Multiple Pathways of Apoptosis Induced by Roscovitine in Leukemic Cell Lines In Vitro

Hairong Song¹, Åke Sidén² and Zuzana Hassan*,¹,³

¹Experimental Cancer Medicine, Clinical Research Center, Department of Laboratory Medicine, ²Department of Neurology, ³Center for allogeneic stem cell transplantation, Karolinska University Hospital, Huddinge, 14186, Stockholm, Sweden

Abstract: Roscovitine is a potent inhibitor of cyclin-dependent kinases (CDKs) that competes with the ATP binding pocket of kinases. Roscovitine has been shown to have cytotoxic effect on cancer cell lines in vitro and also in tumor xenografts in vivo. A strong synergistic effect in combination with conventional cytostatics has been reported in cancer cell lines in vitro. In this study, the mechanisms of rosvositive-induced cell death were investigated in human leukemic cell lines HL-60, Jurkat and K562. Cells were incubated with rosvositive (0.5-200 μmol/L) up to 24 hours and cell viability and proliferation were studied using resazurin and ³H-thymidine incorporation assays, respectively. Cell cycle and mitochondrial membrane potential were analyzed using flow cytometry, apoptosis was assessed using morphological criteria in Giemsa staining and apoptotic pathways using Western blot analysis. Both viability and proliferation were inhibited in a concentration-dependent manner in all cell lines. Estimated IC₅₀ was 17, 24 and 47 μmol/L for HL-60, Jurkat and K562, respectively. Loss of mitochondrial membrane potential, release of cytochrome c, active fragment of caspase-3 and cleaved PARP were observed in all three cell lines. The cleaved fragments of caspase-2 and -8 were observed in HL-60 and Jurkat cells and the order of appearance differed between these two cell lines, while none of these fragments was observed in K562 cells. Thus, rosvositive is a potent inducer of apoptosis in leukemic cells and apoptosis has been mediated through different pathways depending on the cell line.

Keywords: Roscovitine, CDKI, apoptosis, cell cycle, leukemia.

INTRODUCTION

Cell cycle aberration and apoptosis dysregulation are the hallmark of human cancer [1]. Cyclin-dependent kinases (CDKs) are critical regulators of cell cycle progression and cellular transcription, but they are also associated with apoptosis. CDKs have been suggested to be an attractive target for development of novel anticancer drugs [2]. Roscovitine is a 2,6,9- trisubstituted aminopurine analogue that competes with the ATP binding pocket of kinases. Roscovitine has been shown to have cytotoxic effect on cancer cell lines in vitro and also in tumor xenografts in vivo. A strong synergistic effect in combination with conventional cytostatics has been reported in cancer cell lines in vitro. In this study, the mechanisms of rosvositive-induced cell death were investigated in human leukemic cell lines HL-60, Jurkat and K562. Cells were incubated with rosvositive (0.5-200 μmol/L) up to 24 hours and cell viability and proliferation were studied using resazurin and ³H-thymidine incorporation assays, respectively. Cell cycle and mitochondrial membrane potential were analyzed using flow cytometry, apoptosis was assessed using morphological criteria

The primary cause of chemotherapy failure in leukemia is resistant disease due to defects in cell cycle regulation or apoptotic pathways [16]. Chronic myeloid leukemia (CML) is probably the most extensively studied human malignancy. It is characterized by the BCR/ABL fusion gene that is related to resistance to cytostatic drugs [17]. Recently, it has been found that rosvositive or its analogues flavopiridol and olomoucine induce apoptosis in leukemic cells in vitro when combined with different proteasome or signal pathway inhibitors in vitro [18-22]. Thus, rosvositive in combination with other drugs may be the future therapeutic strategy in leukemia.

In our study, we investigated the effect of CDK-inhibitor rosvositive on human leukemia cells. Our results demonstrated that rosvositive rapidly induced apoptosis through activation of caspase-2 or -8 associated with mitochondrial injury in HL-60 and Jurkat cell lines, respectively, while in K562 cells, apoptosis was initiated through the mitochondrial pathway.

