Roscovitine Induces Cell Death and Morphological Changes Indicative of Apoptosis in MDA-MB-231 Breast Cancer Cells

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ABSTRACT

We have previously shown (Mgbonyebi et al., Anticancer Res., 18: 751–756, 1998) that roscovitine, an olomoucine-related purine analogue and a selective inhibitor of cyclin-dependent kinases, inhibited the proliferative activity of human breast epithelial cells in vitro. The purpose of the present study was to identify the cellular processes and targets affected by roscovitine treatment in the estrogen receptor-negative MDA-MB-231 human breast carcinoma cells. Treatment of the cells with 10 μg/ml roscovitine daily for a length of time ranging from 24 to 240 h revealed that the compound inhibited DNA synthesis, induced cell death, and irreversibly inhibited the proliferative activity of the cells. Morphological analysis of roscovitine-treated cells by light and fluorescence microscopy demonstrated that this cyclin-dependent kinase inhibitor induced cell shrinkage, chromatin condensation, reorganization of actin microfilament architecture, and extensive detachment of cells from the cell culture substratum. These cellular events are all known to be associated with apoptosis. Collectively, the data generated from this study suggest that roscovitine induced apoptosis in the estrogen receptor-negative MDA-MB-231 human breast cancer cells. Because the efficacy of many anticancer drugs depends on their ability to induce apoptotic cell death, modulation of this parameter by roscovitine may provide a new chemopreventive and chemotherapeutic strategy for the clinical management of hormone-resistant breast cancers.

INTRODUCTION

Breast cancer is now a common malignant neoplasm in European and North American women. It accounts for more deaths of United States women than any other type of malignancy (1). Despite the combined efforts of scientists and clinicians worldwide, the rate of mortality from the disease is still very high. The cure for breast cancer remains elusive, partly because current treatments for the malignancy are focused on prolonging the survival and improving the quality of life of breast cancer patients rather than cure. Because Beatson demonstrated the effectiveness of ovariectomy for breast cancer treatment (2), estrogen has been identified as the major ovarian hormone responsible for mammary carcinogenesis. Consequently, the clinical management of breast cancer has been based mainly on reducing the effect of estrogens with antiestrogens and aromatase inhibitors (3–6). Although antiestrogens have proved beneficial for palliative care of breast cancer patients (7, 8), it should be realized that only about 30% of breast cancer patients respond to endocrine therapy (9). Moreover, estrogen-responsive tumors initially respond to hormonal treatment but often progress to hormone-independent tumors and become resistant to endocrine therapy (10). Presently, therapeutic choices are limited for hormone-resistant and estrogen receptor-negative breast tumors that are often very aggressive (11). Thus, the treatment of highly invasive tumors that do not respond to endocrine therapy is a major challenge.

The lack of treatment choices for hormone-resistant tumors motivated our search for effective drugs that can be used for the clinical management of both hormone-responsive and nonresponsive breast tumors. Very recently, in vitro screening for anticancer agents in our laboratory led to the discovery that roscovitine, a potent and selective inhibitor of cdk's (12, 13), also inhibits the proliferative activity of human breast epithelial cells (14). Roscovitine is an olomoucine-related purine recently found to inhibit the kinase activity of cdk2/cyclin B, cdk2/cyclin A, and cdk2/cyclin E complexes (15). Micromolar concentrations of roscovitine have been reported to prevent cell cycle progression of mammalian cells at the G1-S and G2-M checkpoints (16, 17). Our experimental studies have demonstrated that roscovitine treatment prevented the exponential growth and decreased the number of viable cells in both estrogen receptor-positive and estrogen receptor-negative breast cancer cells (14). Because the antiproliferative effect of roscovitine is independent of the estrogen receptor status of the breast cancer cells, this potent inhibitor is a potential pharmacological agent for the treatment of both hormone-responsive and -nonresponsive breast cancer cells. To facilitate the translation of the antiproliferative efficacy of roscovitine to the clinical setting, a more detailed study to elucidate the mechanism(s) responsible for its growth inhibitory effect is warranted.

