PHYTOTHERAPY RESEARCH
Phytother. Res. **26**: 528–534 (2012)
Published online 14 September 2011 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.3583

7,7″-Dimethoxyagastisflavone-induced Apoptotic or Autophagic Cell Death in Different Cancer Cells

Chia-Hsiang Hwang,[1,2†] Yu-Ling Lin,[3†] Yen-Ku Liu,[3] Chia-Hung Chen,[3] Hsin-Yi Wu,[4] Cheng-Chang Chang,[2] Chao-Yuan Chang,[2] Yu-Kuo Chang,[2] Yi-Han Chiu,[5] Kuang-Wen Liao[3,4]* and Yiu-Kay Lai[1]*

[1]Department of Life Science, Institute of Biotechnology, National Tsing Hua University, Hsinchu, Taiwan
[2]Yung-Shin Pharmaceutical Industry Co., Ltd, Taichung, Taiwan
[3]Institute of Molecular Medicine and Bioengineering, National Chiao Tung University, Hsinchu, Taiwan
[4]Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan
[5]Department of Life Science, Tzu Chi University, Hualien, Taiwan

7,7″-Dimethoxyagastisflavone (DMGF), a biflavonoid isolated from the needles of *Taxus* × *media* cv. Hicksii, was evaluated for its antiproliferative and antineoplastic effects in three human cancer cell lines. Interestingly, DMGF caused cell death via different pathways in different cancer cells. DMGF induced apoptosis, activated caspase-3 activity and changed the mitochondrial membrane potential in HT-29 human colon cancer cells. However, the apoptotic pathway is not the major pathway involved in DMGF-induced cell death in A549 human lung cancer cells and HepG2 human hepatoma cells. Treatment with 3-MA, an inhibitor of autophagy, significantly decreased DMGF-induced cell death in HepG2 and A549 cells, but did not affect DMGF-induced cell death in HT-29 cells. Following DMGF treatment, the HepG2 cells increased expression of LC3B-II, a marker used to monitor autophagy in cells. Thus, DMGF induced apoptotic cell death in HT-29 cells, triggered both apoptotic and autophagic death in A549 cells and induced autophagic cell death in HepG2 cells. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: 7,7″-dimethoxyagastisflavone; biflavonoid; *Taxus* × *media* cv. Hicksii; apoptosis; autophagy.

INTRODUCTION

Paclitaxel (brand name Taxol), belongs to a class of diterpenoids, known as taxanes, that stabilize microtubules against depolymerization and induce apoptosis (Miller *et al*., 1999; Wang *et al*., 2000). It is produced and isolated from plants of the genus Taxus (yew). In addition to paclitaxel, there are an increasing number of semi-synthetic taxane analogs made from 10-deacetyl baccatin III that are also isolated from Taxus. The companies that make taxane analogs such as docetaxel (brand name Taxotere), larotaxel (XRP9881) and cabazitaxel (XRP6258) are in the process of obtaining regulatory approval or entering late-stage clinical investigation for these drugs. Thus, Taxus can be considered a good resource for the development of new antitumor drugs.

It has been reported that several biflavonoids from plants have biological activities such as cytotoxicity, antitumor activity and anti-angiogenesis (Pang *et al*., 2009; Guruvayoorappan and Kuttan, 2008). 7,7″-Dimethoxyagastisflavone (DMGF), one of the compounds isolated from *Taxus media* var. Hicksii, is a biflavonoid that was first isolated and identified from the leaves of *Araucaria bidwillii* Hooker and *Agathis alba* Foxworthy (Khan *et al*., 1972). It also exists in New Zealand kauri and other species of *Agathis* (Ofman *et al*., 1995). However, only a few studies have described the activity of DMGF. One study indicated that DMGF could inhibit the production of aflatoxin by *Aspergillus flavus* (Goncalez *et al*., 2001). Therefore, the anti-tumor activity of DMGF is unclear.

Though previous researchers have successfully synthesized paclitaxel (Nicolaou *et al*., 1994), the method employed to obtain this compound is not cost-effective. Paclitaxel is currently produced by direct extraction from the biomass of the yew tree or semisynthesis from its precursor 10-deacetyl baccatin III (Baloglu and Kingston, 1999). Traditionally, paclitaxel and its precursors have been extracted from the bark of yew trees, a process that leads to plant death. However, paclitaxel-related compounds may have also been sustainably extracted from the needles and old roots of yew trees, showing that these are also good biomass resources. In addition to paclitaxel or paclitaxel-related compounds, compounds such as flavones can also be isolated from the needles of *Taxus* × *media* cv. Hicksii. Therefore, the needles of yew trees may not only sustainably provide paclitaxel-related compounds without damaging the environment, but are also a good resource for drug discovery.