MATERIALS AND METHODS

Reagents

The following materials were used: RPMI 1640 medium, Dulbecco’s phosphate-buffer saline (PBS), fetal bovine serum (FBS) (Gibco, Invitrogen, Stockholm, Sweden), rosvositive (LC laboratories, Boston, USA), etoposide (Bristol-Myers Squibb, Bromma, Sweden), ³H-Thymidine (Amer sham Pharmacia Biotech AB, Uppsala Sweden), resazurin (R&D systems Inc, Minneapolis, MN, USA), propidium iodide (PI) and ribonuclease A (RNase A) (Sigma-Aldrich Sweden AB, Stockholm Sweden), ethanol (Kemety1, Haninge, Sweden).

Cell Cultures

The HL-60 cell line was purchased from DSMZ (Braunschweig, Germany), Jurkat and K562 were purchased from ATCC (LGC Promochem AB, Boras, Sweden). Cells were grown in RPMI 1640 medium supplemented with 10%...
heat-inactivated FBS (complete medium) at 37°C in 95% humidified 5% CO₂ atmosphere. Cells were seeded at a concentration of 2 x 10⁵ cells/ml. All experiments were run in exponentially growing cells in complete medium without antibiotics.

**Treatment**

The stock solution of roscovitine in a concentration of 50 mmol/L was prepared in DMSO and stored at -20°C. Stock solution was diluted in culture medium immediately before use. Cells were incubated with roscovitine in final concentrations of 0.5, 1, 5, 10, 25, 50, 100 and 200 μmol/L up to 24 h. The cells treated with etoposide (VP16) in a final concentration of 6 μg/ml for 24 h served as a positive control for apoptosis. The final concentration of DMSO in all experiments was 0.2%. The cells grown in complete medium served as controls.

**Viability and Proliferation**

Cell viability was studied using resazurin viability assay. Ten thousands cells were seeded in triplicates on 96 wells black microplates and incubated with roscovitine for 24 and 72 h. Then resazurin was added to each well in a final concentration of 10% and incubated for 2 h at 37°C. Fluorescence was read using Fluostar Optima (BMG Labtech, Offenburg, Germany) at a wavelength 590 nm.

Proliferation was assessed using ³H-thymidine incorporation assay. Aliquots of 0.2 ml of cell suspension were incubated in triplicates in 96 wells microplates with 1 μCi ³H-thymidine for 4 h at 37°C. The activity was measured using scintillation fluid (Optiscint Hisafe, Amersham Pharmacia Biotech AB, Uppsala Sweden) and scintillation counter (WALLAC, EG&G Comp, Turku, Finland).

**Cell Cycle Analysis**

The cells were washed once in PBS, fixed in 70% ethanol in PBS and stored at -20°C until analysis. Then the cells were washed in cold PBS, stained in hypotonic PI solution (20 μg/ml) and incubated with RNase A (100 μg/ml) for 30 minutes. The DNA profile was analyzed using FACScan flow cytometer and CELL Quest software (Becton Dickinson, San Jose, CA, USA).

**Assessment of Apoptosis**

Apoptotic cells were identified with morphological criteria such as condensed chromatin and fragmented nuclei in May-Grünwald-Giemsa staining on cytopsin slides. Apoptosis was estimated as percentage of a minimum of 400 counted cells per slide.

**Assessment of Mitochondrial Membrane Permeability**

Mitochondrial membrane permeability (ΔΨm) was assessed using tetramethylrhodamine methyl ester (TMRM, Molecular Probes, Carlsbad, California, USA), which specifically accumulates into mitochondria depending on ΔΨm. Cells treated with roscovitine and controls were incubated with TMRM in a final concentration of 25 nmol/L at 37°C for 30 min. After washing, the cells were resuspended in 1 ml of PBS, and analyzed using flow cytometry.