Although the in vivo behavior of any given tumor is remarkably influenced by complex interactions between the tumor cells and the host environment, cell culture models are used for the initial characterization of cellular processes and molecules involved in the modulation of tumor cell behavior (18). In breast cancer studies, estrogen-responsive and -nonresponsive breast cancer cell lines as well as in vivo animal models have been extensively used for elucidating the factors responsible for cell growth (19) and for developing new strategies to inhibit cell growth (20). In this study, MDA-MB-231, a highly malignant estrogen receptor-negative human breast epithelial cell line (21), was used to determine whether the effect of roscovitine was reversible or irreversible, i.e., cytostatic or cytotoxic, and to define the cellular targets and processes affected by the potent cdk-inhibitor.

MATERIALS AND METHODS

Cell Culture. The estrogen receptor-negative MDA-MB-231 human breast cancer cells were purchased from the American Type Culture Collection (Rockville, MD). The cells were grown in DMEM/F12 medium supplemented with 10% FCS and antibiotics. All of the media were obtained from the Fox Chase Cancer Tissue Culture Facility. The cells were routinely cultured in T75 flasks (Corning Inc., New York, NY) at 37°C in a humidified atmosphere of 5% CO2.

Experimental Treatments for Cell Growth Determinations. Fully confluent cells were harvested, counted with a hemocytometer, and seeded into 96-well plates (Corning, Inc., New York, NY) in a final volume of 100 μl of culture medium per well.

To determine whether the antiproliferative effect of roscovitine was reversible or irreversible, three sets of identical cells were plated into triplicate wells in 96-well plates. After 24 h in culture, the first set of cells (controls) were treated with various concentrations of roscovitine. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: cdk, cyclin-dependent kinase; BdRd, 5-bromo-2′-deoxyuridine; TRITC, tetramethylrhodamine B Isothiocyanate; ECM, extracellular matrix.
exposed daily to culture medium, and the second set was exposed to 10 μg/ml roscovitine throughout the duration of the experiment (240 h). The third set of cells was initially treated with 10 μg/ml roscovitine for 96 h, washed with serum-free medium, and then exposed to culture medium for 144 h.

To determine whether the effect of roscovitine was cytostatic or cytotoxic, the cells were treated according to the procedure of Kaur et al. (22) with minor modifications. Three sets of identical cells were plated into triplicate wells in 96-well plates. The first set of cells (controls) was exposed to culture medium while the second set was treated daily with 10 μg/ml roscovitine for 240 h. The third set of cells was initially treated with culture medium until the cells were confluent (120 h) followed by daily treatments with 10 μg/ml roscovitine.

**Cell Growth Determinations.** At each initial time point, cell numbers were determined by counting the number of viable cells with a hemocytometer after staining with trypan blue. Cell growth was determined by treating the cells with the Cell Proliferation Reagent WST-1 (Boehringer Mannheim, Indianapolis, IN) as described previously (23). Roscovitine-treated and -untreated cells were incubated with 10 μl of WST-1 Reagent for 4 h, and the absorbance of the wells was spectrophotometrically read at a wavelength of 490 nm with an EL-312 microplate reader (Bio-tek Instruments, Inc., Winooski, VT). Absorbance values were plotted against the number of cells counted with the hemocytometer, and a standard curve was generated.

**Determination of DNA Synthesis.** The effect of roscovitine on DNA synthesis was determined colorimetrically by the measurement of BrdUrd incorporation. MDA-MB-231 cells were seeded in triplicate wells in 96-well plates. After attachment, the cells were treated daily with different concentrations of roscovitine (ranging from 1 to 20 μg/ml) dissolved in DMSO. Control cells were incubated under identical conditions with the same volume of DMSO-containing medium. DNA synthesis was measured with the BrdUrd colorimetric assay kit (Boehringer Mannheim) according to the manufacturer’s instructions.

