Magnolol and honokiol enhance HL-60 human leukemia cell differentiation induced by 1,25-dihydroxyvitamin D_3 and retinoic acid

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Abstract

Magnolol (MG) and honokiol (HK), two lignans showing anti-inflammatory and anti-oxidant properties and abundantly available in the medicinal plants *Magnolia officinalis* and *M. obovata*, were found to enhance HL-60 cell differentiation initiated by low doses of 1,25-dihydroxyvitamin D_3 (VD_3) and all-*trans*-retinoic acid (ATRA). Cells expressing membrane differentiation markers CD11b and CD14 were increased from 4% in non-treated control to 8–16% after being treated with 10–30 μM MG or HK. When added to 1 nM VD_3, MG or HK increased markers expressing cells from approximately 30% to 50–80%. When either MG or HK was added to 20 nM ATRA, only CD11b, but not CD14, expressing cells were increased from 9% to 24–70%. Under the same conditions, adding MG or HK to VD_3 or ATRA treatment further enlarged the G_0/G_1 cell population and increased the expression of p27[Kip1], a cyclin-dependent kinase inhibitor. Pharmacological studies using PD098059 (a MEK inhibitor), SB203580 (a p38 MAPK inhibitor) and SP600125 (a JNK inhibitor) suggested that the MEK pathway was important for VD_3 and ATRA-induced differentiation and also its enhancement by MG or HK, the p38 MAPK pathway had a inhibitory effect and the JNK pathway had little influence. It is evident that MG and HK are potential differentiation enhancing agents which may allow the use of low doses of VD_3 and ATRA in the treatment for acute promyelocytic leukemia.
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Keywords: Magnolol; Honokiol; 1,25-Dihydroxyvitamin D_3; All-*trans*-retinoic acid; Differentiation

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1. Introduction

Acute myeloid leukemia (AML) is characterized by the arrest of differentiation which leads to the accumulation of immature cells. Compounds that can reverse this maturation arrest are potentially useful as therapeutic agents. For example, AML patients can go into clinical remission when treated with all-*trans*-retinoic acid (ATRA) (Warrell et al., 1991) and differentiation of several human myeloid cell lines could be induced by 1,25-dihydroxyvitamin D_3 (VD_3) (Koeffler, Hirji, & Itri, 1985; Miyaura et al., 1981). Unfortunately, the therapeutic effect of ATRA is hampered by its toxicity (Akiyama, Nakamura, Nagasaka, Sakamaki, & Onozawa, 1992; Paydas et al., 2003; Radcliffe & Czajka-Narins, 2000; Sakakibara et al., 1993; Tallman et al., 2000) and VD_3's clinical use is burdened by its hypercalcemic effect (Smith et al., 1999). One approach to overcome these problems is to introduce a second chemical that enhances the differentiation-inducing effects of agents such as VD_3 and ATRA and allows the use of the inducers at lower, non-toxic doses.

Magnolol (MG) and honokiol (HK) are two lignans abundantly available in the medicinal plants *Magnolia officinalis* and *M. obovata* (Fujita, Itokawa, & Sashida, 1973; Teng et al., 1988; Yahara, Nishiyori, Kohda, Nohara, & Nishioka, 1991). A number of pharmacological activities of MG and HK, including anti-tumor (Bai et al., 2003; Yang, Hsieh, Tsai, & Hsu, 2002, 2003; Zhong et al., 2003), anti-platelet aggregation (Pyo, Lee, & Yun-Choi, 2002; Teng et al., 1988), anxiolytic (Kuribara, Stavinoha, & Maruyama, 1999; Maruyama, Kuribara, Morita, Yuzurihara, & Weintraub, 1998) and anti-inflammation effects (Matsuda et al., 2001; Son, Lee, Yun-Choi, & Ryu, 2000) have been described. In addition, MG and HK display antioxidant activities 1000 times more potent than that of α-tocopherol in protecting rat heart mitochondria against lipid peroxidation (Lo, Teng, Chen, Chen, & Hong, 1994).