**Western Blot Analysis**

Cells were lysed in lysis buffer (50 mmol/L Tris pH 7.4, Complete mini protease inhibitor cocktail, 0.1 mol/L PMSF, TritonX 100) on ice for 30 min. The lysate was then centrifuged at 10000 g for 5 min at 4°C, and stored at -70°C. The subcellular fractions were prepared as follows: The cells were suspended in buffer (5 mmol/L Tris-HCl, pH 7.4, 5 mmol/L succinate acid, 10 mmol/L MgCl₂, 0.5 mmol/L EDTA, 147.5 mmol/L KCl, 5 mmol/L KH₂PO₄, 0.005% digitonin), and incubated on ice for 10 min, and then centrifuged at 10000g for 5 min at 4°C. Supernatant containing cytosolic fraction was collected. The remaining pellet was resuspended in Complete mini protease inhibitor cocktail and stored at -70°C.

Protein concentrations were determined with BCA protein assay (Pierce, Rockford, IL, USA) according to the manufacturer’s recommendations. Protein samples (20 μg) were separated with 12% SDS-PAGE and transferred to a PVDF membrane. The PVDF membrane was blocked in 5% non-fatty dry milk solution at 4°C overnight and then incubated with primary antibodies for 2 h at room temperature. The following primary antibodies were used: rabbit antibodies against caspase-2, -3, -8, Bid, Bcl-X L (Becton Dickinson, Stockholm, Sweden) and actin (Sigma, St. Louis, MO, USA), and mouse antibodies against cytochrome c (Becton Dickinson, Stockholm, Sweden), Bcl-2 (Dako Sweden AB, Stockholm, Sweden) and PARP (Oncogene Research Products, Boston, MA, USA). The dilution of primary antibodies was 1:5000 (except for actin and PARP 1:1000). After washing, the membrane was incubated with a peroxidase-conjugated secondary anti-rabbit or anti-mouse antibody in dilution 1:10000 (except for actin and PARP 1:2000) for 1 h at room temperature. The proteins were visualized using ECL Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech AB, Uppsala Sweden).

**Statistical Analysis**

All quantitative experiments were performed three times. Results are expressed as mean ± SD.

**RESULTS**

**Effect of Roscovitine on Cell Viability and Proliferation**

Roscovitine decreased viability of all cell lines in a concentration-dependent manner (Fig. 1). Different sensitivities to roscovitine were observed with IC₅₀ of 17, 24 and 47 μmol/L in HL-60, Jurkat and K562, respectively. Maximum effect of roscovitine was reached within 24 h of incubation and prolonged exposure to roscovitine for 72 h did not further increase cytotoxicity in any of the cell lines. The cytotoxic effect was irreversible, since decrease in viability remained unchanged when the cells were incubated with roscovitine for 24 h, then washed and incubated in roscovitine-free media up to 72 h (data not shown). Incubation with DMSO in the final concentration of 0.2% did not affect viability of cells.

Proliferation decreased in a concentration- and time-dependent way in all cell lines (Fig. 2). However, the effect was most pronounced in HL-60 cells.
The effect of roscovitine on viability of HL-60, Jurkat and K562 cell lines. Cells were seeded in a concentration of 10000 cells per well in triplicates and exposed to roscovitine in concentrations of 0.5 to 100 μmol/L for 24h. Viability was assessed using resazurin assay. Cells incubated with complete medium served as controls. The results were calculated as a percentage of the appropriate control. Experiments were run three times and results are expressed as mean ± SD.

Roscovitine-Induced Apoptosis and Cell Cycle Analysis

Roscovitine induced apoptosis in a concentration- and time-dependent manner in all three cell lines. Roscovitine in concentrations of 25 and 50 μmol/L induced apoptosis in HL-60 and Jurkat cells. Apoptotic morphology was observed already at 3 h and was markedly increased at 6 h of the treatment with roscovitine (Fig. 3a,b). In K562 cell line, apoptotic morphology was induced by concentrations of 100 μmol/L and 200 μmol/L (Fig. 3c).

