Millepachine, a novel chalcone, induces G_{2}/M arrest by inhibiting CDK1 activity and causing apoptosis via ROS-mitochondrial apoptotic pathway in human hepatocarcinoma cells in vitro and in vivo

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In this study, we reported millepachine (MIL), a novel chalcone compound for the first time isolated from Millettia pachycarpa Benth (Leguminosae), induced cell cycle arrest and apoptosis in human hepatocarcinoma cells in vitro and in vivo. In in vitro screening experiments, MIL showed strong antiproliferative activity in several human cancer cell lines, especially in HepG2 cells with an IC_{50} of 1.51 \mu M. Therefore, we chose HepG2 and SK-HEP-1 cells to study MIL’s antitumor mechanism. Flow cytometry showed that MIL induced a G_{2}/M arrest and apoptosis in a dose-dependent manner. Western blot demonstrated that MIL-induced G_{2}/M arrest was correlated with the inhibition of cyclin-dependent kinase 1 activity, including a remarkable decrease in cell division cycle (cdc) 2 synthesis, the accumulation of phosphorylated-Thr14 and decrease of phosphorylation at Thr161 of cdc2. This effect was associated with the downregulation of cdc25C and upmodulation of checkpoint kinase 2 in response to DNA damage. MIL also activated caspase 9 and caspase 3, and significantly increased the ratio of Bax/Bcl-2 and stimulated the release of cytochrome c into cytosol, suggesting MIL induced apoptosis via mitochondrial apoptotic pathway. Associated with those effects, MIL also induced the generation of reactive oxygen species. In HepG2 tumor-bearing mice models, MIL remarkably and dose dependently inhibited tumor growth. Treatment of mice with MIL (20 mg/kg intravenous [i.v.]) caused more than 65% tumor inhibition without cardiac damage compared with 47.57% tumor reduction by 5 mg/kg i.v. doxorubicin with significant cardiac damage. These effects suggested that MIL and its easily modified structural derivative might be a potential lead compound for antitumor drug.

Introduction

Flavonoids constitute the largest, ubiquitous and most important group of polyphenolic compounds in nature and are categorized, according to the chemical structure, into flavonols, flavones, flavanones, iso-flavonoids, catechins, anthocyanidins and chalcones (1,2). They have aroused considerable interest recently for their various bioactivities, including anti-allergic (3), anti-inflammatory (4), antimicrobial (5), anti-tumor (6) and especially anticancer activities (7–9). Allovedicib (flavopiridol), an important semisynthetic flavonoid derived from the indigenous Indian plant Diospyros binectariferum is an important representative flavonoid for cancer therapy for instance. It is an inhibitor of cyclin-dependent kinase (CDKs) by inducing cell cycle arrest, by preventing phosphorylation of CDKs and by downregulating cyclins D1 and D3 expression and resulting in G_{1} cell cycle arrest and apoptosis (10,11). Its combination therapy with bortezomib has now completed Phase I clinical trial against blood cancers successfully after nearly 20 years’ of research (12). Therefore, flavonoids are a group of compounds full of research and development value.

Millettia pachycarpa Benth (Leguminosae), a flavonoids rich traditional Chinese medicine, has been used as a blood tonic in anti-helminthic and in cancer therapy in a preparation called ‘Jixueteng’ in Chinese for many years (13–15). Our previous study has revealed that two of its major components, barbigerone and deguelin, showed significant antitumor activity (16,17). Millepachine (MIL), a novel chalcone, was for the first time isolated from seeds of M.pachycarpa. It has a benzpyrene structure with a 2,2-dimethylbenzopyran motif endowing it with sufficient lipophilicity to ensure good cell membrane penetration (18). According to our literature research, there are only four of these chalcone compounds reported (19,20), and there is no report on their antitumor activity. In our anticancer compounds screening program from medicinal plants, MIL showed significant antiproliferative effect against several cancer cell lines, especially in hepatocarcinoma cells.

Nowadays, hepatocellular carcinoma is the third most common cause of cancer-related mortality in the world (21). Doxorubicin, as the first-line drug for treating hepatocellular carcinoma, is an effective agent (22), which is used with great care because of its irreversible, long-term cardiac damage (23). Therefore, the development of a safe and more effective therapeutic strategy is very necessary.

