Roscovitine Inhibits Differentiation and Invasion in a Three-Dimensional Skin Reconstruction Model of Metastatic Melanoma

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Abstract
The aim of this study was to investigate the therapeutic potential of a cyclin-dependent kinase inhibitor, roscovitine, in cultured melanoma cells and a three-dimensional skin reconstruction model of metastatic melanoma. The modulatory effects of roscovitine on the growth and survival of normal melanocytes and cultured melanoma cell lines were tested. Additionally, we investigated the potential of roscovitine to regulate the growth and differentiation of a metastatic melanoma cell line (A375) in a three-dimensional skin reconstruction culture consisting of A375 cells admixed with normal human keratinocytes embedded within a collagen-constricted fibroblast matrix. We show that roscovitine is able to induce apoptosis in the melanoma cell lines A375, 888, and 624 but not in normal human cultured epithelial melanocytes. The degree of apoptosis within these cell lines correlated with the accumulation of p53 protein and concomitant reduction of X-linked inhibitor of apoptosis protein, with no change in the proteins Bcl-2 and survivin. We also found that roscovitine inhibited the growth and differentiation of A375 melanoma cells within the dermal layer of the skin. The results of this study show that roscovitine has the potential to inhibit the differentiation and invasion of metastatic melanoma and may be useful as a therapy for the treatment of patients with metastatic melanoma.

Introduction
Melanoma is the most aggressive form of skin cancer, responsible for six of seven total skin cancer deaths. Although the incidence of melanoma has increased dramatically in the last few years, there have not been parallel advances in therapies for those with advanced melanoma, highlighting the critical need to evaluate new therapeutic strategies (1-3). The inhibition of the metastatic process is a central focus of research, as the majority of patients with advanced stage III and IV disease will ultimately die of disseminated melanoma (4, 5). The malignant transformation and metastatic potential of melanoma is a complex process involving multiple cellular and molecular mechanisms (6). Previous research has identified several possible involved mechanisms, such as the disruption or interference of the metastatic process involving aberrations in the adhesion and degradation of the extracellular matrix, cell cycle deregulation, and escape from apoptosis. Thus, it is hypothesized that identifying a protecting mechanism capable of preventing a primary tumor cell from metastasizing may require a blockade of one or more of these processes.

Cell cycle deregulation is one of the hallmarks of malignant transformation, with the cyclin-dependent kinase (Cdk) pathway emerging as an attractive target for cancer therapy (7). One class of small-molecule Cdk modulators includes the purine analogue roscovitine, which inhibits Cdk activity directly by competing for the ATP-binding sites of Cdk and causing apoptosis within various tumor cells (8-15). Others have examined the clinical utility of roscovitine in a phase I dose escalation trial, revealing that roscovitine is well tolerated with limited toxicity (16). Compared with the parent compounds (olomoucine and bohemine), roscovitine is metabolized slowly from the plasma with an improved tissue distribution and higher uptake by tumor tissues in mice (17-19). It is currently in phase II clinical trials for patients with lung and breast cancer (16). The mechanism of action involved in the antiproliferative activity of roscovitine remains poorly understood. It is hypothesized that the antiproliferative effect of roscovitine is attributed to the inhibition of Cdk2 and Cdc2, with recent reports indicating that the mechanism responsible for cell death is dependent on the inhibition of transcriptional Cdkks, such as Cdk7/Cdk9, and other signaling pathways (16).

In this study, we tested the therapeutic efficacy of roscovitine in cultured melanoma cells and in a three-dimensional skin reconstruction (3-DSR) model of metastatic melanoma. The latter model consists of a coculture of epidermal keratinocytes and melanoma cells plated onto fibroblast-contracted collagen gels (20-23). In contrast to melanoma cells in monolayer, the growth pattern and biology of melanoma cells in a 3-DSR model closely resemble those...
Apoptosis was further confirmed by analysis of cleavage of caspase-dependent cell death in A375 melanoma cells. Roscovitine treatment induced apoptosis in melanoma cells, resulting in a decreased overall cell survival.