This study investigated the cytotoxicity of 7,7″-dimethoxyagastisflavone (DMGF) isolated from the needles and old roots of *Taxus x media* cv. Hicksii on

* Correspondence to: Professor Kuang-Wen Liao, Department of Biological Science and Technology, National Chiao Tung University, Hsinchu 30050, Taiwan; Prof. Yiu-Kay Lai, Department of Life Science, Institute of Biotechnology, National Tsing Hua University, Hsinchu 30013, Taiwan.
E-mail: liaonms@pchome.com.tw; yklai@life.nthu.edu.tw
†Contributed equally to this study.

Received 13 January 2011
Revised 13 May 2011
Accepted 16 May 2011

human cancer cell lines. Interestingly, it was found that this biflavonoid DMGF triggered different death pathways in different tumor cell types. DMGF could induce apoptotic death in HT-29 colon cancer cells and trigger autophagic death in A549 lung cancer and HepG2 hepatoma cells. Therefore, DMGF may be a good candidate as an anti-cancer drug.

MATERIALS AND METHODS

Plant material. Needles of *Taxus* × *media* cv. Hicksii were collected from Taichung, Taiwan in 2008. The plant was authenticated by the Department of Life Science, National Taiwan University. A voucher specimen is kept at the Herbarium of the same department.

Extraction and isolation of DMGF and other compounds. The dried needles of *Taxus media* var. Hicksii (10 kg) were extracted with methanol (MeOH) at a preferred ratio of 1:1 (w/v) at room temperature to yield a methanol extract. The biomass was extracted in this manner at least two times. The pooled methanol extracts were concentrated *in vacuo* to give a crude extract (780 g) which was then partitioned with water/ethyl acetate (EtOAc) (1:1) at least three times. The combined EtOAc layer resulted in 140 g of black syrup, which was mixed with 350 g silica gel 60 N (Timely, Japan) and packed in an open column, and eluted with hexane and an increasing concentration of EtOAc in hexane to afford five fractions (4:1, 3:1, 7:3, 13:7, 3:2, A–E). Fraction E was further purified by recrystallization from hexane/acetone (3:1) then re-chromatographed with the same elution condition to give 4.2 g (98.5%) of DMGF sample. DMGF and seven taxane derivatives were isolated and the yields of different compounds are shown in Table 1.

HPLC analysis. DMGF was analysed by liquid chromatography on a Waters HPLC system (Waters 2796 Bioseparation Module) composed of an auto sampler and a detector (Waters 2996 photodiode array detector) equipped with C18™ Symmetry column (250 mm × 4.6 mm i.d.) with a particle size of 5 μm. Isocratic elution was monitored at 254 nm under room temperature and performed for methanol and water (30:70, v/v at a flow rate of 0.5 mL/min).

Identification of the compounds. The chemical structure of DMGF and other compounds was confirmed by ^{1}H and ^{13}C NMR spectra. ^{1}H (400 MHz) and ^{13}C (400 MHz) NMR spectra were recorded on a Bruker AV-400 spectrometer using deuterated chloroform as the solvent and tetramethylsilane (TMS) as an internal standard.

Cells and cell cultures. HT-29 human colon cancer, A549 human lung cancer and HepG2 human hepatoma cell lines were purchased from BCRC (Hsinchu, Taiwan, ROC) and maintained in DMEM medium (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with heat-inactivated 10% fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin/amphotericin in 5% CO_2 at 37 °C.

Assay for cell growth inhibition. The cells (1×10^4 cells/well) were seeded overnight in a 96-well plate. After treatment with serial concentrations of the various isolated compounds in dimethyl sulfoxide (DMSO, final concentration of DMSO was 0.1%), the cells were incubated at 37 °C for 48 h. Subsequently, the cell viability was measured by MTT assay. The cell viability ratio (%) was calculated using the following equation:

$$\%\ \text{viability} = \frac{\text{absorbance of test sample}}{\text{absorbance of control}} \times 100\%$$

The IC_{50} was then calculated for each treatment. The results were expressed in duplicate and three independent experiments. Morphological changes in the treated cells were monitored by light microscopy.