In this study, we first found that MIL exhibited strong cytotoxic effect against HepG2 and SK-HEP-1 cells. We discussed the mechanism of MIL’s antiproliferative effect in hepatocarcinoma cells through inducing cell cycle arrest by inhibiting CDK1, and by activating and causing apoptosis via reactive oxygen species (ROS)-mitochondria apoptotic pathway. We also evaluated MIL’s antitumor activity in a HepG2 tumor-bearing xenograft mice model in vivo. Both the in vitro and in vivo experiments showed and confirmed MIL to have significant antitumor effects.

Materials and methods

Reagents and chemicals

3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide, Hoechst 33258, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and antibodies against β-actin, caspase 3 and caspase 8 were purchased from Sigma (St Louis, MO). Antibodies against cyclin B1, cyclin D1, total and phosphory- (Thr14/Tyr15/Thr161) cell division cycle (cdc) 2, checkpoint kinase (Chk) 2, cdc25C, Bcl-2, Bax and cytochrome c were obtained from Cell Signaling Technology (Danvers, MA).

MIL was isolated from M.pachycarpa Benth according to Supplementary 1, available at Carcinogenesis Online. Its structure has been identified using spectroscopic methods and X-ray diffraction experiment (Supplementary 2, available at Carcinogenesis Online). Samples containing MIL of 90% or higher purity were in all experiments unless otherwise indicated. MIL was dissolved in DMSO to produce a 10 mM stock solution and stored at 4°C. Prior to experiment, the stock solution was diluted in cell culture medium at a

Abbreviations: Cdc, cell division cycle; CDK, cyclin-dependent kinase; Chk, checkpoint kinase; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; i.v., intravenous; IC_{50}, 5.5,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolocarbocyanine iodide; MIL, millepachine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl-transferase deoxyuridine triphosphate nick-end labeling; SD, standard deviation.

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final DMSO concentration of 0.05% (v/v). Controls were always treated with the same amount of DMSO as used in the corresponding experiment.

**Cell lines and cultures**

The human hepatocarcinoma cell lines, HepG2 and SK-HEP-1, and the normal hepatocyte cell line, LO2, were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were all cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum and maintained in an incubator with a humidified atmosphere of 5% CO₂ at 37°C.

**Cell proliferation assay**

Cells (1 × 10⁴) were plated in 96-well culture plates and treated with vehicle alone (0.1% DMSO) or various concentrations of MIL for 24, 48 and 72 h, respectively. MTT solution (20 µl of 5 mg/ml) was added into each well and incubated at 37°C for 4 h. The precipitation of each well was dissolved in 150 µl DMSO after aspirating the medium of each well. The absorbance at 570nm was determined in each well with the Spectramax M5 Microtiter Plate Luminometer (Molecular Devices). The growth of the treated cells was compared with vehicle-treated control cells, which were arbitrarily assigned 100% viability.

**Flow cytometry assay**

To analyze the cell cycle distribution, cells were treated with vehicle alone (0.1% DMSO) or various concentrations of MIL for different period. Then, cells were stained with propidium iodide and analyzed by a flow cytometer (TASCC240). Data were analyzed using Modfit 2.8 software.

**Morphological analysis of nuclei**

Treated cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Hoechst 33258 (10 µg/ml) dissolved in phosphate-buffered saline (PBS) was added to the fixed cells, incubated for 5 min at room temperature and washed with PBS twice. Then, images were taken using an inverted fluorescence microscopy (Olympus, Tokyo, Japan).

**Western blot assay**

After being treated with various concentrations of MIL, cells were lysed in cell lysis buffer (Biyotome, Shanghai, China) on ice. About 1 µl of phenylmethyl-sulfonfluoride solution was added to lysis buffer prior to use. The cell lysates were centrifuged at 13 000g for 15 min. Protein concentration was determined by Bio-Rad detergent-compatible protein assay (Bio-Rad, Hercules, CA). Protein samples were then subjected to 8–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto the polyvinylidene difluoride membrane. The membrane was blocked in 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 for 2 h and then incubated with primary antibody and subsequently with appropriate horseradish peroxidase-conjugated secondary antibody. Proteins were visualized with enhanced chemiluminescence (Millipore, Bedford, MA).