It has been suggested that agents with anti-inflammation or anti-oxidant properties can enhance the terminal differentiation of induced leukemia cells. For examples, indomethacin potentiates the response of HL-60 cells to VD_3 and ATRA (Bunce et al., 1996; Sokoloski & Sartorelli, 1998) and vitamin E and several other antioxidants enhance cell maturation induced by VD_3 (Sokoloski, Hodnick, et al., 1997). A number of plant-derived antioxidants such as curcumin (Liu, Chang, Cui, Newmark, & Conney, 1997; Sokoloski, Shyam, & Sartorelli, 1997), ascorbate (Quesada et al., 1996), carnosic acid (Danilenko, Wang, & Studzinski, 2001; Danilenko et al., 2003; Steiner et al., 2001), silibinin (Kang, Lee, Kim, Cho, & Kim, 2001), and carotenoids lycopene (Amir et al., 1999) were found to augment the differentiation inducing activities of VD_3 or ATRA on leukemic cell lines.

We investigated the effects of MG and HK on the differentiation of HL-60 human promyelocytic cells and in particular their potential effects on enhancing the activities of low doses of VD_3 and ATRA. In this paper, we characterized the effects of MG and HK, individually and in combination with VD_3 or ATRA, on the proliferation, differentiation and cell cycle progression of HL-60 cells. Furthermore, evidence was provided to show the MEK/MAP kinase signaling pathway is involved in the actions of MG and HK.

2. Materials and methods

2.1. Materials

ATRA, nitro blue tetrazolium (NBT), phorbol 12-myristate 13-acetate (PMA), propidium iodine (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA); VD_3 was from Alexis Biochemicals (San Diego, CA, USA); magnolol and honokiol (Fig. 1A and B) were from Wako Pure Chemical Industries Ltd. (Japan); MAPK inhibitors were from Calbiochem Ltd. (CA, USA).
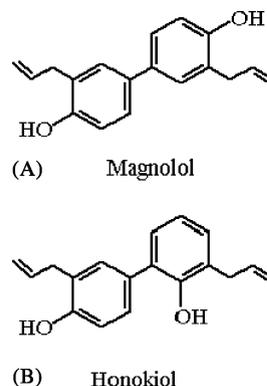
Fig. 1. Chemical structures of: (A) magnolol and (B) honokiol.

2.2. Cell culture

The human promyelocytic leukemia HL-60 cell line was obtained from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, NY, USA) at 37 °C in humidified 5% CO_2 atmosphere. In drug treatment experiments, MG, HK or various MAPK inhibitors were added to cells 2 h before the addition of VD_3 or ATRA. The final concentrations of the solvents (data not shown), ethanol or DMSO, were below 0.2% and 0.1% (v/v), respectively, which did not affect cell growth and differentiation.

2.3. Cell growth, apoptosis and cell cycle analysis

Cells were counted using a hemocytometer and viable cells were identified by the 0.2% trypan blue exclusion assay. For flow cytometry analysis, cells were collected, washed, suspended in cold PBS, fixed in 75% ethanol at −20 °C overnight, washed and resuspended in PBS with RNAase (0.1 mg/ml). Cellular DNA was stained with PI and cell samples were analyzed on a Becton Dickson flow cytometer (BD Biosciences, USA) using CELL Quest software (Verity Software House Inc., Topsham, ME).

2.4. Cell surface CD11b and CD14 antigens

The expression of cell surface markers CD11b and CD14 was estimated by flow cytometry. Samples of 1×10^6 cells were washed twice with PBS, resuspended in 100 µl of PBS and then incubated for 45 min at 4 °C with 5 µl R-phycoerythrin-conjugated anti-human CD11b or CD14 monoclonal antibodies (Caltag Laboratories, USA). Cells were then washed twice with and resuspended in 0.5 ml PBS, and analyzed on a Becton Dickson flow cytometer (BD Biosciences, USA) with CELL Quest software (Verity Software House Inc., Topsham, ME).