Measurement of mitochondrial membrane potential. The mitochondrial membrane potential was determined using a Cell Meter™ JC-10 mitochondria membrane potential assay kit (ABD Bioquest, CA, USA). Briefly, the cells were harvested after treatment with DMGF (2.5 μg/mL) for 48 h, incubated with 50 μL of JC-10 dye-loading solution (25 μL of JC-10 in 5 mL assay buffer) at 37 °C for 30 min and analysed by flow cytometry (Becton Dickinson). For each sample, 10000 events were recorded and used to plot the red/green fluorescence ratio. Data were calculated as: mitochondrial membrane potential index = the mean of the green fluorescence (FL1)/the mean of the red fluorescence (FL2).

Table 1. The cytotoxicity of isolated compounds from *Taxus media* var. Hicksii in different cancer cell lines

Compound	Yield (%)	Purity (%)	IC_{50} HT29	IC_{50} A549	IC_{50} HepG2
Baccatin III	0.02%	98.0	13.0 ± 1.3 μg/mL	21.6 ± 1.1 μg/mL	> 40 μg/mL
Taxachitriene A	0.01%	98.5	> 40 μg/mL	> 40 μg/mL	> 40 μg/mL
5a-Hydroxy-2a,7b,9a, 10b,13a-tetraacetoxy4 (20),11-taxadine	0.01%	98.5	> 40 μg/mL	15.5 ± 2.1 μg/mL	> 40 μg/mL
2a,10b,13a,20-tetraacetoxy-5a-hydroxy-3,8-secotaxa-3,7,11-trien-9-one	0.01%	98.5	> 40 μg/mL	> 40 μg/mL	> 40 μg/mL
7,7"-Dimethoxyagastis-flavone	0.04%	98.5	2.3 ± 0.6 μg/mL	2.8 ± 0.1 μg/mL	3.9 ± 1.7 μg/mL
10-Deacetylpaclitaxel (10-DAT)	0.02%	98.5	0.1 ± 0.1 μg/mL	0.2 ± 0.1 μg/mL	> 40 μg/mL
10-Deacetyl-cephalomannine	0.01%	98.5	0.8 ± 0.1 μg/mL	> 40 μg/mL	2.5 ± 0.3 μg/mL
Paclitaxel	0.03%	99.0	7.5 ± 0.3 ng/mL	7.2 ± 0.9 ng/mL	6.8 ± 1.3 μg/mL

Cell cycle analysis. The cells were treated with 2.5 μg/mL of DMGF for 48 h. After treatment, 10^6 cells were harvested by trypsinization followed by centrifugation. The cell pellets were washed with PBS and fixed in 3 mL of 70% ethanol at 4 °C for 30 min. After centrifugation at 400 × **g**, the fixed cells were resuspended with 1 mL staining buffer (5% Triton-X 100, 0.1 mg/mL RNase A and 4 μg/mL propidium iodide) for 30 min at room temperature. In total 10000 cells were analysed for DNA content by flow cytometry. The distribution of cells in the G0/G1, S and G2/M phases of the cell cycle were determined using Modfit software (Becton Dickinson).

Assay of caspase-3 activity. The cells were seeded into 24-well cultured plates at 1×10^5 cells/well overnight. The cells were then treated with 2.5 μg/mL of DMGF for different durations. Caspase-3 activity was determined with the PE active caspase-3 apoptosis kit (BD Pharmingen, San Jose, CA, USA), following the manufacturer's instructions.

Western blotting for autophagy analysis. The cells were treated with or without DMGF at IC_{50} for 12 h. For immunoblotting, equivalent amounts of cell lysate were resolved by SDS-PAGE (10%) and transferred onto PVDF membranes. After blocking, the membranes were incubated with the anti-LC3BII antibody (GeneTex Inc., Irvine, CA, USA). The membranes were then treated with goat anti-rabbit peroxidase-conjugated antibody, and the immunoreactive proteins were detected using an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

RESULTS

Isolation and identification of compounds from *Taxus* × *media* cv. Hicksii

Compounds were extracted and isolated from the needles and old roots of *Taxus* × *media* cv. Hicksii. The yields of different compounds were determined, and the compounds with the highest yields were further identified. The names of the eight identified compounds and their yields and purity are shown in Table 1.

