Serine palmitoyltransferase inhibitor myriocin induces growth inhibition of B16F10 melanoma cells through G₂/M phase arrest

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Abstract

Objectives: Melanoma is the most aggressive form of skin cancer, and it resists chemotherapy. Candidate drugs for effective anti-cancer treatment have been sought from natural resources. Here, we have investigated anti-proliferative activity of myriocin, serine palmitoyltransferase inhibitor, in the *de novo* sphingolipid pathway, and its mechanism in B16F10 melanoma cells.

Material and methods: We assessed cell population growth by measuring cell numbers, DNA synthesis, cell cycle progression, and expression of cell cycle regulatory proteins. Ceramide, sphingomyelin, sphingosine and sphingosine-1-phosphate levels were analysed by HPLC.

Results: Myriocin inhibited proliferation of melanoma cells and induced cell cycle arrest in the G₂/M phase. Expressions of cdc25C, cyclin B1 and cdc2 were decreased in the cells after exposure to myriocin, while expression of p53 and p21^{waf1/cip1} was increased. Levels of ceramide, sphingomyelin, sphingosine and sphingosine-1-phosphate in myriocin-treated cells after 24 h were reduced by approximately 86%, 57%, 75% and 38%, respectively, compared to levels in control cells.

Conclusions: Our results suggest that inhibition of sphingolipid synthesis by myriocin in melanoma cells may inhibit expression of cdc25C or activate expression of p53 and p21^{waf1/cip1}, followed by inhibition of cyclin B1 and cdc2, resulting in G₂/M arrest of the cell cycle and cell population growth

inhibition. Thus, modulation of sphingolipid metabolism by myriocin may be a potential target of mechanism-based therapy for this type of skin cancer.

Introduction

Malignant/advanced melanoma is the most aggressive form of skin cancer, has poor prognosis, and its incidence is rapidly increasing. Survival of patients with metastatic malignant melanoma ranges from 4.7 to 11 months (median survival 8.5 months) (1). Poor survival in malignant melanoma patients is mainly due to tumours' resistance to radiation or chemotherapy, by dysregulation of apoptotic pathways (2–4). Several postoperative adjuvant immunotherapies have been approved to treat malignant melanoma (5,6), interferon- α and interleukin-2 have been commonly used for advanced disease (5–7), but these cause significant toxicity and have low efficacy.

Candidate drugs for effective anti-cancer treatment, from natural resources, have been sought. Myriocin has been known as either ISP-1 or thermozymocidin and has been produced from *Mycelia sterilia*, *Isaria sinclairii* and *Cordyceps cicadae*. Its chemical structure was identified as (2S, 3R, 4R, 6E)-2-amino-3,4-dihydroxy-2-hydroxy-methyl-14-oxo-6-eicosenoic acid (8,9) (Fig. 1a) and it was shown to suppress proliferation of lymphocytes and IL-2-dependent mouse cytotoxic T cells (10). Myriocin inhibits serine palmitoyltransferase, the first step in a *de novo* sphingolipid biosynthesis pathway, which reduces the intracellular pool of sphingolipid intermediates (11). Myriocin also decreases extracellular sphingomyelin, sphingosine-1-phosphate and glycosphingolipids levels (12,13).

Sphingolipids are highly bioactive and are involved in regulation of apoptosis, cell proliferation, cell migration, cell senescence and inflammation (14–18). Ceramide and sphingosine have been implicated to be lipid signalling molecules in regulation of apoptosis, while sphingosine-1-phosphate has been reported to be a trigger for signal transduction pathways of cell proliferation (19–21).

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Figure 1. Inhibitory effect of myriocin on malignant melanoma cell population growth. (a) B16F10 malignant melanoma cells were seeded at a density of 10^5 cells per well (10 cm^2) on six-well plates and cultured for 24 h. Cells were exposed to myriocin at 0, 0.1, 0.5, 1, 5, and $10 \mu\text{M}$ for 24–96 h, and both morphological appearances and confluence were observed by phase contrast microscopy (magnification, $\times 100$). The cells were then trypsinized, and numbers were counted using a haemocytometer. (b) Monolayers of malignant melanoma cells were scored and preparations cultured in medium for 48 h with or without myriocin. Cell migration into the wound areas was examined by phase-contrast microscopy. (c) After exposure of malignant melanoma cells to myriocin at 1 and $10 \mu\text{M}$ including 4 h treatment with [^3H] thymidine, labelling reaction was terminated and quantified using a liquid scintillation counter. Data expressed as mean $\pm$ SD of three independent experiments in triplicate. Significant differences from the controls, * $P < 0.05$ and ** $P < 0.01$.