**Terminal deoxynucleotidyl-transferase deoxyuridine triphosphate nick-end labeling assay**

Cell DNA damage was evaluated with an *in situ* Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) by using terminal deoxynucleotidyl-transferase to transfer biotin-deoxyuridine triphosphate to the free 3'-OH of the cleaved DNA. After treated with 2 µM MIL for 24 h, and 48 h, cells were fixed with 4% formaldehyde in PBS for one hour at 4°C and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing with PBS, the cells were incubated in the dark at 37°C for one hour with terminal deoxynucleotidyl-transferase deoxyuridine triphosphate nick-end labeling (TUNEL) reaction mixture containing enzyme solution and labeled solution. The changes in DNA damage were observed using fluorescence microscopy (Olympus).

**Measurement of caspase 3 activity**

Caspase 3-like protease activity in cells was analyzed using a colorimetric test system. Briefly, cells were treated with 2.5 µM MIL for 36 h and 1 × 10⁶ cells were lysed with lysis buffer (Biyotome) on ice. The lysates were centrifuged at 14 000g at 4°C for 15 min. Cytosolic protein was mixed with 10 µl caspase 3-specific substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (2 µM) for 2 h at 37°C. The value of absorbance of sample was monitored at 405 nm using a Spectramax M5 Microtiter Plate Luminometer.

**Measurement of mitochondrial membrane potential (ΔΨm)**

Mitochondrial membrane potential was determined by flow cytometry following staining with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylthiopyrinium iodide (JC-1). The cells were treated with MIL at 2.5 µM for different period and then various concentrations (1.25–5.0 µM) for 48 h at 37°C. After being incubated with JC-1 for 30 min at 37°C, the cells were collected and washed twice with cold PBS. The monomeric form emits light at 527 nm after excitation at 490 nm. Thus, the color of the dye changes from orange to green, depending on the mitochondrial membrane potential, and could be analyzed by flow cytometry with orange emission in channel 2 (FL2).

**Immunofluorescence of cytochrome c**

Cells were treated with vehicle alone (0.1% DMSO) or 2 µM MIL for 36 h. After being fixed with 4% formaldehyde polymer for 15 min, the cells were incubated with blocking buffer (PBS containing 3% bovine serum albumin, 2% goat serum and 0.2% Triton X-100) for 1 h. Then, cells were incubated with antibodies against cytochrome c overnight. After washing twice with PBS, the cells were incubated with corresponding fluorescence-conjugated secondary antibodies for 1 h. The changes in cytochrome c level were detected using an inverted fluorescence microscope.

**Measurement of ROS**

DCFH-DA was used to measure ROS formation. After exposure to different concentrations of MIL for 6 h, cells were incubated in 10 µM DCFH-DA containing medium at 37°C for 20 min. Cells were then washed with PBS three times to remove DCFH-DA, which had not entered into the cells. The fluorescence was visualized immediately at wavelength of 485 nm for excitation and 530 nm for emission by an inverted fluorescence microscope. Total green fluorescence intensities of each well were quantified using image-analysis software (Cell Quest Program).

**In vivo tumor xenograft study**

To determine the *in vivo* antitumor activity of MIL, HepG2 cells (1 × 10⁵ in 100 µl saline) were injected subcutaneously into the right flanks of female nude mice (6 weeks old, BALB/cA-nu [nu/nu]). When the tumor reached 100 mm³, mice were randomly divided. The mice in the test group first received intravenous (i.v.) injections of MIL (5, 10 and 20 mg/kg in sodium chloride injection containing 2.5% Tween-80 and 2.5% ethanol in a volume of 200 µl) at 2 day intervals and lasting for 18 days. And then, to investigate whether (i) the mice could tolerate higher doses and (ii) better antitumor activities could be achieved, mice received another i.v. injections of MIL (50 and 100 mg/kg) with the same sample preparation method as MIL (5, 10 and 20 mg/kg) and 250 µl injection volume for MIL (50 mg/kg) and 250 µl injection volume x 2 for MIL (100 mg/kg). The control group was treated with an equal volume of vehicle. The positive group received i.v. injections of doxorubicin (5 mg/kg) every 4 days. Tumor burden was measured every 2 days with a caliper (calculated volume [mm³] = 0.125 × length × width × width). After treatment, mice were killed and some internal organs (heart, liver, spleen, lung and kidney) and tumors were collected. All animals used in the experiments were treated humanely in accordance with institutional animal care and use committee guidelines.

