Enhancement of Radiosensitivity by Roscovitine Pretreatment in Human Non-small Cell Lung Cancer A549 Cells

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Roscovitine has been reported to have anti-proliferative properties and is in process of undergoing clinical trials. In addition to its intrinsic anticancer properties, it has recently been suggested that roscovitine may also enhance the activity of traditional chemo- and radio- therapies in certain cancer cell lines. The purpose of this study was to define the activity of roscovitine in increasing radiosensitivity of human non-small cell lung cancer (NSCLC) cell line A549 cells in vitro. A549 cells were exposed to ionizing radiation (IR) of γ-ray with or without roscovitine pretreatment. Clonogenic assay was performed and cell cycle and apoptosis were analyzed by flow cytometry. Expression of PARP, Ku70 and Ku80 proteins was detected by Western blot. The active form of caspase-3 positive cells were measured by flow cytometry. Our results showed that roscovitine caused dose-dependent apoptosis in A549 cells. Pretreatment with minimally toxic concentration of roscovitine significantly radiosensitized A549 cells by inhibiting colony formation. We then examined potential mechanisms that may contribute to the enhanced radiation response induced by roscovitine. Our results showed that the combination treatment significantly induced apoptosis in A549 cells compared to roscovitine or IR treatment alone. Meanwhile, in the co-treatment group, the percentage of cells with the active form of caspase-3 was markedly increased, while roscovitine or IR alone had little effect. Roscovitine decreased S phase cells when used alone or in sequential combination with IR. Furthermore, this combination treatment blocked DNA repair process after IR, indicated by down regulation of Ku70 and Ku80 proteins, while the singly used treatment did not. Taken together, these results suggest that roscovitine has the potential to act as a radio-sensitizer in A549 cells by promoting caspase-3 activity and increasing apoptosis, affecting cell cycle distribution and impairing DNA repair process.

INTRODUCTION

Lung cancer is the leading cause of cancer mortality in the world, with non-small-cell lung cancer (NSCLC) accounting for up to 80% of total pulmonary malignancies. Despite the fact that early-stage lung cancer is amenable to and may be cured by surgical resection, recurrence rates remain high and it is often diagnosed at late stages and responds poorly to conventional chemotherapy and radiotherapy, most likely because of the emergence of resistance. Hence there is an urgent need to develop novel treatment strategies.

The cyclin-dependent kinase (Cdk) inhibitor roscovitine is under evaluation in clinical trials for its antiproliferative properties. Roscovitine is a small molecule that inhibits Cdns via direct competition in the ATP-binding site. It is particularly active against Cdk1 (Cdc2), Cdk2, and Cdk5 and induces G1 and G2-M cell cycle arrest. Roscovitine has been reported to have anti-tumor effects in some cancer cell lines by inducing apoptosis, including Ewing’s sarcoma family tumor cells, prostate cancer cells, breast cancer cells and leukemia cells. In addition to its intrinsic anticancer properties, roscovitine has been demonstrated to increase the susceptibility of tumor cells such as HCT116 and H1299 cells to doxorubicin treatment by hindering DNA repair processes. Roscovitine also could enhance radiosensitivity in human breast carcinoma in vitro and in vivo.

In the present study, roscovitine was investigated in combination with ionizing radiation (IR) of γ-ray for its potential enhancing effect on the radiation response of NSCLC A549 cells. Our data showed that roscovitine, by affecting cell...
cycle distribution, promoting caspase-3 activity and inhibiting expression of DNA repair proteins, could significantly increase IR-induced apoptosis in A549 cells.