Growth inhibition activity of isolated compounds in tumor cell lines

To assess the cytotoxicity of the eight compounds, the IC_{50} values of each compound in HT-29, A549 and HepG2 cells were determined. The antineoplastic agent paclitaxel was used as a reference compound. Table 1 summarizes the antiproliferative effects of the eight isolated compounds on these cells. The IC_{50} values show that paclitaxel, DMGF and 10-DAT had higher antiproliferative effects, while other compounds showed weak-to-moderate antiproliferative effects.

Even though 10-DAT showed high antiproliferative activity, it is a paclitaxel derivative that is already known to have antitumor activity. Thus, DMGF was selected for further study of its antitumor effects. It was found that DMGF had different antiproliferative effects on different cancer cell lines and primary cells, including human PBMCs ($IC_{50} = 11.3$ μg/mL) and mouse splenocytes ($IC_{50} = 9.9$ μg/mL). In addition, DMGF treatment led to clearly different morphological changes in different cell lines. DMGF significantly induced cell membrane shrink and formed apoptotic bodies in HT-29, whereas the presence of apoptosis in DMGF-treated A549 cells was not as obvious (Fig. 1A). Moreover, treatment with DMGF resulted in the vacuolation of cytoplasm in HepG2 and A549 cells, but not in HT-29 cells (Fig. 1A).

Effects of DMGF on the cell cycle in HepG2, A549 and HT-29 cell lines

As shown in Fig. 1B, DMGF caused an increase in the number of cells in the sub-G1 phase in all cell types, which indicates that cell death is one of the reasons for the decreased proliferation. The proportion of cells in each phase was calculated; DMGF had different effects on the cell cycle in different tumor cells. DMGF decreased the proportion of HT-29 cells in the G0/G1 phase and increased the proportion in the G2/M phase. However, DMGF only had minor effects on the cell cycle in A549 and HepG2 cells (Fig. 1B).

Determination of the effect of DMGF on apoptosis in HepG2, A549 and HT-29 cell lines

To further determine whether the antiproliferative effects of DMGF resulted from induction of apoptosis, activation of caspase-3, an indicator for the late phase of apoptosis, was measured. DMGF treatment increased caspase-3 activity in HT-29 cells in a time-dependent manner. In contrast, DMGF caused the caspase-3 activity to stay constant over time in A549 cells and had no effect on HepG2 cells (Fig. 2A). Mitochondrial membrane potential ($\triangle\Psi_M$), the loss of which is indicative of the induction of the endogenous apoptosis pathway, was measured in all three cell lines following DMGF treatments. Fig. 2B illustrates the different effects of DMGF on different cells; HT-29 cells were the most susceptible, A549 cells were slightly affected and HepG2 cells did not lose $\triangle\Psi_M$ following DMGF treatment.

The effect of DMGF on the induction of autophagy

The results show that intracellular vesicles were increased in DMGF-treated HepG2 and A549 cells, indicating that the autophagy pathway may be involved in DMGF-induced cell death in these two cell lines. Thus, 3-methyladenine (3-MA), an inhibitor of autophagy, was used to examine this hypothesis. Treatment with 3-MA did not affect DMGF-induced cell death in HT-29 cells, but significantly decreased DMGF-induced cell death in HepG2 and A549 cells (Fig. 3A). DMGF was also found to have a greater influence on autophagic death in HepG2 cells compared with A549