**Histological analysis**

The excised tumors and tissues were fixed in 4% neutral-buffered formalin solution for more than 24 h and embedded in paraffin. Sections of the tissues (3–5 µm) were stained with hematoxylin and eosin. The effect of inducing apoptosis of MIL would be evaluated using TUNEL assay (*in situ* Cell Death Detection Kit; Roche).

**Statistical analysis**

All data were expressed as mean ± standard deviation (SD) and statistically compared by one-way analysis of variance with Dunnett’s test or unpaired Student’s t-test in different experiments, and P < 0.05 was taken as statistically significant.

**Results**

**MIL strongly inhibited proliferation and viability of human hepatocarcinoma cells but not of normal hepatocyte cells**

In the pre-experiment of anticancer compounds screening, MIL (Figure 1A) showed strong antiproliferation activity on several cancer cell lines (Supplementary 3, available at *Carcinogenesis* Online). And MIL showed the most sensitivity and best antitumor activity against HepG2 cells (IC₅₀ 1.51 µM). Thus, we assumed MIL might show stronger antiproliferation toward hepatocarcinoma cells and chose HepG2 and SK-HEP-1 cells to further study MIL’s antitumor mechanism.

We compared the proliferation inhibition effects of MIL on the growth of human hepatocarcinoma cells (HepG2 and SK-HEP-1), as well as normal human hepatocyte cells (LO2). As shown in Figure 1B, the treatment of HepG2 and SK-HEP-1 cells with MIL (0.625–10 µM)
resulted in a significant reduction on cell proliferation as assessed by MTT assay, with IC50 values of 1.57 and 2.13 μM, respectively. In contrast, MIL at the concentration of 10 μM only caused 31.6% viability reduction of LO2 cells. These data suggested that MIL did inhibit the proliferation and viability on human hepatocarcinoma cells with minimum cytotoxicity to normal human hepatocytes.

**MIL induced G2/M phase arrest in hepatocarcinoma cells**

To investigate whether MIL could affect the cell cycle regulation, we determined its effect on cell cycle regulation by flow cytometry after staining with propidium iodide. The effects of MIL on HepG2 and SK-HEP-1 cell cycle distribution were shown in Figure 1C. It could be seen that a 24 h exposure of HepG2 cells to MIL caused a significant inhibition of cell cycle progression and resulted in a statistically significant increase in G2/M phase fraction accompanied by a decrease in G0/G1 cells in a concentration-dependent manner. Compared with the vehicle control with the G2/M phase distribution of 19.8%, the percentages of G2/M fraction were increased to 46.6%, 64.4% and 80.4% in HepG2 cells treated with 1.25, 2.5 and 5 μM MIL, respectively. Similar results were observed on the analysis of the effects of MIL treatment on cell cycle progression of SK-HEP-1 cells. Treatment of SK-HEP-1 cells with MIL for 24 h resulted in a significantly higher number of cells in the G2 phase at the concentrations of 1.25 (33.5%), 2.5 (49.6%, P < 0.01) and 5 μM (70.1%, P < 0.01), compared with vehicle control (18.7%) (Figure 1C). Following morphology staining of cells with Hoechst 33258, we could see that the chromosomes were haphazardly scattered in the cell nucleus (Figure 1D). These results indicated that the inhibitory effect of MIL on the proliferation of HepG2 and SK-HEP-1 cells correlated with G2/M phase arrest.

**MIL induced G2/M arrest through inhibiting the activity of CDK1**

The CDK1 with the cyclin B1 and cdc2 complex could drive cell cycle progression (24,25). It is activated by binding cyclin B1 to cdc2 and phosphorylation of cdc2 on the Thr161, and negatively regulated by the rapid dephosphorylation of cdc2 on both Thr14 and Tyr15 (24). To investigate whether MIL affected CDK1 activity of treated cells, the level of CDK1-related proteins was analyzed. As shown in Figure 2A, after exposure to MIL, both HepG2 and SK-HEP-1 cells showed an increase in the protein levels of Chk2, cyclin B1 and Thr14 phosphorylated cdc2, and a significant decrease in the protein levels of cyclin D1, cdc25C, cdc2 and Tyr15, Thr161 phosphorylated cdc2, suggesting that MIL induced a G2/M arrest through inhibiting the activity of CDK1.