Ceramide-1-phosphate has been identified as a direct activator of cytosolic phospholipase A₂, which may be implicated in mediation of inflammatory responses (15). Altered glycosphingolipid profiles in multidrug-resistant ovarian cancer cells suggests that lactosylceramide biosynthesis may be largely uncoupled from glucosylceramide biosynthesis in the Golgi apparatus (22).

The present study has focused on inhibition of cell number expansion by myriocin treatment in malignant melanoma. Cell population growth is tightly coupled to cell cycle progression (23), which in turn is controlled by cyclins that binds to cyclin-dependent kinases (CDK). Progression through G₁ phase to S phase, and G₂ phase to M are cooperatively regulated by several classes of CDK, which are constrained by CDK inhibitors. Transition requires activity of CDK4, CDK6, cdc2, cyclin B1, cyclin D, cyclin E and CDK inhibitors (24–26). Arrest of the cell cycle can be induced by CDK inhibitors bound to cyclin-CDK complexes (27).

In this study, we investigated anti-carcinogenic effect of myriocin and the mechanism by which it works, in murine malignant melanoma cells. We report for the first time that alteration in sphingolipid metabolism by myriocin induces cell-cycle arrest leading to inhibition of cell proliferation, and this may be a potential target for anti-cancer therapy.

Materials and methods

Materials

Myriocin, caffeine and *o*-phthalaldehyde (OPA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and RPMI 1640 medium was purchased from Life Technologies Inc. (Gaithersburg, MD, USA). Sphingolipid ceramide *N*-deacylase (SCDase) was obtained from Takara (Shiga, Japan) and [^3H]-thymidine was purchased

from Amersham Pharmacia Biotech (Buckinghamshire, UK). Primary antibodies against cyclin B1, cdc2, cdc25C, Chk2, PLK1, Wee1, p53, p-p53 and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and p21^{waf1/cip1} antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA). C₁₇ based-sphingosine, C8-ceramide and *N*-oleoyl-D-erythro-sphingosine (C₁₇ base) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Other reagents were of the highest purity available.

Cell culture

B16F10 cells originating from a murine malignant melanoma were obtained from ATCC (Manassas, VA, USA) and were cultured in RPMI 1640 medium containing 10% foetal bovine serum (FBS), 100 units/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine in a CO₂ incubator at 37 °C. Myriocin was added to the cells as an ethanolic solution, and control cells were treated with the same vehicle concentration (which did not exceed 0.05%) in RPMI 1640 medium containing 2% FBS.

Measurement of cell proliferation

B16F10 melanoma cells were seeded into six-well culture plates at 10⁵ cells per well and were grown in RPMI 1640 containing 10% FBS at 37 °C for 24 h. When they reached 70% confluence, the medium was treated with myriocin for 24–96 h. Cells in each well were collected after treatment with trypsin-EDTA, then counted using a haemocytometer.

Migration assay

Cells were cultured in 12-well plates and grown to confluence. Medium was aspirated and cells were incubated with RPMI 1640 medium containing 0.1% FBS for 24 h before each experiment. Tips (200 μ l) were used to score the samples to provide denuded areas. Cells were washed twice in PBS and treated with myriocin for 48 h. Cell migration into wound areas was examined by phase-contrast microscopy. Photomicrographs were taken at 0, 24 and 48 h, and cell migration distance was determined by subtracting values obtained at 0 h from 24 and 48 h. Migration distances were expressed as percentages over control values. A representative experiment of at least three independent experiments is shown in each figure.

DNA synthesis assay

DNA synthesis was determined by [³H]-thymidine incorporation. Briefly, B16F10 cells were cultured and exposed

to myriocin, at 1 and 10 μ M, for 24–96 h including treatment with 2 μ Ci/ml [³H]-thymidine for 4 h. Cells were then washed gently in ice-cold PBS containing 10% trichloroacetic acid and ethanol/ether (1:1, v/v), and released using 0.5 N NaOH. Cell lysates were mixed with liquid scintillation cocktail (Ultimagold; Packard Bioscience, Meriden, CT, USA), and radioactivity was measured using a liquid scintillation counter (LS3801; Beckman, Düsseldorf, Germany).