We examined the role of p53 in sensitizing melanoma cells to roscovitine by comparing the effects of roscovitine in A375 cells, which express wild-type p53, with those in SK-Mel-2, SK-Mel-28, and MeWo cells, which express mutant p53. The results of the MTT assay reveal that roscovitine reduced the viability of A375 cells at an IC50 of 25 μmol/L, whereas it failed to induce apoptosis in SK-Mel-2, SK-Mel-28, and MeWo cells even when treated with 50 μmol/L roscovitine for 72 h (Fig. 3C). Together, these results suggest that the lack of response in SK-Mel-2, SK-Mel-28, and MeWo cells treated with roscovitine could be partially attributed to the expression of mutant p53 in these cells.

Roscovitine was also examined in an organotypic skin culture system using a full-thickness 3-DSR model of A375 melanoma cells (Fig. 4). Single-cell suspensions of normal human keratinocytes and A375 melanoma cells were seeded at a ratio of 1:10 onto a fibroblast-contrainted collagen gel matrix. The resultant cell culture inserts were allowed to grow and differentiate in serum-free medium to form three-dimensional, highly differentiated, full-thickness, skin-like tissues. H&E staining of day 8 cultures revealed a dermis composed of numerous viable fibroblasts and an epidermis consisting of organized basal layer of spinous and granular keratinocytes and stratum corneum. In addition, clusters of A375 melanoma cells were found at the dermal/epidermal junction (data not shown).
On day 8, the 3-DSR inserts were then cultured with medium containing DMSO or 25 μmol/L roscovitine. The roscovitine-containing medium was replenished every other day, and duplicate cultures were fixed, stained, and sectioned on days 10, 17, and 21. The results of H&E staining reveal that cultures treated with DMSO developed nodules of melanoma cells by day 10 (Fig. 4A), gradually invading the dermis by day 17 (Fig. 4B and C). Individual melanoma cells infiltrating the dermis were visible in this culture by H&E staining. In contrast, cultures treated with roscovitine had smaller tumor nodules and remained closer to the epidermis with less invasion of the dermal structures (Fig. 4D-F).

We confirmed the presence of melanoma cells within the 3-DSR culture by immunohistochemical analysis for the expression of the S100 protein, comparing S100 expression in tissue sections prepared from day 17 culture inserts exposed to either DMSO or roscovitine. It is evident that the majority of the cells isolated from the tumor nodules treated with DMSO were strongly positive for S100, with fewer positive cells detected within the epidermis of roscovitine-treated cultures (Fig. 5A and B). These results suggest that roscovitine was able to prevent the growth and differentiation of melanoma cells. We also examined the proliferation of melanoma cells by immunohistochemical analysis with the Ki-67 antibody and found that 50% of DMSO-treated S100-positive cells also expressed the Ki-67 protein, suggesting that melanoma cells in the nodule are proliferating (Fig. 5C). In contrast, none of the S100-positive cells found within the epidermis of roscovitine-exposed cultures expressed Ki-67 (Fig. 5D). There were only a few melanoma cells within the dermis that were positively stained for Ki-67.

Lastly, we used immunohistochemical analysis of activated caspase-3 to examine the inhibition of melanoma cell growth by apoptotic mechanisms. We found higher levels of activated caspase-3 in cell cultures exposed to roscovitine compared with...
DMSO (Fig. 5E and F). A quantitative analysis of the immunohistochemical results depicting the differences in the expression of S100, Ki-67, and active caspase-3 in melanoderm cultures treated with DMSO versus roscovitine is shown in Fig. 5G. Collectively, these results suggest that roscovitine treatment can inhibit the growth and differentiation of melanoma nodules and is also able to induce apoptosis of melanoma cells within the surrounding skin matrix.

Discussion

We have attempted to increase our understanding of the mechanism of action of roscovitine and its role in melanoma metastasis. Our results show that (a) roscovitine induces apoptosis of A375, 888-Mel, and 624-Mel cells but not of normal human epithelial melanocytes; (b) the level of apoptosis in these cells directly correlates with the accumulation of p53 and reduction of XIAP but not of Bcl-2 and survivin; and (c) roscovitine is able to inhibit the growth and differentiation of A375 melanoma nodules in the dermal layer of skin.