**MIL induced DNA damage in human hepatocarcinoma cells**

Tumor cells would undergo cell cycle arrest or apoptotic death in response to DNA damage (26,27). In previous experiments, we demonstrated that MIL could induce cellular G2/M arrest. To investigate whether MIL induces DNA damage in treated cells, HepG2 and SK-HEP-1 cells were exposed to 2 μM MIL for 24 h. Following TUNEL assay, significant DNA damages were observed in both hepatocarcinoma cells treated with MIL for 24 h (Figure 2B).

**MIL induced apoptosis in hepatocarcinoma cells**

To determine whether reduction of proliferation capacity and cell viability of the hepatocarcinoma cells induced by MIL was associated with cells apoptosis, HepG2 and SK-HEP-1 cells were treated with MIL as described previously. Then, apoptotic cells were assessed using both qualitative and quantitative methods. As shown in Figure 3A, morphology of the cells treated with MIL for 48 h changed completely with the characteristics of nucleus breaking up, cell shrinking and breaking into apoptotic bodies. To quantitatively analyze the apoptotic effect of MIL, we used flow cytometry to detect the amount of apoptotic cells. The percent of apoptotic HepG2 cells treated by MIL increased from 40.0% to 77.1% along with the concentration increasing from 1.25 to 5 μM at 48 h, whereas the percent of apoptotic cells was merely 7.5% when treated with vehicle control (Figure 3B). Also there was little apoptosis of MIL-treated cells for 24 h as control (data not shown). Treatment of SK-HEP-1 cells with MIL for 48 h also resulted in a significant dose-dependent apoptosis: the vehicle control (4.6%), 1.25 μM (28.3%, P < 0.01), 2.5 μM (56.8%, P < 0.01) and 5 μM (66.4%, P < 0.001). Significant DNA...
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MIL induced apoptosis through regulating the mitochondria-mediated apoptotic pathway

Caspases play a central role in many forms of apoptosis (28). To determine if caspase 3 activation was induced by MIL treatment, both HepG2 and SK-HEP-1 cells were exposed to 2.5 μM MIL for 36 h and subjected to a colorimetric caspase 3 activity assay. The result showed that caspase 3 activity was significantly increased from 0.5 μM pNA of vehicle control cells to 7.5 μM pNA in 2.5 μM MIL-treated HepG2 cells. And the activity of caspase 3 in SK-HEP-1 cells treated with 2.5 μM MIL increased to 5.34 μM pNA, compared with 1.12 μM pNA of vehicle control (Figure 4A). Western blot analysis also revealed that the levels of pro-caspase 3 and pro-caspase 9 decreased, whereas that of cleaved caspase 3 and cleaved caspase 9 increased, in a dose-dependent manner, in the two hepatocarcinoma cells treated with MIL (Figure 4B).

The activation of caspase 9 and caspase 3 hinted that the molecular mechanism of apoptosis might be via the mitochondrial pathway (29). To investigate whether the mitochondria-mediated apoptotic pathway is involved in MIL-induced apoptosis, we investigated the levels of Bcl-2 and Bax. Figure 4C showed that exposure of HepG2 and SK-HEP-1 cells to MIL resulted in a decrease in Bcl-2 and an increase in Bax proteins levels, thereby leading to increased Bax/Bcl-2 ratio. Suggesting that MIL induced apoptosis might be via the mitochondrial apoptotic pathway (30) (Figure 4C). To verify that MIL induces the disruption of the mitochondrial membrane potential, we used the lipophilic cation JC-1 to detect alterations in the mitochondrial membrane potential in HepG2 and SK-HEP-1 cells. Once the mitochondrial membrane potential is disrupted, the fluorescence emission of JC-1 changes from orange to green. As shown in Figure 4D, treatment of HepG2 cells with MIL resulted in a time- and dose-dependent increase in the number of green fluorescence-positive cells from 18.84% of treatment with MIL for 24 h to 86.34% for 72 h, and 37.63%, 51.19% and 66.07% on treatment with 1.25, 2.5 and 5 μM MIL for 48 h, compared of vehicle control. Similarly, the number of SK-HEP-1 cells with the loss of mitochondrial membrane potential also increased from 9.21% after treatment for 24 h to and 87.53% for 72 h. Furthermore, treatment of SK-HEP-1 cells with 1.25, 2.5 and 5 μM MIL for 48 h increased the loss of the mitochondrial membrane potential by 12.49%, 41.91% and 57.17%, respectively (Figure 4D).