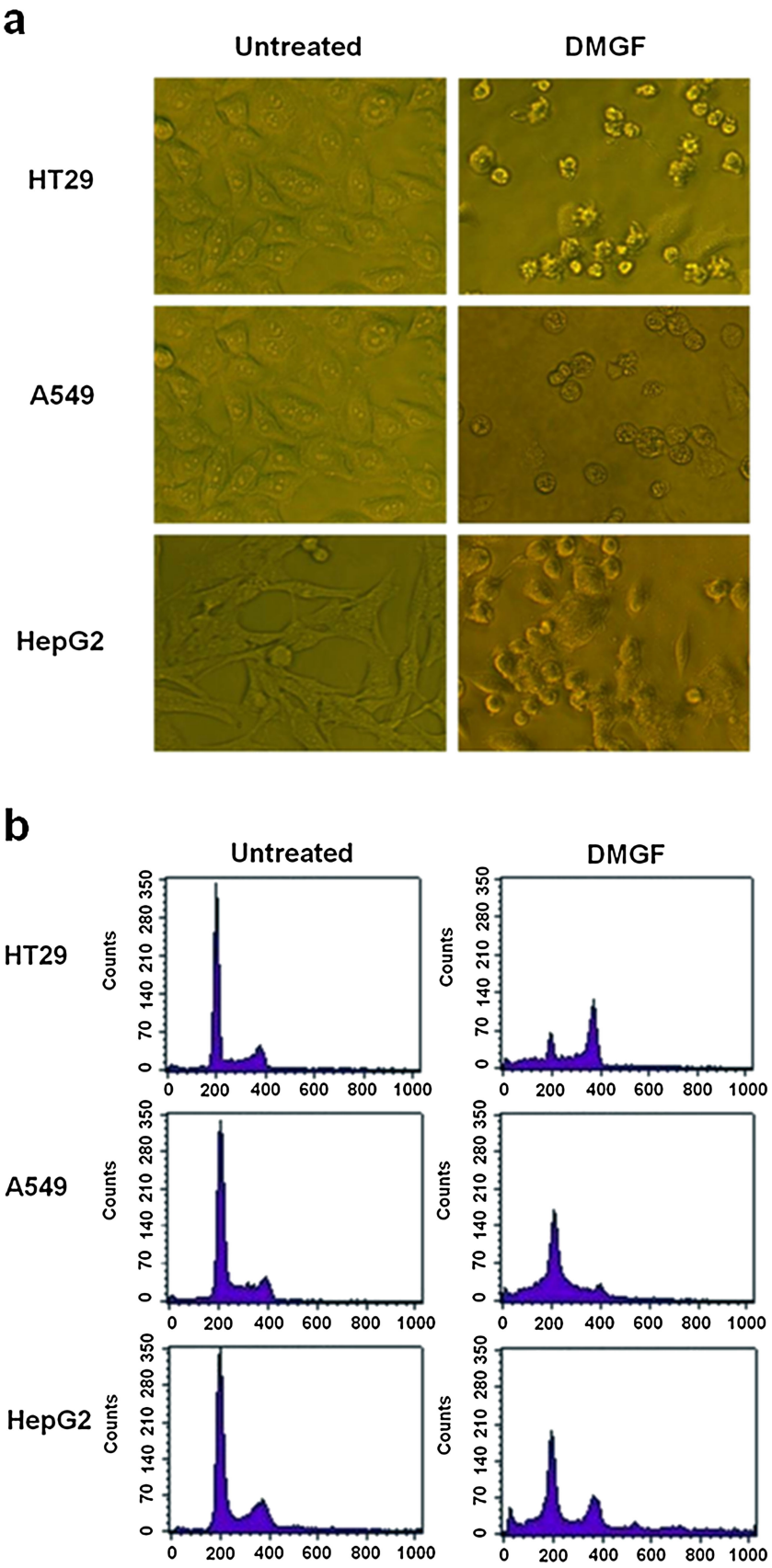

Figure 1. Effects of DMGF on (a) morphological changes (b) cell cycle in HT-29, A549 and HepG2 cells. Cell morphology photographed under a microscope (200× magnification). The cell cycle distributions and the percentage of cells in each phase were obtained with Modfit software. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

cells. Furthermore, the three cell lines were monitored for changes in levels of autophagy regulators after DMGF treatment. The results show that DMGF increased the levels of LC3-II in HepG2 cells, maintained levels of LC3-II in A549 cells and lowered levels of LC3-II in HT-29 cells (Fig. 3B).