2.5. Nitroblue tetrazolium blue reduction (NBT) assay

Samples containing 1×10^6 cells were washed twice and incubated for 30 min at 37 °C in 1 ml serum free RPMI-1640 medium containing 0.1% (w/v) NBT and 100 ng/ml PMA. After incubation, cells were washed with 0.3 ml chilled 70% methanol and lysed in 0.5 ml 2 M potassium hydroxide overnight. Precipitated formazan was dissolved by the addition of 0.6 ml DMSO and the absorbance was read at 570 nm.

2.6. Western blot analysis

Samples containing 1×10^7 cells were pelleted, washed twice with ice-cold PBS, then lysed in 200 µl of cell lysis buffer (20 mM Tris–HCl, 1% (v/v) NP-40, 1.5 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH7.5) supplemented with 1 mM DTT, 10 µg/ml each of aprotinin, leupeptin and pepstatin A for 20 min at 4 °C. Supernatants after centrifugation at $14,000 \times g$ for 15 min at 4 °C were collected. Samples containing 50 µg of protein were separated on SDS–polyacrylamide gel and then transferred onto nitrocellulose membrane (0.45 µm, Bio-Rad). Membranes were immunoblotted with anti-p27^{Kip} antibody (1:200, Oncogene Research Products, #NA35) or anti-β-tubulin antibody (1:500, Santa Cruz Biotechnology Inc., #sc-9104), followed by horseradish peroxidase-conjugated secondary antibodies (1:5000) and visualized by ECL (Amersham Biosciences) according to manufacturer's instructions. Densitometric data of Western blot results were obtained using a Fluor-S Multiimager equipped with Multi-Analyst software (Bio-Rad Laboratories Inc., Hercules, USA).

2.7. Drug enhancement

Two compounds (A and B) were considered enhancing each other's actions if the effect of combined treatment (AB) was larger then the sum of their individual effects (AB > (A + B)) after subtraction of the respective background control valves. Statistical analyses were preformed using an unpaired two-tailed Student's t-test.

3. Results

3.1. Magnolol and honokiol enhanced cell differentiation initiated by VD_3 and ATRA

CD11b and CD14 antigens are undetectable on myeloid precursors. CD11b antigen increases
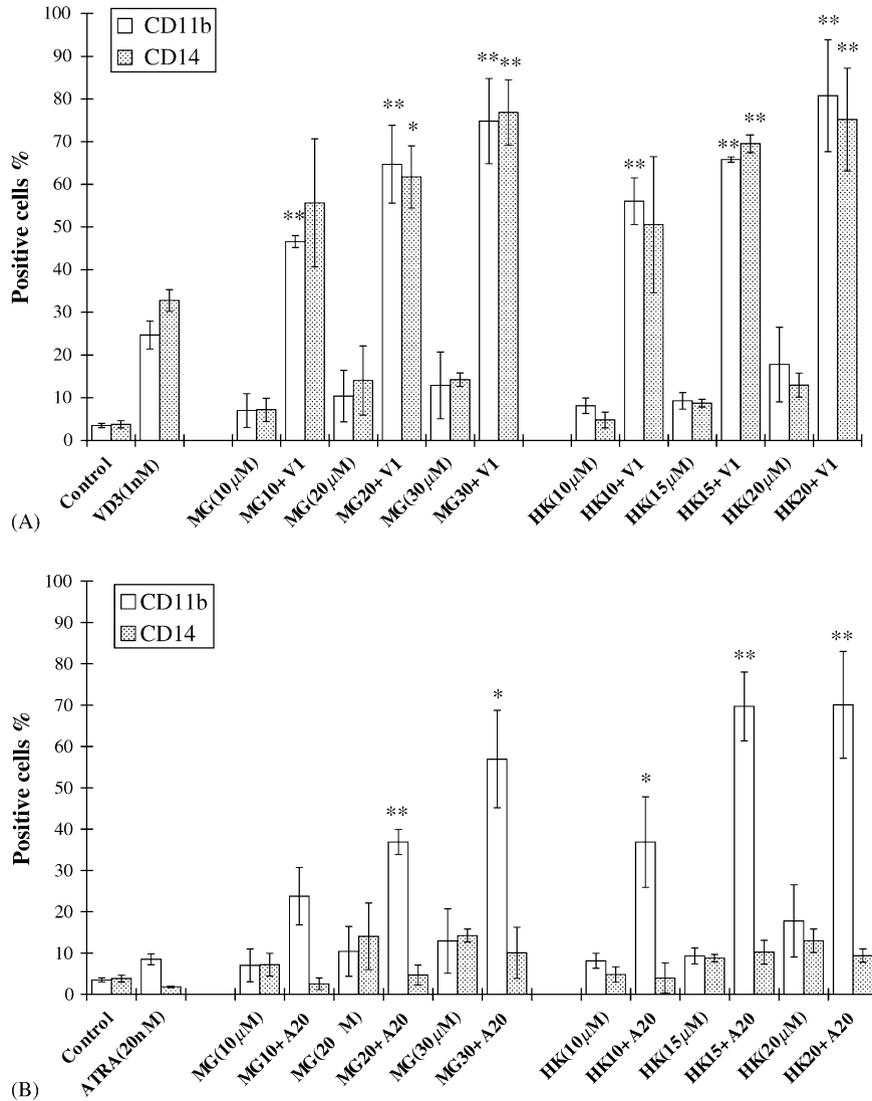
Fig. 2. Magnolol and honokiol enhance VD_3- and ATRA-induced expression of cell surface differentiation markers. HL-60 cells were incubated for 96 h with various concentrations of MG and HK with (A) 1 nM VD_3 or (B) 20 nM ATRA. Cell surface markers expressions were determined by flow cytometry. Results are mean ± standard derivation of triplicate determinations. Statistically significant differences from the sum of the individual effects of drugs: $^*p < 0.05$ and $^{**}p < 0.01$.