**Experimental Treatments for Light and Fluorescence Microscopy.** MDA-MB-231 cells were grown in two-well Lab-Tek chamber slides (Nunc, Inc., Naperville, IL) in a final volume of 1 ml of culture medium per well under standard conditions. After 24 h in culture, the cells in one of the wells were treated daily with 10 μg/ml roscovitine (Calbiochem, La Jolla, CA) dissolved in DMSO, and incubated for lengths of time ranging from 24 to 120 h. Control cells in the second chamber were incubated under identical conditions with the same volume of DMSO-containing medium.

**Evaluation of Apoptotic Morphology by Light Microscopy.** At the indicated times after roscovitine treatments, the cells were washed with PBS, and changes in cell morphology were examined by staining slides with the Leukostat staining reagent (Fisher Scientific, Pittsburgh, PA) as described previously by Cotter et al. (24) with some modifications. After staining, the slides were dried thoroughly, rinsed in deionized water, and mounted with aqueous/dry mounting medium (Biomeda Corp., Foster City, CA). The slides were observed under a phase-contrast light microscope and photographed.

**Analysis of Chromatin Condensation by Fluorescence Microscopy.** Cells grown in two-well Lab-Tek chamber slides were washed in PBS after treatment with 10 μg/ml roscovitine. The cells were fixed for 10 min in 3.7% paraformaldehyde in PBS, washed, and then stained for 2 min with 0.5 μg/ml Hoechst 33342 in PBS (Sigma, St. Louis, MO). The slides were washed in PBS, dried thoroughly, rinsed in deionized water, and mounted with FlourSave Mounting Reagent (Calbiochem, La Jolla, CA). The cells were visualized with a fluorescence microscope at an excitation wavelength of 340–380 nm and emission wavelength of 435–485 nm and photographed. The Hoechst dye stains morphologically normal nuclei dimly blue, whereas apoptotic nuclei demonstrate condensed, smaller, and very intensely bright blue nuclei.

**Analysis of Actin Microfilament Organization.** At the indicated times after roscovitine treatments, MDA-MB-231 cells were washed in PBS, fixed for 10 min in 3.7% paraformaldehyde in PBS at room temperature, and incubated for 5 min in 0.1% Triton X-100. The cells were then washed in PBS and incubated in 0.6 μM of phalloidin labeled with TRITC (Sigma) for 20 min. The slides were dried, rinsed in deionized water, and mounted with FlourSave Mounting Reagent. The cells were analyzed by laser confocal microscopy and photographed.

**RESULTS**

**Irreversible Effect of Roscovitine.** As illustrated by the growth curve in Fig. 1, MDA-MB-231 control cells started to grow exponentially after 48 h in culture and attained confluence after another 100 h of incubation. Compared with the controls, it is evident that 10 μg/ml roscovitine prevented the exponential growth of the cells. When the cells were exposed daily to roscovitine for 96 h, washed, and treated with the culture medium without roscovitine for 144 h, the cells failed to grow despite the absence of the drug. These results demonstrate that the growth inhibitory effect of roscovitine on MDA-MB-231 breast cancer cells was irreversible.

**Cytotoxic Effect of Roscovitine.** As shown in Fig. 2, MDA-MB-231 cells that were exposed to culture medium without roscovitine remained in the lag phase of growth for 48 h, grew exponentially for about 100 h, and became fully confluent after 168 h in culture. In contrast, daily treatment with 10 μg/ml roscovitine prevented the exponential growth of the cells. When nondividing confluent cells were treated with roscovitine after 168 h in culture, a time-dependent inhibition of cell growth was observed after 24 h of treatment. Compared with the controls, the number of viable cells measured by the WST-1 colorimetric assay was reduced by about 50% at the end of the experiment. Because the reduction of WST-1 Reagent to formazan product requires viable cells with functional mitochondrial dehydrogenases (25), these results suggest that roscovitine is cytotoxic to confluent or resting cells.