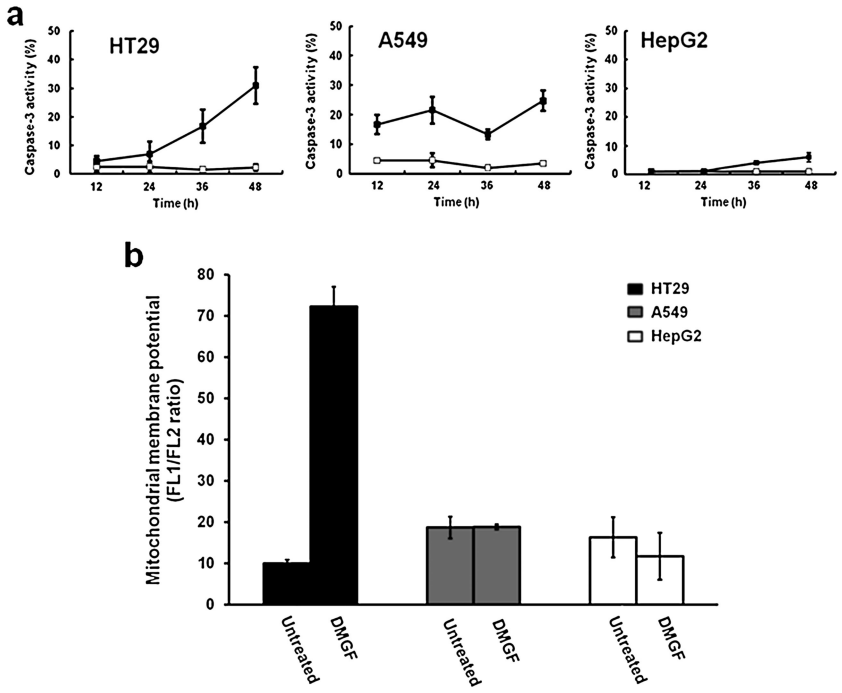

Figure 2. The apoptotic activity of DMGF in different cancer cells. (a) Caspase-3 activity was determined after treatment with DMGF for different potential was calculated by dividing the FL1 value by the FL2 value. Data are shown as the mean ± SD of three independent experiments ($n = 6$).

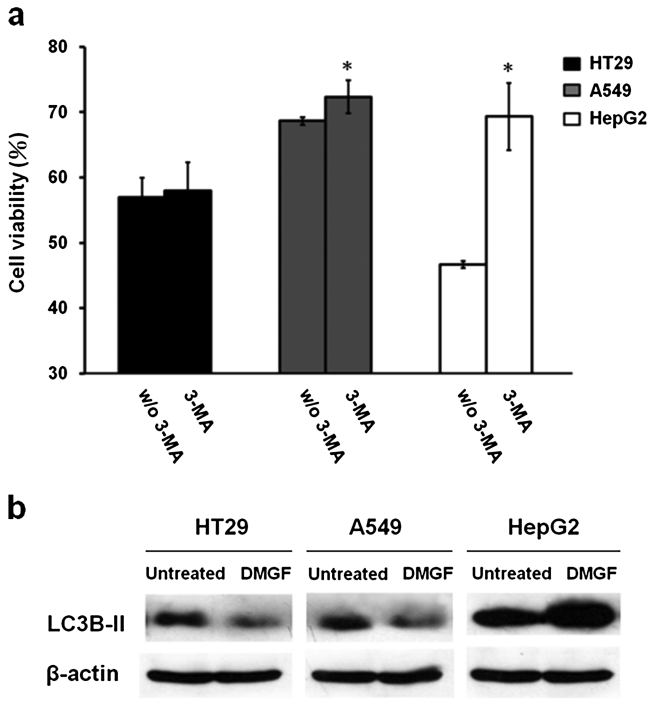

Figure 3. The autophagic activity of DMGF in different cancer cells. (a) Cells were treated with 2 μg/mL DMGF and co-incubated with or without 3-MA (57 μM). Cell viability was then analysed by MTT assay. The asterisk indicates a significant difference between control and DMGF-treated cells by the Student's *t*-test. (b) The effects of DMGF on LC3B-II levels. The levels of LC3B-II were determined in DMGF-treated HT-29, A549 and HepG2 cells using anti-LC3B-II antibodies. β-actin was used as the normalized control. Results shown are representative of three independent experiments.

DISCUSSION

This study examined the effects of compounds isolated from *Taxus media* var. Hicksii on the cytotoxicity of tumor cells. The genus *Taxus* has been studied extensively for its taxoid compounds, particularly paclitaxel and its precursor 10-deacetyl baccatin-III. Many taxoids with antineoplastic, cytotoxic or antipromastigote activities have been isolated and investigated from the different organs of the *Taxus* genus (Chattopadhyay *et al*., 2006; Dai *et al*., 2006; Georgopoulou *et al*., 2007; Jiang *et al*., 2008).

One of the compounds isolated from *Taxus media* var. Hicksii, DMGF, is a biflavonoid that has yet to be characterized in the literature. Only a few studies have described the activity of DMGF. One study indicated that DMGF could inhibit the production of aflatoxin by *Aspergillus flavus* (Goncalez *et al*., 2001). Our results show that DMGF not only has therapeutic potential against the growth of cancer cell lines but also induced cell death differently in different tumor cell lines. HT-29 cells were the most susceptible to DMGF-induced apoptosis. Accordingly, HT-29 cells also showed significant changes in $\triangle\Psi_M$, an important marker of endogenous apoptosis (Krysko *et al*., 2008) (Fig. 2B). In addition, early- and late-phase apoptosis, phosphatidyl-serine externalization and an increase in caspase-3 activity were present in HT-29 cells following treatment with DMGF (Fig. 2A). It was also shown that DMGF treatment resulted in the formation of apoptotic bodies, cell shrinkage and bleb formation (Rello *et al*., 2005; Cotter, 2009), which are all classic morphological changes that occur in HT-29 cells during apoptosis.