dramatically during the differentiation of the myeloid precursors into granulocytes or monocytes, while the increase of CD14 antigen is only found in monocytes. The two markers are found on approximately 4% of untreated HL-60 cells (Fig. 2A and B). Treatment with MG or HK individually had a small effect and increased marker expressing cells to 8–15%. VD_3 at 1 nM increased CD11b and CD14 expressing cells to 24.71%

and 32.80%, respectively. The addition of 10–30 μM MG or 10–20 μM HK to VD_3 treatment increased markers expressing cells to approximately 50–80%, as shown in Fig. 2A.

MG and HK also enhanced ATRA-induced HL-60 cell differentiation (Fig. 2B), but only CD11b, and not CD14, expressing cells was increased. This would suggest that MG or HK enhanced ATRA-induced
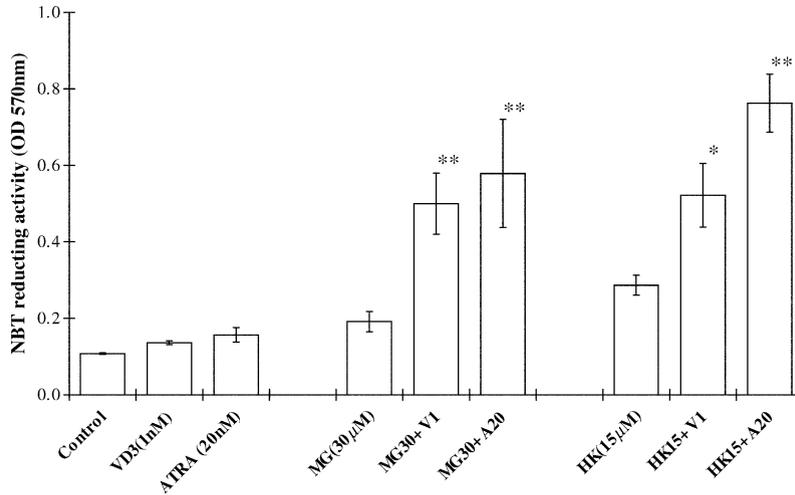
Fig. 3. Magnolol and honokiol enhance VD_3- and ATRA-induced nitroblue tetrazolium reduction. HL-60 cells were incubated for 72 h with various concentrations of MG and HK with 1 nM VD_3 or 20 nM ATRA. NBT reducing activity was determined and results are mean ± standard derivation of triplicate determinations. Statistically significant differences from the sum of the individual effects of drugs: $^*p < 0.05$ and $^{**}p < 0.01$.

differentiation predominantly along the granulocytic lineage.