**Effect of Roscovitine on DNA Synthesis.** Measurement of BrdUrd incorporation during DNA synthesis by proliferating MDA-MB-231 breast cancer cells showed that roscovitine inhibited DNA synthesis in a dose-dependent manner. As illustrated in Fig. 3, treatment of the cells with 1 μg/ml roscovitine had no measurable effect on BrdUrd incorporation compared with the controls, but exposure of the cells to 5 μg/ml roscovitine reduced BrdUrd incorporation by about 25%. Furthermore, exposure of the cells to 10 μg/ml roscovitine inhibited DNA synthesis by 60%, whereas the inhibition induced by treatment of the cells with 20 μg/ml roscovitine was about 90%. These results demonstrate that roscovitine acts as an antimitogenic agent in MDA-MB-231 breast cancer cells.
Effect of Roscovitine on Cell Morphology. Differences in cell morphology were observed between roscovitine-treated and control MDA-MB-231 cells by light microscopy after staining with the LeukoStar reagent. As shown in Fig. 4, the most conspicuous changes observed in roscovitine-treated cells included cell shrinkage and extensive detachment of the cells from the cell culture substratum. These changes, which are characteristics of apoptotic cell death (26), became visible after 24 h of roscovitine treatment but were absent in control cells. The morphological changes became more remarkable with increased time of drug treatment. These observations suggested that cells treated with roscovitine detached from the substratum and died by apoptosis.

Effect of Roscovitine on Chromatin Condensation. The occurrence of apoptosis was further verified by Hoechst staining, which detects chromatin condensation, one of the hallmarks of apoptotic cell death (27). Some differences were observed in the nuclei of roscovitine-treated and -untreated MDA-MB-231 breast cancer cells after staining with Hoechst 33342. (Fig. 5). The Hoechst 33342 dye stained morphologically normal nuclei dimly blue, whereas roscovitine-treated cells demonstrated bright blue and smaller nuclei. These changes in nuclear morphology, which were initially observed after 24 h of roscovitine treatment and increased thereafter, reflected chromatin condensation and nuclear shrinkage. As shown in Table 1, apoptotic cell death assessed by chromatin condensation occurred in 13.8% of MDA-MB-231 breast cancer cells after 24 h of treatment with roscovitine, in comparison with 3.6% in the cells that were treated with the vehicle alone. After 48 h of treatment with roscovitine, 35.7% of the cells were apoptotic as compared with 3.5% of the control cells. Exposure of the cells to roscovitine for 72 h induced apoptosis in 93.8% of the cells, whereas only 6.7% of the cells exposed to the vehicle died by apoptosis. Although chromatin condensation occurred in 4.1% of vehicle-treated cells after 96 h of incubation, this phenomenon was observed in 86.5% of roscovitine treated cells. Moreover, contrary to the absence of condensed chromatin in vehicle-treated cells, 88.2% of cells exposed to roscovitine for 120 h exhibited chromatin condensation. These results demonstrate that roscovitine induces morphological changes characteristic of apoptotic cell death.

Effect of Roscovitine on the Organization of Actin Microfilaments. Because the early breakdown of actin microfilaments has been reported to be a prerequisite for cell-shape alterations and cell death in mammalian cells (28), we investigated the effect of roscovitine treatments on the organization of actin microfilaments in MDA-MB-231 breast cancer cells. As depicted in Fig. 6, staining of the cells with TRITC-labeled phalloidin showed that DMSO-treated control cells exhibited a well-defined F-actin network that was mainly organized into stress fibers. Exposure of the cells to 10 μg/ml roscovitine resulted in disruption of the actin microfilament network. The time course of cytoskeletal modifications that was examined by fixing cells at various time intervals after roscovitine treatments revealed that distinct changes occurred in the architecture of actin microfilaments. The actin fibers of roscovitine-treated cells were disorganized, disassembled, or disrupted. These results clearly indicated that roscovitine induces the reorganization of actin microfilament architecture in MDA-MB-231 breast carcinoma cells.