Preceding the loss of mitochondrial membrane potential would be the release of cytochrome c from the mitochondria to the cytosol (31). The results of the immunofluorescence detection of cytochrome c showed the levels of cytochrome c to have significantly increased in the cytosols of HepG2 and SK-HEP-1 cells treated with 2 μM MIL (Figure 5A). Likewise, western blot analysis with anticytochrome c showed a dose-dependent increase of cytosolic cytochrome c in the two cell lines (Figure 5B). Thus, these data suggested that the mitochondrial pathway was involved in the MIL-induced apoptosis in the HepG2 and SK-HEP-1 cells.

MIL dose dependently elevated ROS level

Mitochondria are the major source of ROS and ROS could determine the fate of cancer cells through regulating a number of cellular pathways (32). In our experiment, DCFH-DA was applied to detect the change of intracellular ROS level after MIL treatment. The results of flow cytometry analysis showed that the ROS generation had a significant increase in both HepG2 and SK-HEP-1 cells treated with varying concentrations of MIL (1.25–5 μM) (Figure 5C).

MIL inhibited HepG2 xenograft growth in nude mice

To determine whether MIL inhibited tumor growth in vivo, we examined the effect of MIL on the growth of HepG2 cells in nude mice. In HepG2 xenograft model, the mean tumor volume for control increased from 162.64 ± 29.42 to 1155.07 ± 156.88 mm3, whereas those of MIL-treated groups at 5, 10 and 20 mg/kg i.v. treatment increased from 152.07 ± 32.01 to 800.33 ± 100.34 mm3, 170.87 ± 25.96 to 654.31 ± 116.99 mm3 and 158.85 ± 37.81 to 527.46 ± 54.04 mm3, respectively, and the doxorubicin group from 162.42 ± 25.36 to 736.88 ± 116.52 mm3 (Figure 6A). At the end of the experiment, the mean tumor weight of mice from MIL-treated groups (5, 10 and 20 mg/kg, i.v.) were 0.56 ± 0.06, 0.44 ± 0.08 and 0.26 ± 0.05 g, respectively, compared with 0.76 ± 0.08 g for the control group and 0.40 ± 0.06 g for the doxorubicin-treated group (Figure 6B). Tumor growth inhibition was most evident in mice treated with MIL at 20 mg/kg for 20 mg/kg/2 days. Compared with control, treatment with MIL at 5, 10 and 20 mg/kg resulted in the inhibitory rate of 26.53%, 41.88% and 65.93%, respectively, compared with 47.57% of mice treated with doxorubicin.

In addition, during the experiment, no sign of toxicity, which was measured by parallel monitoring of body weight and hematoxylin and eosin dyeing of tissue sections (data not shown), was observed in MIL-treated mice. Nevertheless, strong pathologic changes were observed in the heart tissue of doxorubicin group, demonstrating obvious cardiac...
damages by doxorubicin (Figure 6C). Our results revealed that MIL was an effective antitumor lead compound with less toxicity. The immunohistochemical analysis by TUNEL showed that MIL induced significant apoptosis in tumor, whereas the vehicle-treated mice revealed minimum apoptotic effect (Figure 6D). We also performed additional higher doses such as 50 and 100 mg/kg administrations to investigate whether (i) the mice could tolerate such doses and (ii) better antitumor activities could be achieved. As shown in Figure 6E and F, 50 and 100 mg/kg treatment with MIL achieved better antitumor activity than that of 20 mg/kg, the mean tumor volumes and tumor weights from MIL-treated groups (50 and 100 mg/kg, i.v.) were significantly decreased compared with control group. Treatment with MIL at 50 and 100 mg/kg i.v injection resulted in better antitumor
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Activity with respective 79.10% and 86.65% tumor inhibitory rates compared with 73.49% at 20 mg/kg. Although higher dose of administration should be performed, the poor solubility of MIL limited its further higher dose treatment by i.v injection. Further modification of MIL, or suitable pharmaceutics formation work would be performed in future.