HL-60 cell differentiation was also assessed by the NBT reduction assay, a typical marker of myeloid maturation (Hozumi, 1983). At low doses of PMA, differentiated HL-60 cells (both granulocytes and monocytes) rapidly generate superoxide, which in turn reduces the NBT dye to blue–black deposits in the cytoplasm (Collins, Ruscetti, Gallagher, & Gallo, 1979; Newburger, Chovaniec, Greenberger, & Cohen, 1979). HL-60 cells were treated with 30 μM MG, 15 μM HK and low doses of VD_3 (1 nM) or ATRA (20 nM). NBT reduction assay was performed on day 3. Low doses of VD_3 or ATRA did not cause significant changes in NBT reduction. The addition of MG or HK to low doses of VD_3 or ATRA markedly enhanced NBT reduction (Fig. 3). It is evident that both MG and HK enhanced HL-60 cell maturation initiated by low doses of VD_3 or ATRA.

3.2. Effects of MG, HK, VD_3 and ATRA on cell proliferation and apoptosis

The IC_{50} values (concentration causing 50% growth inhibition) of MG and HK on HL-60 cells were determined by MTT assay (data not shown). The IC_{50} values for 96 h MG and HK treatments were 99.41 μM and 32.99 μM, respectively. During all subsequent experiments, we employed 10–20 μM MG/HK since concentrations within this range significantly enhance VD_3- or ATRA-induced HL-60 cell differentiation (Fig. 2A and B).

As shown in Fig. 4A, starting with 5.0×10^4 cells/ml, MG at 10 μM or 20 μM alone or in combination with 1 nM VD_3 did not significantly affect the growth of HL-60 cells. On the other hand, when added to ATRA at the above concentrations, MG caused a clear growth inhibition (Fig. 4B). HK at 10 μM and 20 μM, alone or in combination with VD_3 or ATRA, inhibited HL-60 cell growth at days 3 and 4 of treatment (Fig. 4C and D), and these effects appeared to be due to HK alone rather than in concert with VD_3 or ATRA. DNA content of treated cells was analyzed by flow cytometry and our results showed no evidence suggesting the involvement of apoptotic cell death (data not shown).