DISCUSSION

Recent in vitro and in vivo studies have demonstrated that roscovitine inhibits the proliferative activity of a number of mammalian cells (14, 29, 30). Data presented here indicate that roscovitine induces an irreversible inhibitory effect on the proliferation of MDA-MB-231 human breast carcinoma cells. It is evident from the results presented herein that cells exposed to 10 μg/ml roscovitine lose their capability to proliferate after 96 h of treatment with the drug. This finding suggests that this selective inhibitor of cdk's has the potential to irreversibly suppress the growth of breast tumors. From a therapeutic point of view, the clinical relevance of this observation is that roscovitine does not need to be continuously present in the system to be effective in suppressing tumor growth. In addition to its irreversible effect on cell growth, a substantial reduction in the number of viable
cells was observed in confluent nondividing cells treated with roscovitine—results that suggest that the antiproliferative effect of this compound may be partly due to its ability to induce cell death. Moreover, the measurement of BrdUrd incorporation revealed that roscovitine inhibited DNA synthesis in MDA-MB-231 breast cancer cells. The inhibition of DNA synthesis by roscovitine is probably due to its direct effect on the DNA replication machinery, as suggested in a recent report that showed that DNA synthesis was suppressed by roscovitine in homogenized tissues of rat cerebral cortex (31).

The mechanisms responsible for the antiproliferative effect of roscovitine in human breast cancer cells is not yet defined; however, the irreversible cytotoxic effect exerted on the cells by the drug, coupled with the finding that this purine analogue induced cellular shrinkage and chromatin condensation, indicated that apoptosis was induced by roscovitine in MDA-MB-231 breast cancer cells. Cell death by apoptosis results from the action of a genetically encoded suicide program that leads to cellular, morphological, and biochemical changes that include cell volume loss, mitochondrial depolarization, activation of caspase, chromatin condensation, and nuclear fragmentation. Our analyses of roscovitine-treated cells by both light and fluorescence microscopy are the first evidence demonstrating that morphological changes characteristic of apoptotic cell death was induced by this cdk inhibitor. Although induction of apoptosis by a variety of cytotoxic and chemotherapeutic drugs has been reported by some laboratories (32–34), data regarding the involvement of roscovitine in the apoptotic cell death of breast cancer cells are not available. Herein, we have demonstrated for the first time, that apoptotic cell death is induced in hormone-independent breast cancer cells by roscovitine. It was reported recently that overexpression of p27Kip1, a natural inhibitor of cdks was toxic to MDA-MB-231 breast cancer cells (35), but cell death by apoptosis was not demonstrated. Moreover, Wang et al. (36) have demonstrated that overexpression of p27Kip1 induced apoptosis in several mammalian cells. Roscovitine and p27Kip1 kinase inhibitor are both known to inhibit the kinase

Fig. 4. Representative phase contrast photomicrographs of Leukostat-stained MDA-MB-231 breast cancer cells (×40). A, morphology of cells treated with vehicle-containing medium without roscovitine. B, morphology of cells treated with roscovitine showing fewer numbers of cells and cell shrinkage.
activity of cdk2/cyclin E complex (14). These observations, combined
with our current results suggest that inhibition of cdk2/cyclin E
activity may trigger apoptosis in breast cancer cells. Growing evi-
dence now indicates that the efficacy of many anticancer drugs is
related to their ability to induce apoptosis (37); as such, induction of
apoptosis by roscovitine may open new strategies for improving
breast cancer therapy and prevention.