Fig. 5. MIL increased the releasing of cytochrome c into cytosol and induced ROS elevating in HepG2 and SK-HEP-1 cells. (A) Immunofluorescence of cytochrome c. HepG2 and SK-HEP-1 cells were treated with vehicle or 2 μM MIL for 36h. Cytochrome c was detected by a fluorescence microscopy as described in Materials and methods. Data from a typical experiment were shown, repeated three times with similar results. (B) MIL increased the releasing of cytochrome c into cytosol. HepG2 and SK-HEP-1 cells were treated with varying concentrations of MIL (0, 1.25, 2.5 and 5 μM) for 36h. The protein levels of cytochrome c were determined by western blot. (C) Measurement of ROS. HepG2 and SK-HEP-1 cells were treated with 1.25, 2.5 and 5 μM MIL for 6h. The level of ROS was measured by DCF fluorescence with flow cytometry.

Fig. 6. MIL inhibited tumor growth and induced apoptosis in HepG2 xenograft model. (A) Tumor volume-time and body weight-time curve. Treatment was initiated when the average size of the tumor reached 100 mm³. Three test groups were treated with indicated dosage in 200 μl of physiological saline of MIL every 2 days. The positive group received doxorubicin at a dosage of 5 mg/kg every 4 days, and the control group received injection of physiological saline alone as test groups. The mice (n = 6 per group) were treated for 18 days. (B) The bar charts of tumor weight. (C) The hematoxylin and eosin stain of tissue section of tumor and heart tissue of MIL- and doxorubicin-treated nude mice. (D) TUNEL assay of tumor. The apoptosis of tumor were determined by using TUNEL kit. (E) Tumor volume-time and body weight-time curve of the additional HepG2 xenograft model. The injection volumes of the treatment groups (n = 7 per group) were 200, 250 and 250×2, respectively for 20, 50 and 100 mg/kg. (F) The bar charts of tumor weight of the additional HepG2 xenograft model. *P < 0.05, **P < 0.01 and ***P < 0.001, significantly different compared with control by t-test.
Discussion

Hepatocellular carcinoma is one of the most common malignancies worldwide, but conventional antineoplastic drugs are typically non-selective cytotoxic molecules with significant systemic untoward effects. Therefore, it is important to develop safer and more effective therapeutic drugs.

Plants have been a main source of highly effective conventional drugs for the treatment of many forms of cancer. Although the actual compounds separated from plant often may not serve as the drugs, they provide leads for the development of potential novel agents (33). In our anticancer agents screening program from the natural products, MIL (Figure 1A), first separated from M. pachycarpa, has a novel structure that a 2,2-dimethylbenzopyran motif collected to the C2 and C3 of ring C of a typical chalcone skeleton (19,20). Up to now, MIL was the fifth of these compounds with this special structure. In the antiproliferation experiment, MIL showed strong antiproliferation effect on several human cancer cell lines, especially on HepG2 cells. Thus, we assumed MIL might show stronger antiproliferation in hepatocarcinoma cells. Hence, in this study, we revealed MIL induced significant G2/M arrest and apoptosis in two hepatocarcinoma cell lines, HepG2 and SK-HEP-1 cells. Moreover, it had little toxicity effect on normal hepatocyte cells LO2. Our work here focused on MIL’s mechanism of causing G2/M arrest via inhibiting CDK1 activity and inducing apoptosis through ROS-mitochondrial apoptotic pathway.