Cell cycle analysis

Cell cycle progression was determined by flow cytometric analysis after propidium iodide (PI) staining. For determination of cell cycle progression, suspensions of cells at density of 10⁵ cells per well were fixed overnight in 70% ethanol at 4 °C then further incubated overnight with PI staining reagent. After staining, 10 000 cells per experiment were analysed using FACS Calibur apparatus and cell cycle progression was determined using Modifit LT program (Verity Software House, Topsham, ME, USA).

Western blot analysis

Cells were incubated in RPMI 1640 containing myriocin for 24–48 h. For assay of cyclin B1, cdc2, cdc25C, Chk2, PLK1, Wee1, p53, p-p53 and p21^{waf1/cip1} expression, cells were resuspended in protein lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing proteinase inhibitors (1 mM aprotinin, 1 mM leupeptin, 1 mM PMSF) and protease inhibitors (1 mM NaOV₃, 1 mM NaF) at 4 °C followed by BCA protein assay. Protein samples were electrophoretically separated by 10–15% SDS-PAGE and were then transferred to polyvinylidene difluoride membranes (GE Healthcare Life Sciences, Piscataway, NJ, USA). Membrane samples were incubated with primary and secondary antibodies and were then developed using enhanced chemiluminescence (Amersham Pharmacia Biotech) before exposure to X-ray film (Eastman-Kodak, Rochester, NY, USA). Primary antibodies and their dilution factors were as follows: cyclin B1 (1:500); cdc2 (1:200); cdc25C (1:1000); p21^{waf1/cip1} (1:1000); Chk2 (1:1000); PLK1 (1:1000); Wee1 (1:500); p53 (1:1000); p-p53 (1:1000); and β -actin (1:1000).

Sphingolipid analysis

Quantification of ceramide and sphingomyelin was performed as previously described with modification (28,29). Briefly, lipids were extracted from samples for 1 h at 37 °C after spiking with ceramide (C₁₇ base) or dihydrosphingomyelin (C₁₈ base) as internal standard, and cera-

amide and sphingomyelin were separated by TLC after development in either diisopropylether/methanol/29% NH_4OH (40:10:1, v/v/v) or chloroform/methanol/29% NH_4OH (65:25:4, v/v/v), respectively. Ceramide was deacylated by SCDase to produce sphingosine. Sphingomyelin was converted to sphingosine by simultaneous reaction with both SCDase and SMase. Released sphingosine from ceramide and sphingomyelin was analysed by HPLC. For measurement of sphingosine and sphingosine-1-phosphate (30), lipids were extracted for 1 h at 37 °C after addition of sphingosine (C_{17} base) and sphingosine-1-phosphate (C_{17} base), an internal standard. Sphingosine-1-phosphate was dephosphorylated using alkaline phosphatase to release sphingosine, which was analysed by HPLC.

Statistical analysis

Experimental results were expressed as mean $\pm$ SD and one-way analysis of variance (ANOVA) was used for multiple comparisons, using Sigma Stat® (Jandel Co., San Rafael, CA, USA). Differences with $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ were considered statistically significant.

Results

Inhibitory effects of myriocin on cell population growth of malignant melanoma cells

We initially examined how myriocin influenced number expansion of the malignant melanoma cells. Attainment of confluence after treatment with myriocin was much less for malignant melanoma cells than of controls; however, after exposure to myriocin floating cells were not observed. Treatment of our B16F10 cells with myriocin at 0, 0.1, 0.5, 1, 5 and 10 μM , until maximal 96 h, resulted in population growth inhibition in both concentration- and time-dependent manners (Fig. 1a). Cell numbers began to decrease at 48 h after exposure, and cell proliferation at 1, 5 and 10 μM for 96 h was inhibited by approximately 70% compared to that of controls. Cell migration was determined by a wound-healing assay (Fig. 1b) and those treated with myriocin at 1 and 10 μM for 48 h were significantly inhibited by in the region of 69% and 73%, respectively, compared to those of concurrent controls.