**MATERIALS AND METHODS**

**Cell culture and treatments**  
NSCLC cell line A549 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum, 10 mg/ml antibiotics (penicillin and streptomycin) and 2 mmol/L L-glutamine at 37°C under 5% CO₂ and saturated moisture. Roscovitine (Sigma, St Louis, Missouri, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma), final concentration of 5 μM, 10 μM, 20 μM and 40 μM was used to treat A549 cells and proper amount of DMSO was used as vehicle control. For combination treatment, cells were pretreated with 10 μM roscovitine for 24 h before undergoing IR with 5 Gy γ-ray. Both adherent and non-adherent cells were harvested by trypsin digestion and washed twice with PBS. The experiments were performed using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, USA) according to the manual. Briefly, cell pellets were re-suspended in 100 μl binding buffer (10 mM HEPES [N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid], 140 mM NaCl, and 2.5 mM CaCl₂, pH 7.4), and stained with 5 μl Annexin V–FITC and 5 μl propidium iodide (PI) staining solution in the dark at room temperature (RT) for 15 minutes. The cell samples were analyzed by flow cytometry on a FACScan station with Cell Quest software using the FL1 and FL2 range for Annexin V FITC and PI, respectively.

**MTT Assay**  
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma) was used to determine cell survival in a quantitative colorimetric assay. A549 cells were plated in 96-well tissue culture plates and allowed to attach for 24 h. The cells were radiated with 2 Gy, 5 Gy and 10 Gy with or without roscovitine 10 μM pretreatment for 24 h. 20 μl MTT (5 mg/ml) was added to the culture medium 4 h before harvesting. The medium was then aspirated carefully without disturbing the blue formazan crystals. Then 150 μl DMSO was added to each well to dissolve the formazan crystals while slightly agitating the cells on an automated shaker.

**Clonogenic Assay**  
The effectiveness of the combination of roscovitine and IR was assessed by clonogenic assay. Briefly, A549 cells were treated with DMSO as vehicle control or roscovitine 10 μmol/L for 24 hours and then irradiated with 5 Gy γ-ray. Following treatment, cells were incubated for 24 hours. Thereafter, cells were trypsinized and seeded in triplicate 60-mm dishes (500 cells per dish) and incubated for 14 days to allow for colony growth. Colonies of > 50 cells were counted manually using a microscope.

**Flow cytometry analysis of active caspase-3**  
Cells were collected after indicated treatments mentioned above. The experiment was performed following the manual. Briefly, cells were digested with trypsin and washed once in PBS, then fixed and permeabilized using the Cytofix/Cytoperm™ Kit (BD Pharmingen) for 20 min at RT, and pelleted and washed with Perm/Wash™ buffer (BD Pharmingen). Cells were then stained with FITC labeled anti-caspase-3 active form (BD Pharmingen) for 60 min at RT in the dark. Following incubation with the antibody, cells were washed in Perm/Wash™ buffer, re-suspended in Perm/Wash™ buffer and analyzed by flow cytometry on a FACScan station with Cell Quest software using the FL1 for FITC labeled Caspase-3 active form.

**Western blotting**  
Cells were rinsed in PBS and then lysed in lysis buffer containing 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS and 50 mM Tris (pH 8.0). The lysates were kept on ice for 30 min before centrifuging at 14,000 rpm to remove any cellular debris. Protein concentrations of the lysates were determined by the Bradford protein assay system (Bio-Rad, Hercules, CA). Equal amounts of protein (20 μg protein each lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond C, Amersham, UK). Immunoblots were blocked with 5% skim milk in TBS/Tween 20 (0.05%, v/v) for 1 hour at RT. The membrane was incubated with primary antibody overnight at 4°C. The antibody for PARP was from BD Pharmingen, Ku70 and Ku80 antibodies were from BD transduction laboratories, and β-Actin antibody was purchased from Sigma. Following several washes with PBS containing 0.1% Tween-20, the membrane was incubated with corresponding secondary antibody conjugated with horseradish peroxidase (Sigma), diluted in 5% skim milk (1: 5000) at RT for 1 h. The blots were developed using an enhanced chemiluminescence Western blotting detection system (Amersham Bioscience, UK).

The absorbance of the suspension was measured spectrophotometrically at 490 nm on an ELISA reader. The results were expressed as a percentage of the absorbance present in treated cells compared to control cells.