Roscovitine inactivates Cdk2, Cdc2, Cdk7, and Cdk9. Roscovitine induced G1 and/or G2-M cell cycle arrest of both melanocytes and melanomas, which is consistent with its inhibition of Cdk2 and Cdc2 activity, as indicated by the analysis of cell cycle profiles and cell growth. These results are in part agreement with others that suggest a critical role for Cdk2 in supporting melanoma growth (24). In contrast to other tumor types, melanocytes and primary melanoma express higher levels of Cdk2 mRNA and protein, with the latter maintained by a melanoma-specific transcription factor, MITF, expressed in the majority of human melanomas. The depletion of Cdk2 with small interfering RNA or overexpression of a dominant-negative mutant arrests melanoma cells at the G1 phase and strongly suppresses their colony growth without inducing apoptosis (24). These studies provide further evidence that roscovitine is an inhibitor of the cell cycle in both melanocytes and melanoma cells.

Cdks, such as Cdk7 and Cdk9, which are additional targets of roscovitine, regulate transcription by phosphorylating the COOH-terminal domain of RNA polymerase II (25). Recent reports indicate that tumor cells may have increased dependence on RNA polymerase II transcriptional activity and roscovitine can induce cell death by inhibiting its activity (25). Accordingly, dephosphorylation of RNA polymerase II by roscovitine induced sustained DNA damage and apoptosis of melanoma cells, but not of melanocytes, as determined by H2AX phosphorylation, DNA fragmentation, and PARP cleavage. Further, inactivation of Cdk7 and Cdk9 leads to inhibition of transcription and may account for the reduced expression of short-lived proteins with short-lived transcripts, such as the antiapoptotic proteins XIAP and Mcl1. We found that roscovitine treatment specifically down-regulated expression of XIAP but not of Mcl1. However, a direct link between RNA polymerase activity and loss of XIAP expression has not been firmly established. Although Bcl-2, a MITF regulated gene, is overexpressed in melanoma cells, its expression did not change in roscovitine-treated melanoma cells, suggesting that roscovitine may induce apoptosis, in part, through the down-regulation of XIAP. This, in turn, may allow cells to escape from the inhibitory activity on caspase-3 and caspase-9.

The p53 tumor suppressor protein plays a key role in the chemosensitivity and radiosensitivity of cancer cells. We previously reported that maximum apoptosis of prostate cancer cells by roscovitine required the accumulation of wild-type p53 (14). Coincidentally, we observed a significant degree of apoptosis in roscovitine-treated A375, 888-Mel, and 624-Mel...
cells that harbor wild-type p53 but not in SK-Mel-2, SK-Mel-28, and MeWo, previously shown in prostate cancer (14). Because melanoma cells have a very low frequency of p53 mutations, with genetic alterations found in only 1% to 5% of all primary melanomas and in 11% to 25% of metastatic lesions (26, 27), roscovitine may seem to be a more attractive therapeutic agent for patients with melanoma compared with other tumor types where >50% tumors express mutant p53.

We also show that roscovitine is able to modulate the growth and differentiation of melanoma cells in a three-dimensional skin culture. Routine cell culture techniques generally fail to reproduce drug effects on intact human skin, which comprises complex epithelial-mesenchymal interactions that are poorly understood (20-23). In an attempt to overcome this barrier, others have developed a 3-DSR model that mimics the normal in vivo tumor microenvironment (20, 21, 28, 29). The 3-DSR melanoma model has several advantages over melanoma cells on monolayer: (a) the culture system results in an epidermal morphology and differentiation; (b) it includes cellular interactions that take place within the skin involving melanoma cells, keratinocytes, and fibroblasts; and (c) it has the potential to reveal not only drug efficacy but also the molecular mechanisms underlying the mode of action.