DNA synthesis of the malignant melanoma cells was determined by [^3H] thymidine incorporation assay; those treated with myriocin at 1 and 10 μM for 48–96 h had lower levels of radioactivity compared to controls (Fig. 1c). Incorporation of [^3H] thymidine into DNA of control cells was approximately 5150, 8499, 13194 and 19819 cpm per well for 24, 48, 72 and 96 h, respectively.

However, [^3H] thymidine uptake in test cells treated at 10 μM myriocin was in the region of 5481, 5614, 7759 and 10238 cpm per well, respectively. DNA synthesis of myriocin-treated cultures at 1 μM for 96 h was inhibited by around 50% compared to concurrent controls. These results indicate that myriocin-induced DNA synthesis inhibition appeared to be correlated with cell population growth in a time-dependent manner.

Myriocin arrests malignant melanoma cell population growth at G_2/M phase

Effects of myriocin on the various phases of cell cycle progression were investigated (Fig. 2). Population of apoptotic cells in the sub- G_0 fraction (subdiploid DNA content) was not detected, and accumulation of cells in G_2/M occurred after addition of myriocin (Fig. 2). Cells cultured for 48 h resulted in 59.8% synchronization of the cell cycle in G_0/G_1 and 33.5% in S phase. Proportions of myriocin-treated cells at 0, 1 and 10 μM in G_2/M were in the order of 6.7%, 18.4% and 18.7%, respectively (Fig. 2a). Proportions of myriocin-exposed cell populations at 1 μM for 0, 24, 48 and 72 h in G_2/M were 4.7%, 8.3%, 16.2% and 18.6%, respectively. Myriocin treatment of malignant melanoma cells resulted in a significantly higher population in G_2/M in a time-dependent manner (Fig 2b). Thus, percentages of our B16F10 malignant melanoma cells in G_2/M were significantly higher after myriocin treatment compared to controls, indicating that myriocin may inhibit cell proliferation by G_2/M arrest during cell cycle progression.

Effect of myriocin on cell cycle regulatory proteins

To elucidate mechanisms of myriocin-induced cell cycle arrest in the G_2/M phase, effects of myriocin on cell cycle regulatory factors were determined (Fig. 3). Myriocin treatment of our malignant melanoma cells at 1 and 10 μM for 48 h inhibited expression of cyclin B1, cdc2 and cdc25C compared to control cells, whereas myriocin treatment for 24 h did not change expression levels of these regulator proteins (data not shown). Expression of p53 and p21^{waf1/cip1}, upstream signalling molecules of cyclin B1 and cdc2, was determined after myriocin exposure. Cells treated with myriocin for 24 h exhibited elevation of p53 and p21^{waf1/cip1} expression (Fig. 3), but expression of p53 was not changed during the earlier time period (data not shown). Expression of Chk2, upstream signalling molecule for cdc25C, PLK1 Wee1, cdc2/cyclin B1 complex inhibitor, was not changed after myriocin exposure. Caffeine has been known to inhibit phosphorylation of p53 (31). Malignant melanoma cells treated with caffeine at 0, 3 and 5 mM plus 1 μM myriocin showed signifi-

Figure 2. Inhibitory effect of myriocin on malignant melanoma cell cycle progression. (a) B16F10 cells were treated with myriocin at 0, 1, and 10 μM for 48 h and harvested by trypsinization. (b) Cells were exposed to myriocin at 1 μM for 0–72 h and harvested, then stained with PI solution, and 10 000 events per experiments were analysed by flow cytometry. Data representative of at least three independent experiments with similar results. Cell cycle progression was determined using the Modifit LT program.

cantly lower expression of p53, p-p53 and p21^{waf1/cip1} (Fig. 4), confirming that inhibition of cell proliferation by myriocin may occur through activation of p53. Thus, these results demonstrate that myriocin-induced inhibition of cell proliferation may occur through both inhibition of cdc25C and activation of p53 and p21^{waf1/cip1} signalling pathways leading to cell cycle arrest at G₂/M phase.