Cell cycle analysis
A549 cells were treated with DMSO or roscovitine 10 μM for 24 h, part of the cells were collected as control and roscovitine 24 h groups, the rest cells were exposed to IR of 5 Gy γ-ray and were collected at 6 h and 24 h after IR treatment. At the time of harvesting, cells were digested with 0.25% trypsin (Gibco) and re-suspended in phosphate-buffered saline (PBS), fixed in 70% ethanol at 4°C overnight. When analyzing, cells were washed with PBS and treated with 20 μg/ml ribonuclease A (RNase A, Sigma) at 37°C for 30 min. Cells were then stained with 50 μg/ml propidium iodide (PI, Sigma) for 30 min and DNA content was analyzed by flow cytometry with FACSscan (Becton Dickinson, Mountain View, CA, USA) using the CELLQuest program (Becton Dickinson). Cell cycle distribution was analyzed by WinMDI software.

Statistical analysis
All data represent at least three independent experiments. Statistical comparisons were made using Students’ t-test. P < 0.05 was considered to represent a statistically significant difference.

RESULTS
Roscovitine causes apoptosis in a dose-dependent manner in A549 cells
Since roscovitine could induce apoptosis in certain tumor cells,8,9) we first set out to assure that roscovitine is efficient in A549 cells. In early stage of apoptosis, membrane phospholipid phosphatidylserine (PS) translocates from the inner to the outer leaflet of the plasma membrane and is exposed to the external cellular environment.10) Annexin V has a high affinity for PS and therefore is a sensitive probe for identifying cells that are undergoing apoptosis. Cells were cultured in proper amount of DMSO as vehicle control and different concentrations of roscovitine (5 μM, 10 μM, 20 μM and 40 μM) for 24 hours. Annexin V-PI staining was performed and Annexin-V positive cells were indicated as apoptotic cells. As shown in Fig. 1A and 1B, roscovitine 5 μM, 10 μM, 20 μM and 40 μM were used as experimental groups. Western blot with anti-PARP and anti-Actin antibodies were performed. For PARP, both intact form (116 kDa) and cleaved fragment (85 kDa) were detected.
μM has no effect on apoptosis, roscovitine 10 μM modestly induced apoptosis, but with no statistical significance compared to control cells. Roscovitine 20 μM and 40 μM could effectively induce apoptosis and this effect increased with drug concentration.

To further verify the induction of apoptosis, we did western blot to detect PARP protein cleavage. PARP is a target of the caspase protease activity associated with apoptosis. During apoptosis, PARP is cleaved from its 116 kDa intact form into 85 kDa and 25 kDa fragments and PARP cleavage is considered to be a marker of apoptosis. Western blot (Fig. 1C) using anti PARP antibody which could recognize both the intact 116 kDa form and the 85 kDa fragment of PARP showed that the cleaved fragment of PARP, the 85 kDa-band was nearly undetectable in vehicle control, roscovitine 5 μM and 10 μM groups. Roscovitine 20 μM caused a strong 85 KDa-band, and the band is even stronger in roscovitine 40 μM-treated cells. These results indicated that roscovitine could effectively cause apoptosis in A549 cells in a dose-dependent manner.

Pretreatment with roscovitine enhances radiosensitivity of A549 cells

We first did MTT assay to determine cell survival after different doses of radiation with or without roscovitine pretreatment. We chose the suboptimal concentration of roscovitine (10 μM) to pretreat cells, and 2 Gy, 5 Gy and 10 Gy were used to radiate cells. As shown in Fig. 2A, pretreatment with roscovitine for 24 h significantly decreased cell viability compared to cells with radiation alone.

Colony formation ability was then tested in A549 cells exposed to combinations of roscovitine and IR using clonogenic assay to further determine cell survival. A549 cells were pretreated with 10 μmol/L roscovitine or DMSO for 24 hours, following which the cells were radiated with 5 Gy and plated for clonogenic cell survival assay. Figure 2B shows that roscovitine pretreatment suppressed the clonogenic survival of A549 cells after IR compared to roscovitine or IR treatment alone.

Roscovitine enhances IR-induced apoptosis in A549 cells

IR-induced DNA damage followed by cell death is considered to be the mechanism for cancer cell elimination by radiotherapy. We chose roscovitine 10 μM to treat A549 cells for 24 h before IR with 5 Gy γ-ray. Annexin-V positive cells were used to indicate apoptotic cells. As shown in Fig. 3A, when A549 cells were treated with roscovitine 10 μM for 48 h or IR alone for 24 h, the apoptotic rates were 7.4% and 12.6%, respectively. Enhanced apoptosis (34.6%) was observed when cells were exposed to both roscovitine and radiation, suggesting that roscovitine renders A549 cells more susceptibility to radiation-induced apoptosis. Apoptosis was further confirmed with western blot by detecting PARP and its cleaved fragment shown in Fig. 3B. These results indicated that pretreatment with a minimally toxic dose of roscovitine could enhance sensitivity to subsequent low dose of IR treatment.