In contrast, the effects of DMGF on the induction of apoptosis, including phosphatidyl-serine externalization, increased caspase-3 activity and changes in $\triangle\Psi_M$, were minor or did not influence cell death in A549 or HepG2 cells (Fig. 2B). However, it was found that DMGF interfered with the growth rate of these cells compared with untreated cells and that the IC_{50} values were in the range 2–5 μg/mL (Table 1). It was also observed that the

integrity of the plasma membrane in A549 and HepG2 cells was disrupted following DMGF treatment (Fig. 1A). A). In addition, DMGF treatment resulted in an increased number of cells in the sub-G1 phase in these two cell lines (Fig. 1B). These results indicate that the apoptotic pathway is not the major pathway involved in DMGF-induced cell death. In addition, Annexin-V staining indicated that necrosis was also not the major pathway for DMGF-induced death of A549 or HepG2 cells (results not shown). It was found that autophagy, an apoptosis-independent mechanism of cell death, was likely involved in DMGF-induced death in A549 and HepG2 cells (Fig. 3). Using 3-MA, an inhibitor of autophagy, it was shown that autophagy is the major pathway for DMGF-induced cell death in HepG2 and A549 cells. Following DMGF treatment, HepG2 cells showed increased expression of LC3B-II, a marker used to monitor autophagy in cells (Barth *et al*., 2010; Choi *et al*., 2010). These findings suggest that DMGF can induce autophagy in HepG2 cells. In addition, DMGF treatment decreased the expression of LC3B-II in HT-29 cells, suggesting that DMGF-induced apoptosis could suppress the expression of this autophagy marker. According to our results, DMGF induced both apoptotic and autophagic death in A549 cells. Thus, there was no significant change in the expression of LC3B-II in these cells (Fig. 3B).

Unlike apoptosis, autophagy is a reversible process that can contribute to both tumor cell death and survival (Amaravadi and Thompson, 2007). Recently, autophagic agonists have been described as anticancer drugs (Choi *et al*., 2010; Hung *et al*., 2009; Ko *et al*., 2009). Furthermore, our results are consistent with the findings of these studies, in which 6-shogaol and ginsenoside Rk1 were found to induce autophagic death in A549 and HepG2 cells, respectively. In addition, many compounds have been shown to induce death in tumor cells with defective apoptotic machinery (Reed, 2003; Yu *et al*., 2003). Thus, inducers of autophagy combined with inducers of apoptosis may provide a better antitumor effect. Because DMGF is able to induce both the apoptotic and autophagic pathways, it may be a good candidate for antitumor treatment.

In summary, DMGF isolated from the needles of *Taxus media* var. Hicksii has promising anticancer properties due to its ability to induce different apoptotic or autophagic effects in different cell lines. This biflavonoid is likely a contributor to the anticancer properties described here and may act to limit cancer cell progression by inhibiting proliferation, inducing apoptosis, evoking autophagy and/or suppressing inflammation and tumor cell migration. *In vivo* and human clinical studies are needed to determine the efficacy of this compound as a cancer chemoprevention agent.

Acknowledgements

The authors acknowledge the financial assistance provided by Yung-Shin Pharmaceutical Industry Co., Ltd under the cooperative grant scheme (Project No. 98 C117).

Conflict of Interest

The authors have declared that there is no conflict of interest.

REFERENCES

Amaravadi RK, Thompson CB. 2007. The roles of therapy-induced autophagy and necrosis in cancer treatment. *Clin Cancer Res* **13**: 7271–7279.

Baloglu E, Kingston DG. 1999. A new semisynthesis of paclitaxel from baccatin III. *J Nat Prod* **62**: 1068–1071.

Barth S, Glick D, Macleod KF. 2010. Autophagy: assays and artifacts. *J Pathol* **221**: 117–124.

Chattopadhyay SK, Pal A, Maulik PR, Kaur T, Garg A, Khanuja SP. 2006. Taxoid from the needles of the Himalayan yew *Taxus wallichiana* with cytotoxic and immunomodulatory activities. *Bioorg Med Chem Lett* **16**: 2446–2449.