Involvement of sphingolipid synthesis in myriocin-induced cell population growth inhibition

Myriocin specifically inhibits serine palmitoyltransferase in *de novo* sphingolipid biosynthesis and can be expected to decrease levels of cell ceramide and sphingosine. Malignant melanoma cells treated with myriocin at 1 μM for 24–72 h showed marked decrease in ceramide, sphingomyelin, sphingosine and sphingosine-1-phosphate levels compared to concurrent controls (Fig. 5). Ceramide concentration in malignant melanoma cells exposed to myriocin for 24 h was reduced to ~ 0.3 nmol/mg protein from 2.2 nmol in concurrent controls, that is, around sevenfold (Fig. 5a). Sphingomyelin concentration in

malignant melanoma cells treated with myriocin was approximately 3.7 nmol/mg protein compared to 8.7 nmol in controls, at 24 h; this was maintained up to 72 h (Fig. 5b). Sphingosine and sphingosine-1-phosphate concentrations in myriocin-treated malignant melanoma cells for 24 h were 15.2 and 6.8 pmol, respectively, whereas its concentration in concurrent controls was approximately 119.3 and 11.0 pmol/mg protein (Fig. 5c,d). Reduced levels of ceramide and sphingosine in myriocin-treated cells were maintained up to 72 h. Significant reduction in cell ceramide and sphingosine began to occur at 3 h after exposure of test cells to myriocin (Fig. 5a,c). These results suggest that myriocin-induced decrease in cell sphingolipids may be closely involved in inhibition of malignant melanoma cell proliferation.

C8-ceramide, a synthetic cell-permeable sphingolipid, reversed myriocin-caused inhibition of cell population growth in concentration- and time-dependent manners (Fig. 6a). Cell proliferation under conditions of C8-ceramide (10 and 20 μM) plus myriocin is similar to that of controls. Under the same conditions, C8-ceramide was used to enrich myriocin-treated malignant melanoma cells

Figure 3. Effect of myriocin on expression of cell cycle regulatory proteins. B16F10 cells were exposed to increasing concentrations (0–10 μ M) of myriocin for 24–48 h. Cells for p53, p21 and Chk2 expression were harvested at 24 h after myriocin treatment, while samples for cdc25C, PLK1, cyclin B1, cdc2 and Wee1 expression were collected at 48 h. Cell lysates were analysed using 10% SDS–PAGE and immunoblotting. After densitometric quantification, data are expressed as the mean $\pm$ SD of three different experiments with duplicate (* P < 0.05 and ** P < 0.01 versus vehicle control).

to increase endogenous ceramide levels. Elevation of endogenous ceramide levels at 20 μ M C8-ceramide plus 1 μ M myriocin was maximal for 24 h, decreased to concurrent control levels for 48 h and was maintained by 50% of the controls for 72 h (Fig. 6b). C8-ceramide treatment of malignant melanoma cells at 0, 0.1, 1, 10 and

Figure 4. Effect of caffeine on the myriocin-induced increase in p53, p-p53 and p21 expression. B16F10 cells were exposed to caffeine (0, 3 and 5 mM) plus myriocin (1 μ M) for 24 h. Cell lysate was analysed using 10% SDS–PAGE and immunoblotting. After densitometric quantification, data are expressed as the mean $\pm$ SD of three different experiments with duplicate (** P < 0.01 versus myriocin alone-treated group).

20 μ M along with myriocin at 1 μ M for 24 h increased expression of cdc2 and cyclin B1 compared to myriocin-alone treatment (Fig. 6c). These results indicate that exogenous ceramide appears to cause recovery of malignant melanoma cells from inhibition of cell proliferation induced by myriocin, by release from cell cycle arrest. In summary, myriocin inhibited malignant melanoma cell proliferation through G₂/M phase arrest, which resulted from modulation of cell cycle regulatory proteins by blocking *de novo* sphingolipid biosynthesis.

Discussion

Malignant melanoma is the most aggressive form of skin cancer and it is resistant to anti-cancer treatments including radiation and chemotherapy. To solve the problem of chemoresistance, natural products are important sources for its possible treatment. Myriocin, isolated from *Mycelia sterilia*, *Isaria sinclairii* and *Cordyceps cicadae*, is a specific inhibitor of serine palmitoyltransferase in *de novo* sphingolipid biosynthesis (8,11,32–34). Myriocin has been shown to inhibit proliferation of a certain IL-2-dependent mouse cytotoxic T-cell line (11). The study recounted here has demonstrated that myriocin inhibited population growth, migration and DNA synthesis in malignant melanoma B16F10 cells (Fig. 1), while