3.3. Effect of MG, HK, VD_3 and ATRA on the cell cycle and $p27^{Kip1}$ expression

It has been reported that VD_3- and ATRA-induced HL-60 cell differentiation is associated with G_0/G_1 arrest and a concomitant reduction of S phase cells (Daniel, Parreira, Goldman, & McCarthy, 1987; Yen,
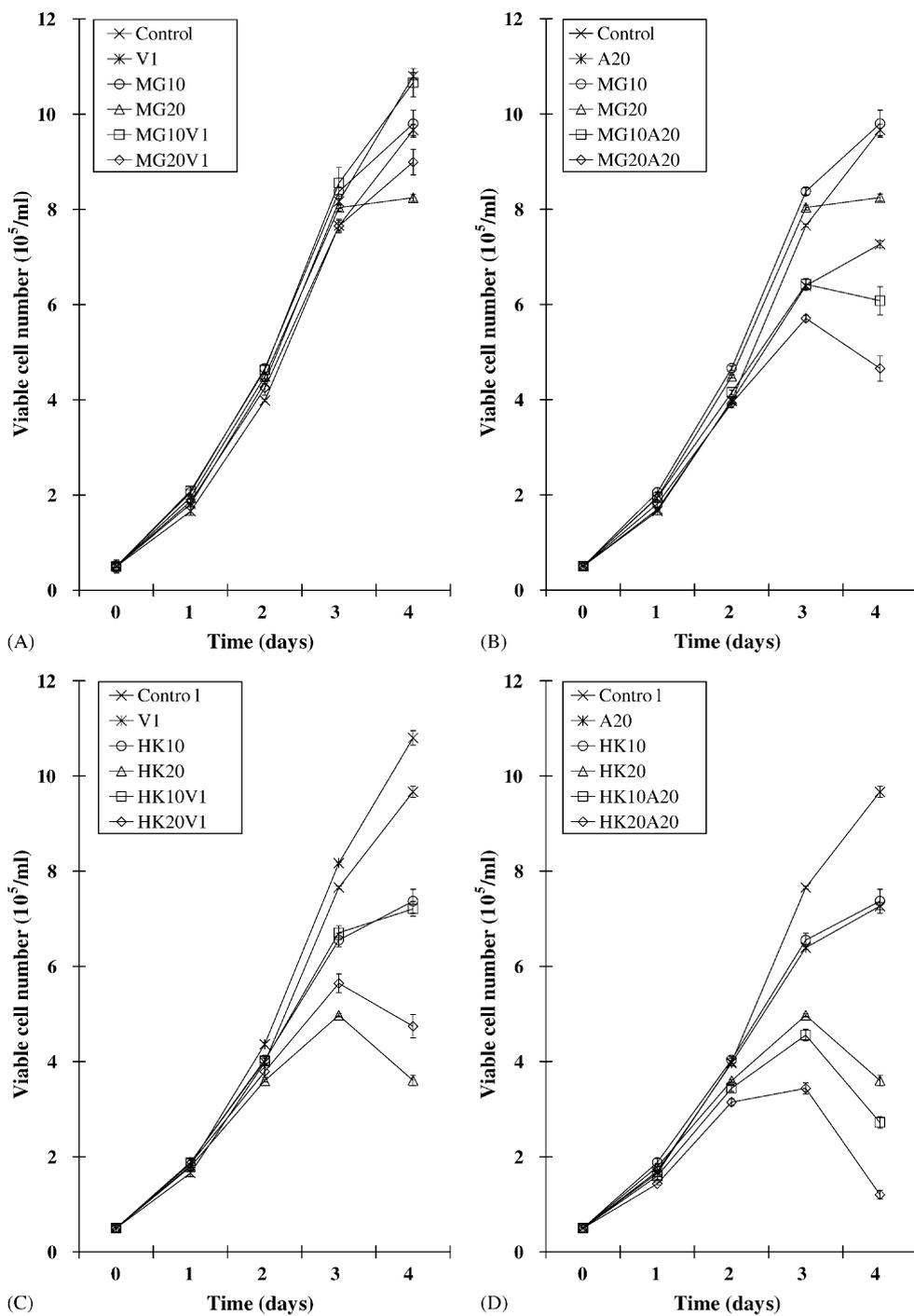
Fig. 4. Effects of magnolol and honokiol on HL-60 cell growth in the presence of VD$_3$ or ATRA. HL-60 cells were incubated for indicated time with various concentrations of MG and HK with (A and C) 1 nM VD$_3$ or (B and D) 20 nM ATRA. Aliquots of sample were taken on the days indicated to perform viable cell counts. Results are mean ± standard derivation of triplicate determinations.

Table 1

Flow cytometric analysis of cell cycle distribution of cells treated with various concentrations of MG, HK, VD$_3$ or 20 nM ATRA for 96 h

Treatment (96 h)	Cell cycle distribution (%)		
	G$_1$	S	G$_2$/M
Control (no treatment)	41.17 ± 3.65	42.53 ± 3.01	16.30 ± 0.91
MG (10 μM)	32.87 ± 1.15a	47.40 ± 2.40	19.74 ± 2.02
MG (20 μM)	35.43 ± 2.30	43.25 ± 1.47	21.32 ± 1.13b
HK (10 μM)	43.30 ± 1.55	40.90 ± 1.64	15.80 ± 0.49
HK (20 μM)	51.45 ± 5.97	35.78 ± 4.21	12.77 ± 1.76a
VD$_3$ (1 nM)	42.81 ± 2.62	40.27 ± 1.96	16.92 ± 1.38
MG10 + V1	39.64 ± 2.58	37.83 ± 1.76	22.52 ± 1.09
MG20 + V1	49.76 ± 2.55c	30.73 ± 0.91d	19.51 ± 1.64d
HK10 + V1	53.31 ± 2.28d	32.36 ± 1.61d	14.33 ± 0.69c
HK20 + V1	64.24 ± 3.87d	21.39 ± 3.68d	14.38 ± 0.72c
ATRA (20 nM)	51.83 ± 1.36	33.45 ± 1.01	14.70 ± 0.39
MG10 + A20	53.46 ± 0.71	31.29 ± 0.46e	15.26 ± 0.62
MG20 + A20	55.17 ± 1.00e	28.26 ± 0.06f	16.57 ± 0.96e
HK10 + A20	61.46 ± 1.69f	22.00 ± 0.40f	16.53 ± 1.30
HK20 + A20	52.30 ± 1.15	15.37 ± 0.70f	32.33 ± 1.85f