Roscovitine induced apoptosis without preceding phase arrest at 6 h and 24 h in HL-60 and Jurkat cells. The maximum sub-G₁ peak was detected at 24 h (Fig. 4a,b), simultaneously with morphological changes. However, in K562 roscovitine in a concentration of 50 μmol/L arrested the cells at G2/M, while a concentration of 200 μmol/L resulted in the sub-G₁ population increase (Fig. 4c).

Mitochondrial Membrane Potential

Loss of ΔΨm was concentration- and time-dependent (Fig. 5). A marked loss of ΔΨm was observed already after exposure to roscovitine in concentrations of 25 and 50 μmol/L for 2 h in Jurkat and HL-60 cell lines. Roscovitine in a concentration of 5 μmol/L did not induce a loss of ΔΨm in Jurkat or HL-60 cells (data not shown). In K562, a marked loss of ΔΨm was firstly observed after exposure to roscovitine in concentrations of 100 and 200 μmol/L for 24 h. Only slight loss of ΔΨm was observed after incubation with roscovitine in a concentration of 50 μmol/L for 24 h, lower concentrations (5 and 25 μmol/L) did not affect ΔΨm (data not shown). DMSO exposure did not affect ΔΨm compared to control group in any of the cell lines. Thus, the mitochondrial pathway is involved in roscovitine-induced apoptosis.

Effect of Roscovitine on Apoptotic Pathways

In HL-60, initiator caspase-2 was cleaved to 15 kDa fragment and caspase-8 to 23 kDa fragment already at 6 h. The effector caspase-3 was cleaved to 17 kDa fragment. PolyADP-ribose polymerase (PARP) was cleaved to 85 kDa, thus confirming caspase-3 activation. Fragment of Bcl-2 of 23 kDa was found together with cytochrome-c release and tBid translocation to mitochondrial compartment. In Jurkat cells, the cleavage of caspase-8 preceded cleavage of caspase-2. Full-length Bcl-X₁ disappeared, while Bcl-2 remained intact. Also in Jurkat cell line, the active fragment of caspase-3, together with PARP cleavage, cytochrome-c release and tBid translocation were observed (Fig. 6a,b).
Fig. (3). Roscovitine-induced apoptosis. Cells were incubated with roscovitine in concentrations of 25 to 200 µmol/L up to 24 h. DMSO in final a concentration of 0.2% served as a control for solvent toxicity, while cells incubated with complete medium served as a control. Apoptosis was assessed using morphological criteria in minimum of 400 cells on cytospined slides in Giemsa staining. All experiments were run three times. Results are expressed as means ± SD. The images represent the intact and apoptotic cells of appropriate cell line. (a) HL-60 cell line; (b) Jurkat cell line; and (c) K562 cell line.

Neither caspase-8, nor caspase-2 was cleaved in K562 cells treated with roscovitine in concentrations of 100 µmol/L and 200 µmol/L for 24 h. Caspase-3 cleavage was observed at 24 h incubation with 100 and 200 µmol/L roscovitine. Full length Bcl-XL protein disappeared after the treatment with roscovitine for 24 h implicating the cleavage of the protein simultaneously with cytochrome-c release (Fig. 6c). No such effects were observed after incubation with roscovitine in a concentration of 50 µmol/L (data not shown). Actin remained intact in all three cell lines treated with roscovitine.