Analysis of actin cytoskeleton of MDA-MB-231 human breast

Fig. 5. Representative fluorescence photomicro-
graphs of MDA-MB-231 breast cancer cells stained
with Hoechst 33342 (×40). A, nuclear morphology
of cells treated with vehicle-containing medium
without roscovitine. B, nuclear morphology of cells
treated with roscovitine. The Hoechst 33342 dye
stains morphologically normal nuclei dimly blue,
whereas apoptotic nuclei demonstrate condensed, in-
tensely bright blue, and smaller nuclei.
cancer cells with TRITC-conjugated phalloidin revealed that actin filaments of cells that were not treated with roscovitine were organized into a dense, dynamic meshwork of actin fibers, whereas the actin fibers of roscovitine-treated cells were either disorganized, disassembled, or disrupted. These observations suggest that roscovitine promotes the breakdown of actin microfilaments. To our knowledge, this is the first demonstration that roscovitine causes the reorganization of actin cytoskeleton. Although the mechanism of action of roscovitine on the cytoskeleton is not yet known, the dramatic effect of the drug on actin filaments indicates that this cdk inhibitor may be involved in cytoskeletal regulation, possibly by reducing the polymerization of actin microfilaments. Recent reports have shown that the reorganization of actin cytoskeleton that is induced by extracellular factors requires phosphatidylinositol 3-kinase activation and subsequent rise in the concentration of intracellular free calcium (38, 39). Thus, it is very likely that the reorganization of actin microfilament induced by roscovitine in our model system may involve the activation of the phosphatidylinositol 3-kinase signaling pathway and induction of calcium influx. In addition, roscovitine may prevent the assembly of actin fibers by modulating the expression and/or activity of Rho GTPases, which have been reported to be involved in the regulation of actin microfilament organization and other associated activities (40–42). Disruption of actin microfilament architecture by roscovitine has some biological implications. In view of the role

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<td>Roscovitine</td>
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% of cells that were apoptotic.

**Table 1** Roscovitine-induced apoptosis in MDA-MB-231 cells

![Fig. 6. Representative confocal photomicrographs of MDA-MB-231 breast cancer cells stained with TRITC-conjugated phalloidin (×60). A, actin microfilament organization of cells treated without roscovitine. B, actin microfilament organization of roscovitine-treated cells showing increased disruption of actin fibers relative to time of exposure.](image-url)
played by actin microfilaments in various aspects of cellular physiology such as cell-cell interactions, cell migration, proliferation, and secretion (42), it can be argued that all of these cellular activities could be affected in breast tumors after roscovitine treatment.

Another notable observation from our morphological analysis was the extensive detachment of cells from the cell culture substrate after exposure to roscovitine. Twenty h after treatment, a progressive loss of cell attachment was observed in roscovitine-treated cells that was not observed in the untreated set of cells. In adherent cells, detachment from the substrate is associated with morphological changes characteristic of apoptosis such as cellular shrinkage and chromatin condensation (43). Recent evidence suggests that cellular attachment to the substrate is mediated by the interactions of integrins with ECM components such as fibronectin, collagen, and vitronectin (26). Binding of integrins to these adhesion molecules results in the activation of focal adhesion kinase (44, 45) accompanied by phosphorylation and recruitment of a number of related cytoskeletal and signaling molecules, thereby transducing anchorage and survival messages to the nucleus (46–48). Conversely, the uncoupling of integrins from ECM proteins leads to disruption of integrin-mediated signal transduction, inactivation of focal adhesion kinase, detachment of cells from the ECM, and apoptotic cell death (49–51). Our data suggest that after roscovitine treatments, MDA-MB-231 cells detach from cell culture substrate and die via apoptosis. This notion is consistent with previous reports that demonstrated that cells deprived of matrix attachment undergo apoptosis (52, 53). Thus, the extensive detachment of cells from the cell culture substrate and the apoptotic cell death observed in our experimental system may be due to the uncoupling of integrin-mediated signaling and/or disruption of cell-matrix interactions induced by roscovitine. In addition to facilitating apoptosis, the loss of adhesion induced by this cdk inhibitor may deny the cells the anchorage and traction necessary for growth and migration and thus prevent breast cancer invasion and metastasis, the major cause of death in breast cancer patients. Because adhesion and invasion are crucial to the initiation of metastatic growth (54), additional studies on the effect of roscovitine on cell adhesion to extracellular matrix components as well as the anti-invasive potential of the drug could be extremely rewarding. Such studies are now going on in our laboratory.

REFERENCES


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