Results are mean ± standard derivation of triplicate determinations. (a and b) Statistically significant differences from no treatment control at $p < 0.05$ and $p < 0.01$, respectively. (c and d) Statistically significant differences from 1 nM VD$_3$ alone at $p < 0.05$ and $p < 0.01$, respectively. (e and f) Statistically significant differences from 20 nM ATRA alone at $p < 0.05$ and $p < 0.01$, respectively.

Reece, & Albright, 1985). We analyzed cell cycle distribution in PI-labeled HL-60 cells by flow cytometry. Treatment with 10 μM or 20 μM MG for 96 h reduced G$_0$/G$_1$ cells with a concomitant increase in S phase cells (Table 1) whereas HK at 20 μM induced a marginal increase in the G$_0$/G$_1$ fraction. Combining VD$_3$ or ATRA with MG (20 μM) or HK (10 μM or 20 μM) also increased the percentage of G$_0$/G$_1$ cells with a concomitant reduction of S phase cells.

p27^{Kip1} is an inhibitor of both cyclin E-cdk2 and cyclin A-cdk2 that regulates G$_1$/S transition and is involved in G$_0$/G$_1$ phase arrest during haematopoietic cell differentiation (reviewed in Furukawa, 2002). Western blot analysis showed a substantial increase in p27^{Kip1} protein levels when 20 μM MG or HK was added to 1 nM VD$_3$ (Fig. 5). The higher doses of MG and HK for the increase in p27^{Kip1} protein expression were probably the result of the difference in the degree of maturation. In ATRA-treated cells, a less prominent effect on p27^{Kip1} protein levels was shown with either MG or HK.

3.4. Effects of MEK inhibitors

In mammals, all three major mitogen-activated protein kinases (MAPK) pathways have been suggested to be associated with myeloid cell differentiation (Barr & Bogoyevitch, 2001; English et al., 1999; Martin-Blanco, 2000). We investigated the involvement of the MEK (extracellular signal-regulated protein kinases), JNK (c-Jun NH$_2$-terminal kinases), and p38 MAPK pathways using their respective inhibitors PD098059, SP600125 and SB203580. Cell differentiation was assessed by the NBT reduction assay. As illustrated in Fig. 6A, PD098059 significantly blocked both drug-induced differentiation and the enhancing effects of MG or HK. SB203580, on the other hand, augmented NBT reduction under almost all conditions of drug treatment (Fig. 6B). SP600125 did not show any significant effect (Fig. 6C). Results in Fig. 7 demonstrate a dose-dependent inhibitory effect of PD098059.

Taken together, our data suggested that while the MEK pathways are important for drug-induced differentiation and the enhancement effects of MG or HK, the p38 MAPK pathway is inhibitory and the JNK pathway is unimportant.