We examined the potential of roscovitine to modulate the growth and differentiation of A375 melanoma cells in a full-thickness 3-DSR model. Our results suggest that roscovitine (a) significantly reduced the growth and proliferation of A375 melanoma nodules as determined by a reduction in tumor nodule size, (b) clearly blocked the invasion of A375 cells to the dermis and in the roscovitine-treated cultures (the remaining S100-positive cells were found closer to the epidermis with less invasion of the dermis), and (c) induced apoptosis of A375 melanoma cells as determined by increased activity of caspase-3 in drug-treated cultures compared with that of DMSO. Collectively, these studies show for the first time that roscovitine is effective in reducing the growth of melanoma cells and inducing apoptosis.

Roscovitine is highly specific; of 151 kinases examined, it binds to Cdks and pyridoxal kinase only (30). A phase I single-agent dose escalation trial has previously revealed that roscovitine is well tolerated with limited toxicity (17-19). Currently, phase II trials for lung and breast cancers are under way (17-19). We believe that there may be a role for using roscovitine in the treatment of patients with advanced melanoma. Further studies, in the clinical setting, to assess the effects of roscovitine as a new therapeutic adjuvant in the treatment of melanoma are warranted. Previous experience with single-agent chemotherapy, however, has yielded only limited success, with response rates well below 20% (31, 32). Given our poor therapeutic modalities and even poorer understanding of the molecular and immunologic events of melanoma, a multimodal approach may be justified, possibly combining roscovitine with an inhibitor of either mitogen-activated protein kinase or phosphatidylinositol 3-kinase/Akt, if higher response rates can be achieved.

Materials and Methods

Cell Lines

The human melanoma cell lines A375, 888-Mel, and 624-Mel were cultured in RPMI 1640 containing 10% fetal bovine serum (33). These lines were derived from patients with metastatic melanoma. The SK-Mel-2, SK-Mel-28, and MeWo cell lines were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Eagle’s MEM supplemented with 2 mmol/L l-glutamine and Earle’s balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, and 10% fetal bovine serum. HEMA-Lp cells were purchased from Cascade Biologics (Portland, OR) and cultured in standard cell culture medium (254 media; Cascade Biologics) supplemented with phorbol 12-myristate 13-acetate–free human melanocyte growth supplement-2 as per the manufacturer’s instruction.

Cell Proliferation and Apoptosis Assays

Cell cycle distribution assays were done using established methods (33). DNA synthesis was measured by BrdUrd incorporation assay. Briefly, cells were cultured on chamber
slides with increasing concentrations of roscovitine (Calbiochem, San Diego, CA) and pulsed with BrdUrd during the last 2 h of treatment. Slides were then fixed with 4% paraformaldehyde for 10 min at 4°C, denatured for 1 h with 2 N HCl at 37°C, and renatured with 1 mol/L Tris (pH 11) for 10 min. Slides were washed and incubated with phycoerythrin-conjugated anti-BrdUrd antibody overnight at 4°C, washed, counterstained with PicoGreen DNA stain before mounting, and then examined for BrdUrd-positive (yellow) or BrdUrd-negative (green) nuclei by fluorescent microscopy. Cell viability was measured using the MTT assay. Briefly, melanoma cells were seeded in flat-bottomed 96-well microtiter plates at a density of 10⁵/mL, incubated for 24 h at 37°C, and then exposed to increasing concentrations of roscovitine followed by an additional 72 h of incubation. MTT (20 μL of a 5 mg/mL solution) was added to each well and incubated for 5 h. After removal of the medium, the precipitated formazan crystals were dissolved in DMSO (100 μL) and the plates were analyzed at 570 nm using a spectrophotometer. DNA fragmentation was determined using previously described methods (14).