Choi IK, Cho YS, Jung HJ, Kwon HJ. 2010. Autophagonizer, a novel synthetic small molecule, induces autophagic cell death. *Biochem Biophys Res Commun* **393**: 849–854.

Cotter TG. 2009. Apoptosis and cancer: the genesis of a research field. *Nat Rev Cancer* **9**: 501–507.

Dai J, Bai J, Hasegawa T *et al*. 2006. A new taxoid from a callus culture of *Taxus cuspidata* as an MDR reversal agent. *Chem Pharm Bull (Tokyo)* **54**: 306–309.

Georgopoulou K, Smirlis D, Bisti S, Xingi E, Skaltsounis L, Soteriadou K. 2007. *In vitro* activity of 10-deacetylbaccatin III against *Leishmania donovani* promastigotes and intracellular amastigotes. *Planta Med* **73**: 1081–1088.

Goncalez E, Felicio JD, Pinto MM. 2001. Biflavonoids inhibit the production of aflatoxin by *Aspergillus flavus*. *Braz J Med Biol Res* **34**: 1453–1456.

Guruvayoorappan C, Kuttan G. 2008. Amentoflavone stimulates apoptosis in B16F-10 melanoma cells by regulating bcl-2, p53 as well as caspase-3 genes and regulates the nitric oxide as well as proinflammatory cytokine production in B16F-10 melanoma cells, tumor associated macrophages and peritoneal macrophages. *J Exp Ther Oncol* **7**: 207–218.

Hung JY, Hsu YL, Li CT *et al*. 2009. 6-Shogaol, an active constituent of dietary ginger, induces autophagy by inhibiting the AKT/mTOR pathway in human non-small cell lung cancer A549 cells. *J Agric Food Chem* **57**: 9809–9816.

Jiang S, Zu Y, Fu Y, Zhang Y, Efferth T. 2008. Activation of the mitochondria-driven pathway of apoptosis in human PC-3 prostate cancer cells by a novel hydrophilic paclitaxel derivative, 7-xylosyl-10-deacetylpaclitaxel. *Int J Oncol* **33**: 103–311.

Khan NU, Ilyas M, Rahman W, Mashima T, Okigawa M, Kawano N. 1972. Biflavones from the leaves of *Araucaria bidwillii* Hooker and *Agathis alba* Foxworthy (Araucariaceae). *Tetrahedron* **28**: 5689–5695.

Ko H, Kim YJ, Park JS, Park JH, Yang HO. 2009. Autophagy inhibition enhances apoptosis induced by ginsenoside Rk1 in hepatocellular carcinoma cells. *Biosci Biotechnol Biochem* **73**: 2183–2189.

Krysko DV, Vanden Berghe T, D'Herde K, Vandenabeele P. 2008. Apoptosis and necrosis: detection, discrimination and phagocytosis. *Methods* **44**: 205–221.

Miller MC, 3rd, Johnson KR, Willingham MC, Fan W. 1999. Apoptotic cell death induced by baccatin III, a precursor of paclitaxel, may occur without G(2)/M arrest. *Cancer Chemother Pharmacol* **44**: 444–452.

Nicolaou KC, Yang Z, Liu JJ *et al*. 1994. Total synthesis of taxol. *Nature* **367**: 630–634.

Ofman DJ, Markham KR, Vilain C, Molloy BPJ. 1995. Flavonoid profiles of New Zealand kauri and other species of Agathis. *Phytochemistry* **38**: 1223–1228.

Pang X, Yi T, Yi Z et al. 2009. Morelloflavone, a biflavonoid, inhibits tumor angiogenesis by targeting rho GTPases and

extracellular signal-regulated kinase signaling pathways. *Cancer Res* **69**: 518–525.
Reed JC. 2003. Apoptosis-targeted therapies for cancer. *Cancer Cell* **3**: 17–22.
Rello S, Stockert JC, Moreno V *et al*. 2005. Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. *Apoptosis* **10**: 201–208.
Wang TH, Wang HS, Soong YK. 2000. Paclitaxel-induced cell death: where the cell cycle and apoptosis come together. *Cancer* **88**: 2619–2628.
Yu J, Tiwari S, Steiner P, Zhang L. 2003. Differential apoptotic response to the proteasome inhibitor Bortezomib [VELCADE, PS-341] in Bax-deficient and p21-deficient colon cancer cells. *Cancer Biol Ther* **2**: 694–699.