Roscovitine promotes IR-induced caspase-3 activity

To understand the molecular mechanisms responsible for the increased activation of apoptotic pathway with roscovitine pretreatment, we investigated the active form of caspase-3 which is the executor during apoptotic event. The
results (Fig. 4A and 4B) showed that cells in vehicle control group were primarily negative for active caspase-3; there were 3.2% and 7.2% of positive cells with active form of caspase-3 at 48 h after roscovitine treatment alone, and 24 h after IR alone, respectively. However, pre-treatment of roscovitine followed by IR treatment greatly increased this percentage to 24.0%. These results showed that pretreatment with roscovitine increased IR-induced apoptosis in A549 cells by triggering caspase-3 pathway.

Roscovitine causes cell cycle re-distribution before and after IR treatment in A549 cells

Cell cycle was measured by flow cytometry after PI staining. As shown in Fig. 5A and 5B, roscovitine 10 μM treatment for 24 hours lowered the percentage of cells in S phase compared to cells in vehicle control group. The two groups of cells were then exposed to 5 Gy γ-radiation. Cell cycle distribution at 6 h and 24 h after IR treatment was compared with those before IR treatment. Without roscovitine pretreatment, cells were arrest in S phase 6 h after radiation, while roscovitine pretreatment caused more cells accumulated in G2/M phase, with little changes in S phase. Twenty-four hours after IR treatment, cells with roscovitine pretreatment had a lower percentage of S phase cells compared to cells with IR treatment alone. Taken together, the response to IR
by arresting cells in S phase was blocked by roscovitine pre-treatment.

**Roscovitine pretreatment inhibits the expression of DNA repair proteins Ku70 and Ku80**

It has been reported that suppressed levels of the repair proteins enhance the radiosensitivity of human tumor cells. To determine whether roscovitine exerted its radiosensitive effects by affecting these proteins, the expression of Ku70 and Ku80, which are known to be involved in the repair of radiation-induced double-strand breaks, was analyzed by western blot in cells treated with roscovitine alone, IR alone and both. We first did western blot to see the changes of Ku70 and Ku80 expression with different concentrations of roscovitine. As shown in Fig. 6A, roscovitine 20 μM or 40 μM
Roscovitine Acts as a Potent Radio-sensitizer in A549 Cells

μM caused down regulation of Ku70 and Ku80 after 24 h's treatment, while roscovitine 5 μM and 10 μM had no detectable effects. These data indicated that roscovitine suppresses Ku70 and Ku80 expression in a dose-dependent manner, which is in constant with the results for flavopiridol, a synthetic Cdk inhibitor. We then chose the suboptimal dose of roscovitine (10 μM) to demonstrate its radiosensitizing effects. As shown in Fig. 6B, the level of Ku70 and Ku80 in cells with roscovitine or IR treatment alone didn’t change much compared to vehicle control, while pretreatment of roscovitine followed by IR resulted in lower level of both proteins. The combined treatment didn’t make detectable repression of Ku70 and Ku80 until 24 h after radiation (Fig. 6C). These results suggest that inhibition of DNA repair may be a mechanism by which roscovitine enhances radiosensitivity in A549 cells.

DISCUSSION

Radiotherapy by gamma-radiation plays a major role in the local treatment of NSCLC patients by inducing DNA damage, triggering cell cycle arrest and apoptosis. It affects cells that are rapidly dividing, such as cancer cells, much more than those that are not. However, many cancer cells including NSCLC cells, developed resistance toward radiation therapy. Roscovitine has been proved to be an effective apoptosis inducer in some malignant tumor cells. The results of present study show that roscovitine could also effectively induce apoptosis in A549 cells in a dose-dependent manner. Based on the results, we used a minimally toxic concentration of roscovitine (10 μM ) to pre-treat A549 cells for 24 h before radiation. This combination of treatment significantly radiosensitizes A549 cells by decreasing cell viability and inhibiting colony formation compared to either of the treatment alone, showing that roscovitine is a potent enhancer of radiotherapy in A549 cells. We further show that this inhibitory effect of cell growth is related to much more apoptotic cells caused by the combined treatment.