3-DSR Culture

Day 7 culture inserts of full-thickness 3-DSR model of A375 melanoma cells were purchased from MatTek (Ashland, MA). These cultures were prepared by plating single-cell suspensions of normal human epidermal keratinocytes and A375 melanoma cells on inserts. DMSO or roscovitine (25 μmol/L; D-F) was added every other day. C. DMSO-treated cultures developed nodules by day 10 (arrow) and gradually invaded the dermis by day 21. B and C. Arrows, melanoma cell clusters at the epidermal-dermal junction; arrowheads, melanoma cells infiltrating the dermis. In roscovitine-treated cultures, nodules were smaller in size and melanoma cells remained close to the epidermis (arrows, D) or moved up into the epidermis (arrowheads, E). F. By day 21, separation of the dermal/epidermal junction occurred. Each treatment was done in duplicate. Representative section from each treatment.

![FIGURE 4. Roscovitine inhibits invasion of A375 melanoma in a 3-DSR model. A to F. H&E staining of A375 melanoma 3-DSR cultures. At day 7, 3-DSR inserts were cultured in the presence of DMSO (A-C) or roscovitine (25 μmol/L; D-F). Inserts were supplemented with fresh medium containing roscovitine every other day. C. DMSO-treated cultures developed nodules by day 10 (arrow) and gradually invaded the dermis by day 21. B and C. Arrows, melanoma cell clusters at the epidermal-dermal junction; arrowheads, melanoma cells infiltrating the dermis. In roscovitine-treated cultures, nodules were smaller in size and melanoma cells remained close to the epidermis (arrows, D) or moved up into the epidermis (arrowheads, E). F. By day 21, separation of the dermal/epidermal junction occurred. Each treatment was done in duplicate. Representative section from each treatment.](image)

![FIGURE 5. Roscovitine inhibits proliferation and induces apoptosis of melanoma cells in a 3-DSR model. A to F. Sections of day 17 culture inserts treated with DMSO or roscovitine were examined for S100 (A and B) and Ki-67 by immunohistochemistry (C and D). E and F. Sections of day 21 culture inserts were examined for expression of active caspase-3 by immunohistochemistry. Each treatment was done in duplicate. Representative section from each treatment. Arrow, positive staining. G. Quantitative analysis of the immunohistochemical results (A-F) depicting the differences in the expression of S100, Ki-67, and active caspase-3 in cultures treated with DMSO (D) versus roscovitine (R). Positively stained cells were counted in three different fields (at ×40 magnification) of each slide. Columns, mean; bars, SD. The p represents significance of the difference in the treatment group compared with the control.](image)


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cells at a 1:10 ratio on fibroblast-contracted collagen gels within cell culture inserts. They were allowed to grow and differentiate in DMEM-based serum-free medium, forming three-dimensional, highly differentiated, full-thickness, skin-like tissues. On day 8, culture inserts were incubated in duplicate with serum-free medium containing either DMSO or roscovitine. Medium was replenished every other day, and cell cultures were collected on days 10, 17, and 21 and fixed with 10% formalin. For immunohistochemistry, culture inserts were paraffin embedded, serial sectioned, and analyzed by H&E staining. The paraffin-embedded sections were deparaffinized, rehydrated in PBS, and treated with proteinase K for 5 min at 37°C for antigen retrieval. The sections were then stained with antibodies specific for the detection of S100, Ki-67, and active caspase-3 (Ventana Medical Systems, Tucson, AZ). Appropriate positive and negative controls were included for each antibody test.

**Western Blotting and Kinase Assay**

Cells were harvested, lysed, and analyzed by Western blotting as described previously (12). We used primary antibodies against Cdk2, Cdc2, Cdk7, Cdk9 (Santa Cruz Biotechnology, Santa Cruz, CA), PARP, p53 (Cell Signaling Technology, Beverly, MA), RNA polymerase II, phosphorylated RNA polymerase II, γH2AX (Upstate Biotechnology, Charlottesville, VA), Bcl-2, XIAP (BD Biosciences, San Jose, CA), and β-actin (Sigma, St. Louis, MO). The kinase assay was done using established methods (34).

**Statistical Analysis**

Results were expressed as the mean ± SD. Levels of significance of the differences between control and treatment groups were determined by the Student’s t test. Values of $P < 0.05$ were considered statistically significant.

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**References**

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