Cyclin D1 is an important protein required for cell cycle progression in G1/S phase (34) and cyclin B1 is critical for the completion of G2/M transition. The results of downregulation cyclin D1 and upregulation cyclin B1 (Figure 2A) revealed that HepG2 and SK-HEP-1 cells treated with MIL had completed G1/S phase and entered into G2/M phase. CDK1 kinase plays a crucial role in regulation of cells from G2 phase into M phase (25). Activation of CDK1 kinase is a highly ordered process. First, binding of cd2 to cyclin B1 is essential for activation of this kinase. Then, phosphorylations of Thr14, Tyr15 and Thr161 of cd2c, which are mediated by upstream kinases such as WEE1, Myt1 and CDK7, are required for activation of the cd2-cyclin B1 complex. Finally, dephosphorylation of Thr14 and Tyr15 of cd2c by cyclin B1 cell division cycle protein (cd2c5C) phosphatase activates the cd2-cyclin B1 complex and triggers the initiation of mitosis (25,35,36). Hence, cd2c is very important in the process of activation of CDK1. In this study, similar cyclin B1 level in Figure 2 hinted that MIL might inhibit CDK1 activity through inactivation of cd2c. Moreover, a case report had demonstrated that G2/M arrest was in response to cd2c inactivation (37). Many factors could induce the inactivation of cd2c, such as inhibiting the production of total cd2c protein, and the phosphorylation of Thr161 and the dephosphorylation of Thr14 and Tyr15 of cd2c. In this study, the inhibitory effect of MIL on cd2c activity was demonstrated in the following three ways: (i) MIL inhibited the synthesis of cd2c protein (Figure 2A). (ii) MIL inhibited the dephosphorylation of cd2c at Thr14 (Figure 2A). Further, the inhibitory effect of dephosphorylation of cd2c at Thr14 was associated with the decrease in cd2c5c protein, which was translocated from the cytoplasm to nucleus at the onset of mitosis to activate cd2c–cyclin B1 complex by dephosphorylation of the Thr14 and Tyr15 residues (37,38). And the downregulation of cd2c5c was related to Chk2 activation and DNA damage, which prevented cells from initiating mitosis (39,40). Further study showed that the cells treated with MIL have an activation of Chk2 as determined by western blot (Figure 2A) and a significant DNA damage shown by TUNEL assay (Figure 2B).

Mitochondrial apoptotic pathway has been described as an important signaling of cell death for mammalian cells (41). The ratio of Bax/Bcl-2 is critical for the induction of apoptosis. An increase in the ratio of Bax/Bcl-2 stimulates the release of cytochrome c from mitochondria into the cytosol (42,43). Cytochrome c then binds to Apaf-1, participating in formation of the apoptosome–deoxyadenosine triphosphate-dependent complex, which activates caspase 9 and caspase 3. Activated caspase 3 is the key executor of apoptosis (44). After the treatment of HepG2 and SK-HEP-1 cells with MIL, we found a decrease in the level of Bcl-2 and an increase in the level of Bax and a significant increase in the ratio of Bax/Bcl-2 (Figure 4C). This induces a time- and dose-dependent decrease in the mitochondria membrane potential (Figure 4D) and an increase in the release of cytochrome c from the mitochondria to the cytosol (Figure 5A). In the cytosol, cytochrome c triggers Apaf-1–mediated activation of caspase 9. Caspase 9 could then activate the effector caspase 3 to induce cells apoptosis. Western blot analysis demonstrated that both caspase 9 and caspase 3 were activated in a dose-dependent manner (Figure 4B). This was supported by observed increase in caspase 3 activity in both hepatocarcinoma cells when treated with MIL. (Figure 4A).

Mitochondria are the main source of ROS (32). The increased levels of ROS may result in the oxidations of amino acids in proteins, of polydesaturated fatty acids in lipids, as well as cause DNA damage and apoptosis (45). In our experiment, we found the ROS level to increase in a dose-dependent manner in both HepG2 and SK-HEP-1 cells when treated with MIL (Figure 5C). TUNEL assay also confirmed that when the cells were treated with MIL, their DNA was significantly damaged (Figure 2B), which could induce cell cycle arrest if it was not repaired within 24 h (27). In addition, mitochondria are the major target of ROS damaging and where increase in ROS level can damage mitochondrial membrane (46). And this is consistent with our result of the dose-dependent decrease in the mitochondria membrane potential (Figure 4D). From these results, we demonstrated that MIL induced cells apoptosis through ROS-mediated-mitochondrial apoptotic pathway.

In conclusion, this study showed a novel chalcone MIL from M. pachycarpa Benth inhibited the proliferation of hepatocarcinoma cells both in vitro and in vivo. In vitro, MIL inhibited CDK1 activity through inducing cd2c inactivation caused by downregulation of cd2c5C, activation of Chk2 and increased DNA damage to induce G2/M arrest in HepG2 and SK-HEP-1 cells. Further, MIL activated ROS-mediated-mitochondrial apoptotic pathway to induce apoptosis in tumor cells. In the HepG2 xenograft model, MIL showed very good antitumor activity and less toxicity than doxorubicin, a first-line drug for hepatocarcinoma. With the great antitumor activity, with safety and with further structural modification, MIL may be a new lead compound as cancer drug candidate.

Supplementary material

Supplementary 1–3 can be found at http://carcin.oxfordjournals.org/

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References

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