional cell cycle control is a common problem in numerous malignancies, CDK inhibitors are considered to inhibit the uncontrolled proliferation in cancer cells. Roscovitine was the first orally bioavailable CDK inhibitor to enter clinical trials for breast, lung, B-cell lymphoma and advanced solid tumors (17-19). Previous studies established that roscovitine predominantly inhibits CDK2 and induces cell cycle arrest at G2/M phase. In the present study, it was identified that roscovitine decreases cell viability in a dose-dependent manner in HeLa cells. A moderately cytotoxic concentration of the drug (20 μM) prevented cell proliferation and induced apoptosis by 2-fold compared with the untreated control cells within 24 h. As demonstrated in previous studies, roscovitine induced apoptosis mainly through modulating MMP and activating caspase-9, -3 and poly(adenosine diphosphate-ribose) polymerase cleavage in different cancer cells (4,18,20-28). Similar to these observations, it was determined in the present study that 20 μM roscovitine decreased the MMP in a time-dependent manner in HeLa cells [MMP loss, 45% (24 h) and 65% (48 h) vs. the control group, respectively]. Although roscovitine (20 μM) upregulated Bax and Puma expression levels time-dependently, anti-apoptotic Bcl-2 was downregulated following long-term exposure of cells to the drug for 48 h. By contrast, Bcl-xL was downregulated in a time-dependent manner following roscovitine treatment. Therefore, it was concluded that roscovitine exerts its apoptotic effect on HeLa cells by altering the MMP and modulating Bcl-2 family members in HeLa cells. A previous study revealed that roscovitine (20 μM) induced apoptosis via reducing the cell proliferation caused by G2/M cell cycle arrest in tamoxifen therapy-resistant breast cancer cell lines. In addition, roscovitine successfully reduced breast tumor volume, which was evaluated in an in vivo xenograft tumor model (26). Although roscovitine mostly activated caspsases to induce the apoptotic cell death mechanism (4), it was established that roscovitine may successfully induce apoptosis in caspase-3 null MCF-7 cells through upregulating the p53 signaling pathway (29). In response to this finding, the present study aimed to examine the potential role of roscovitine on autophagy, which is also accepted as a caspase-independent cell death type in HeLa cells. Previously, it was demonstrated that roscovitine treatment enhanced doxorubicin-induced apoptosis as well as autophagy in sarcoma cell line models (30). In addition, roscovitine downregulated mitogen-activated protein kinase 1/3, which exhibits a high activity in mature oocytes of mammals, frogs or invertebrates, such as starfish, to maintain cell cycle arrest. By this way, mature oocytes underwent apoptosis if they were not fertilized. Roscovitine increased calcium-dependent mitotic cell entry and delayed cell death in sea urchins by downregulating MAPK1/3. Based on these observations, it was suggested that autophagy with a survival-promoting function may be activated when cell cycle arrest was triggered by a specific CDK inhibitor (31). The molecular targets of roscovitine on the autophagic regulation processes have not been fully elucidated yet. To examine its possible molecular targets, the present study first conducted experiments to clarify the potential effect of roscovitine on the key converging molecules between apoptosis and autophagy. Beclin-1 is an important mediator between cell death and survival mechanisms due to its binding ability to anti-apoptotic Bcl-2 family members. Pro-apoptotic Bcl-2 family members have a crucial role in the dissociation of the complex of Beclin-1 and Bcl-2. It was suggested that upregulation of several pro-apoptotic Bcl-2 family members, including BimL, Bcl-2-interacting killer, Bcl-2 homology domain 3 interacting-domain death agonist, Bcl-2-associated death promoter, Puma, but not Bax or Bak, competitively bind to hydrophobic grooves on Bcl-2 and lead to autophagy by inducing the dissociation of Beclin-1 (32). Beclin-1 homodimerization due to Bcl-2 interaction renders autophagic regulation in the cells by preventing heterodimerization of Beclin-1 with Atg14 or other complex proteins. In addition, the interaction between Bcl-2 and Beclin-1 is regulated by c-Jun N-terminal kinase and extracellular signal-regulated kinase (ERK) led to phosphorylation of Bcl-2 or Beclin-1, respectively (33). Death-associated protein kinase is one of the crucial targets of ERK, which consequently induces the phosphorylation of Beclin-1 (34). It was identified that, although HeLa cells were lacking basal Beclin-1 expression, roscovitine treatment for 48 h upregulated the expression levels of Beclin-1. Concomitantly, Bcl-2 downregulation due to roscovitine treatment was marked. It was concluded that the upregulation of pro-apoptotic Puma and Bax was noteworthy following drug treatment for up to 48 h, which may dissociate the Beclin-1 and Bcl-2 complex and lead to heterodimerization of autophagy complex proteins. Additionally, LC3, a cytosolic soluble protein, was cleaved during autophagic induction and its involvement in the autophagic vacuole membrane formation was determined. The 18 and 16 kDa forms of LC3 were called LC3-I and LC3-II, respectively. Amino acid starvation-induced increases in the levels of LC3-II were found to be associated with membrane compartments, whereas LC3-I was localized in the cytoplasm (35). Previous findings stated that roscovitine may synergize both apoptosis and autophagy by enhancing the DNA-damaging effect of doxorubicin in different sarcoma cell lines (30). The number of green fluorescence protein-tagged LC3 puncta was increased due to prolonged cell cycle arrest at G2/M phase triggered by roscovitine treatment in the presence of doxorubicin, a DNA damage-inducing agent. AO staining, which was concentrated in acidic vacuoles, also confirmed that roscovitine synergized the doxorubicin-induced autophagy.
gosome formation. In addition to Beclin-1 upregulation, LC3 cleavage was increased following roscovitine treatment of HeLa cells for 12 h. This suggested that when pro-apoptotic Bcl-2 family members were upregulated in a parallel manner, the autophagy-inducing signaling molecules were also upregulated. p62 has also been demonstrated to be an autophagic marker, which is depleted when autophagosome complexes are engulfed with lysosomes. Exposure of HeLa cells to roscovitine for 48 h eliminated the p62 expression levels. Therefore, lysosomes induced the degradation of p62 and LC3-II. To confirm lysosome-mediated degradation of p62 and LC3-II (16 kDa) in HeLa cells, AO staining was performed. The perceptible acidic vacuoles were distinguished gradually in a time-dependent manner following the exposure of cells to roscovitine. By contrast, as an early response to roscovitine treatment at 12 h, MDC staining indicated acidic vacuole formation in HeLa cells. Therefore, it was concluded that roscovitine (20 µM) induced both apoptosis and autophagy, with each process following one another in a time-dependent manner following the exposure of cells to roscovitine.

Acknowledgements

The authors are grateful to the Istanbul Kultur University Scientific Projects Support Center.

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