Figure 5. Effect of myriocin on sphingolipid biosynthesis. Malignant melanoma cells were exposed to myriocin at 1 μ M for 0–72 h. (a) Ceramide and (b) sphingomyelin levels were analysed by HPLC following lipid extraction and either deacylation by SCDase or dephosphorylation along with deacylation by SCDase plus SMase, respectively. (c) Sphingosine concentration was determined by HPLC analysis following lipid extraction. (d) Sphingosine-1-phosphate was dephosphorylated by alkaline phosphatase to release sphingosine, which was analysed by HPLC. Values are expressed as the mean $\pm$ SD of three different experiments ($n = 3$ –5).

apoptotic cell death did not occur (data not shown). Inhibition of cell growth by myriocin resulted from cell-cycle arrest at G₂/M phase (Fig. 2).

The mechanism by which myriocin induces G₂/M arrest in these cells may be mediated by cell cycle regulatory proteins. Cdc2 and cyclin B1, key factors regulating transition from G₂ to M phase, are maintained in an inactive form when phosphorylated by Wee1 kinase (35). Dephosphorylation of these proteins activates cdc2/cyclin B1 complex and triggers the initiation of mitosis. In this study, myriocin decreased the expression of cdc2 and cyclin B1 in B16F10 malignant melanoma cells (Fig. 4), and this appeared to induce G₂/M phase arrest, then inhibition of cell proliferation.

Tumour suppressor protein p53 can be activated by DNA damage, hypoxia, or aberrant oncogene expression to promote arrest at cell-cycle checkpoints (36). Expression level of p53 in our malignant melanoma cells treated with myriocin was high (Fig. 3). Activated p53 can alter the pattern of gene expression by activating or reducing transcription of many genes that mediate its downstream functions (25). One of the transcription targets of p53 is p21^{waf1/cip1}, a cyclin-dependent kinase inhibitor (CIK). p21^{waf1/cip1} binds to and inhibits activity of the cyclin B1/cdc2 complex to cause arrest in G₂ phase (27). To verify that the observed p53 induction had biological significance in B16F10 cells, p21^{waf1/cip1} protein level was found to be expressed at higher levels than in untreated cells (Fig. 3). Cdc25C can be modulated by PLK1 or Chk2 to stimulate cdc2/cyclin B1 complex, which can be directly inhibited by Wee1 (37,38). However, expression of Chk2, PLK1 and Wee1 in B16F10 cells exposed to myriocin, was not found to be altered, indicating that myriocin-induced cell cycle arrest may occur through either cdc25C or p53 signalling pathways (Fig. 7). Caffeine, an inhibitor of p53 phosphorylation, induced decreased expression of p53, p-p53 and p21^{waf1/cip1} in B16F10 cells treated with myriocin (Fig. 4), suggesting that cell cycle arrest at G₂/M by myriocin may occur through the p53-p21 signalling pathway.

To investigate whether caffeine could reverse effects of myriocin on cell population growth, we treated our

Figure 6. Effect of C8-ceramide on cell population growth inhibition and decreased ceramide content by myriocin. (a) B16F10 malignant melanoma cells were exposed to 1 μ M myriocin and C8-ceramide (0, 0.1, 1, 10 and 20 μ M) for 24–72 h, and cell numbers were counted using a haemocytometer. (b) Ceramide level was determined by HPLC analysis following lipid extraction and deacylation by SCDase. (c) B16F10 cells were exposed to myriocin (1 μ M) and C8-ceramide (0, 0.1, 1, 10 and 20 μ M) for 24 h. cdc2 and cyclin B1 expression were analysed using 10% SDS-PAGE and immunoblotting. Values are expressed as the mean $\pm$ SD of three different experiments in triplicate. (* P < 0.05 and *** P < 0.001 versus myriocin alone-treated group).

malignant melanoma cells with caffeine plus myriocin. However, caffeine did not reverse the effect of myriocin on cell proliferation (data not shown). We observed many

Figure 7. Proposed signalling pathways involved in cell cycle arrest induced by myriocin in malignant melanoma cells.

floating cells when they were treated with caffeine at 3 and 5 mM plus myriocin at 1 μ M. Several studies have reported that caffeine either induces apoptosis in HeLa, PC12D, SH-SY5Y, HaCaT, C2ABR, AT3ABR cells or inhibits proliferation in HepG2, HLF, Huh7 and PLC/PRF/5 cells (39–41). Thus, apoptosis and population growth inhibition after caffeine appear to overcome reversal effects of caffeine on cell expansion, although caffeine inhibits myriocin-induced increase in expression of p53 and p21.