Resistance to apoptosis is known to be a hallmark of various cancers and abnormalities of apoptosis regulation have been shown to contribute to the development of resistance to chemotherapy and radiotherapy in cancer. Caspase-3 is a key protease of caspase family which plays an important role in apoptosis. In the present study, although roscovitine 10 μM treatment or 5 Gy IR alone had little effects on caspase-3 activity, when they were combined sequentially, the percentage of cells with active form of caspase-3 was significantly increased.

Roscovitine also had effects on cell cycle distribution which may influence the outcome after IR. Our data showed that roscovitine treatment for 24 hours decreased S phase cells compared to vehicle control. It has been proved that cells in S phase are the least sensitive to IR-caused DNA damage. Since roscovitine drives more cells leave S phase and arrest in G2/M phase, which is considered to be the most sensitive stage of cell cycle to IR, this re-distribution of cell cycle positioned more cells in the radiosensitive phase at the time of radiation, which may be a mechanism by which roscovitine augmented radiosensitivity of A549 cells.

Chemoresistance and radioresistance are considered to be one of the primary reasons for therapeutic failure in tumors. The activation of DNA repair activity of cancer cells after sublethal DNA damage caused by IR might be one reason for the resistance. IR-caused DNA damage leads to the activation of intra-S and S/M checkpoints and subsequent arrest of cell cycle, to allow time for DNA repair. We also observed S phase arrest at 6h after IR treatment, however, roscovitine pretreatment caused decreased S phase cells compared to IR alone. Ku proteins, including Ku70 and Ku80, are subunits of DNA-dependent protein kinase (DNA-PK) complex that are required for the DNA-binding activity of the complex on DNA breakage to facilitate DNA repair process. It has been demonstrated that cells defective in any of the DNA-PK subunits (i.e., DNA-PKcs, Ku70 and Ku80) are highly sensitive to radiation because they cannot repair DSBs efficiently. We showed that roscovitine in combination with IR could down regulate the expression of Ku70 and Ku80 proteins, thus may block the formation of DNA-PK complex and subsequent DNA repair activity. As a result, more cells with damaged DNA underwent apoptosis.

Lim et al found that inhibition of constitutive NF-κB and cyclooxygenase-2 (COX-2) suppressed Ku70 and Ku80 expression in human gastric cancer cells. Ku70 or Ku80 overexpression by transfection with the Ku70 or Ku80 expression gene, respectively, enhanced proliferation of cells with low NF-κB levels. They concluded that Ku70 and Ku80 expression is mediated by constitutively activated NF-κB and constitutively expressed COX-2 in gastric cancer cells. Recently, Dey et al reported that Seliciclib (R-roscovitine) simultaneously causes p53 activation and NF-κB suppression in A549 cells, showing implications in cancer therapy. Thus, the possible mechanism of down regulation of Ku70 and Ku80 after the combination treatment might be that roscovitine pretreatment for 24 h causes NF-κB inhibition and subsequent suppression of Ku70 and Ku80. Further study needs to be done to reveal the precise mechanisms.

Roscovitine has been demonstrated to enhance radiation response in human breast cancer cell line MDA-MB21, which is p53 mutated. Here we reported that roscovitine increases radiosensitivity in A549 cells, in which functional wt p53 status was confirmed. Taken together, functional P53 does not seem to play an important role in this process, however, more cell lines need to be tested to make a conclusion.

In summary, the current data suggest that roscovitine has the potential to increase radiosensitivity in NSCLC A549 cells most likely by affecting cell cycle distribution, pro-
motivating caspase activity and blocking sublethal DNA damage repair process. It warrants future investigation to evaluate the utility of roscovitine as radio-sensitizer in advanced NSCLC lung cancer and other malignancies.

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REFERENCES