DISCUSSION

Roscovitine is a potent CDK inhibitor that has been developed as a targeted anticancer drug. It has been shown to induce apoptosis in several cancer cell lines in vitro and in
However, after incubation with roscovitine in a concentration of 200 μmol/L, apoptosis remained without a prior cell cycle arrest in HL-60 and Jurkat cells. In both HL-60 and Jurkat cells, tBid was detected by Western blot analysis after exposure to roscovitine (Fig. 6a,b). It has been suggested that caspase-2, -8, and granzyme-B cleave Bid at different sites of Asp in DNA damage pathway [27]. Cleaved caspase-3 contributes, as an effector caspase, to the morphological and functional changes in apoptosis, and is also responsible for the inactivation of the PARP processed during the later stages of apoptosis [33].

Another mechanism of apoptotic regulation is via the mitochondrion. Its dysfunction including the loss of ΔΨm, permeability transition and release of cytochrome c into cytosol induces apoptosis [34]. Disruption of ΔΨm has been shown at 2 h of exposure to roscovitine in HL-60 and Jurkat cells (Fig. 5), indicating the involvement of the mitochondrial pathway in roscovitine-induced death. Cytochrome c was released in roscovitine-treated Jurkat and HL-60 cells (Figs. 6a,b). In turn, cytochrome c binds to Apaf 1, resulting in the recruitment and activation of caspase-9 which directly cleaves pro-caspase-3 and -7 [33]. Bcl-2 family regulates the permeability of the mitochondrial outer membrane. Bcl-2 and Bcl-XL are members of the anti-apoptotic group which are cleaved in HL-60 and Jurkat cells treated with roscovitine (Fig. 6). Overexpression of Bcl-2 or Bcl-XL protein have been associated with drug resistance in hematological malignancies [34], since they exert their inhibitory effect on apoptosis by blocking the release of cytochrome c and mitochondrial ΔΨm [35]. Downregulation of the function of anti-apoptotic proteins is a method to overcome this resistance. Roscovitine synergistically interacted with Bcl-2/Bcl-XL antagonist and induced apoptosis in leukemia cells (U937, HL-60 and Jurkat) [21]. In K562 cells, oncogene BCR/ABL encodes a constitutively active form of ABL tyrosine kinase. Substrates of BCR/ABL kinase include Ras, PI3K/Akt and Src family kinase that play an important role in signaling pathways regulating cell survival and death. Moreover, BCR/ABL kinase activates STAT5, and thus contributes to transcriptional activation of Bcl-XL resulting in inhibition of apoptosis [36,37]. We treated the cells with roscovitine in concentrations of 100 and 200 μmol/L for 24 h. Loss of full-length Bcl-XL together with loss of ΔΨm, cytochrome c and cleavage of caspase-3 were observed (Figs. 5,6c), but caspase-2 and -8 remained intact. This indicates that roscovitine-induced apoptosis in K562 cells is related to mito-
Fig. (6). Protein analysis in roscovitine-induced apoptosis. Cells were incubated with roscovitine in concentrations of 5 to 200 μmol/L for 6 and 24 h. Cells treated with DMSO in a final concentration of 0.2% served as a control for solvent toxicity (D), while cells incubated with complete medium served as a control (C). Cells incubated with etoposide (V) in a final concentration of 6 μg/ml for 24 h served as a positive control for apoptosis. Level of expression and/or integrity of proteins were investigated by immunoblotting. Blots were subsequently stripped and probed with antibody directed against actin to ensure equivalent loading and transfer. (a) HL-60 cell line; (b) Jurkat cell line; and (c) K562 cell line.
chondrial pathway. It has been reported that PI3K/Akt signaling pathway rather than MEK1/2/MAPK, plays a major role in regulating the apoptotic response of human leukemia cells (U937, CCRF, NB4, HL-60 and Jurkat) to CDK inhibitors roscovitine and flavopiridol [20]. However, no study reported the mechanisms of roscovitine induced apoptosis in K562 cells.

In summary, roscovitine exerts its cytotoxicity by inducing mitochondrial injury, caspase activation, and apoptosis in human leukemia cells, however, the kinetics of the events differs depending on the cell type. CDKI might pave a new way of the treatment of CML for which ABL tyrosine kinase is generally a therapeutic target.

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REFERENCES