Sphingolipids are involved in regulation of cell proliferation, adhesion and migration as revealed in cancer pathogenesis and therapy (16,42,43). Exogenously added C2-ceramide induces apoptosis of human colon cancer HT-29 cells through regulating apoptosis-related gene expression (44), and resveratrol can exert anti-proliferative and pro-apoptotic effects in association with elevation of endogenous ceramide in androgen receptor-negative PC3 cells (45). Endogenous ceramide can be generated by a recycling process of the sphingosine backbone, by deacylation of exogenous short-chain ceramides followed by reacylation (46). Pharmacological manipulation of sphingolipid metabolism to modulate tumour cell sphingolipids has received much attention as a novel approach to cancer chemotherapy. Fenretinide (4-HPR) has been shown to act by increasing tumour cell ceramide by *de novo* synthesis (47). Combinations of 4-HPR and modulators of ceramide metabolism have demonstrated increased anti-tumour activity in pre-clinical models, with minimal toxicity (48). In this study, we propose a new approach to increase anti-tumour activity by depleting tumour cell sphin-

golipids. Myriocin, a specific inhibitor of serine palmitoyltransferase in *de novo* pathways, decreased cell levels of ceramide, sphingomyelin, sphingosine and sphingosine-1-phosphate in malignant melanoma cells leading to tumour cell proliferation inhibition (Fig. 5). Sphingosine-1-phosphate induced prostate cancer cell migration (43), and decreased levels of sphingosine-1-phosphate after myriocin exposure may have contributed to inhibition of cell migration and inhibition of proliferation (Figs 1 and 5d). Myriocin-induced decrease in endogenous ceramide levels was reversed by adding C8-ceramide to our B16F10 melanoma cells, and their proliferation and cdc2/cyclin B1 expression related to G₂/M phase were reversed to those of control cultures, indicating that endogenous sphingolipids, primarily ceramide, are essential for tumour cell replication (Fig. 6).

Apparent reduction in cell ceramide in our cells began to occur at 3 h and increased expression of p53, was shown to occur 24 h after myriocin treatment, indicating that regulation of p53 or cdc25C signalling pathways may be preceded by inhibition of sphingolipid biosynthesis (Figs 3 and 5). Several studies have reported that ceramide triggers p53 up-regulation, leading to apoptosis and inhibition of cell proliferation. Ceramide-induced reduction in Bcl-2/Bax ratio, increase in caspase activity, and apoptosis are dependent on increases in p53 levels in neuroblastoma SKN-SH cells (49). Ceramide induces selective arrest of MCF-7 cells in G₁, which is associated with increased expression of p53 and p21 (50). However, lower ceramide level of our malignant melanoma cells induced by myriocin treatment was also shown to contribute to up-regulation of p53, which leads to cell replication inhibition by G₂/M arrest (Fig. 7). ERK signalling plays a major role in tumourigenesis (51), and myriocin inhibits ERK phosphorylation (52) and myriocin may also modulate PKC signalling (53). Thus, myriocin may impact other signalling pathways related to anti-tumour activity. Ceramide acts as a key mediator for malignant melanoma differentiation-associated gene-7/interleukin-24 induction of apoptosis (54), and myriocin may protect against ceramide-induced cell death, with relevance for malignant melanoma. Ganglioside GD3 highly expressed in malignant melanoma cells enhances their properties (55). Given that myriocin also depletes gangliosides of cells and tissues from apoE(−/−) mice (13), the gangliosides may play a role in anti-tumour activity of myriocin in malignant melanoma.

In conclusion, our current data demonstrate that myriocin inhibits malignant melanoma cell proliferation by arresting cell cycle progression at the G₂/M phase. This was induced by myriocin-mediated down-regulation of sphingolipid biosynthesis and subsequent up-regulation of p53 and p21 expression, followed by down-regulation of

cdc2/cyclin B1 complex proteins (Fig. 7). Thus, this study suggests that sphingolipid metabolism may become a promising target for malignant melanoma therapy.

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