3.5. Co-operative effects of MG and HK in the promotion of drug-induced differentiation

MG and HK coexist in Magnoliae cortex and often coexist in other plants (Fujita et al., 1973; Teng et al., 1988; Yahara et al., 1991). Since they may share common biological activities, we investigated whether
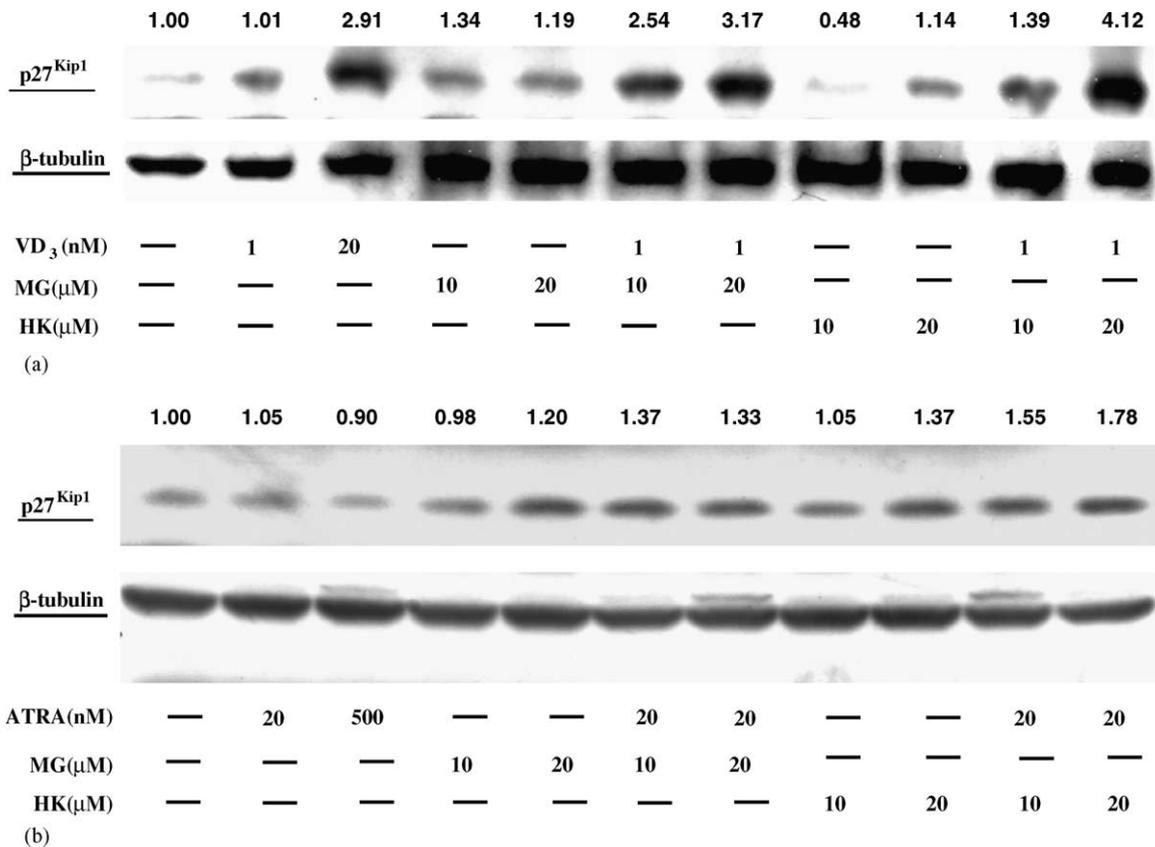
Fig. 5. Effects of magnolol, honokiol, VD_3 or ATRA on p27^{Kip1} protein level. HL-60 cells were treated for 96 h, followed by Western blot analysis with anti-p27^{Kip1} antibody. Densitometric values for the p27^{Kip1} (normalized to β-tubulin and the vehicle control) are shown *above each lane*. These results are representative of two independent experiments.

there is any synergy between them. Cells were treated with MG and/or HK for 72 h and differentiation was assessed by NBT reduction assay. The addition of 10 μM MG or 10 μM HK to VD_3 or ATRA treatment enhanced cell differentiation as before (Fig. 8). However, a more pronounced enhancement effect on differentiation was observed when 10 μM MG and 10 μM HK were simultaneously added to VD_3 or ATRA (Fig. 8). The enhancement effect could be observed at MG:HK ratios of 2:1, 1:2, 4:1, 1:4, 6:1, and 1:6 (data not shown).

4. Discussion

We have demonstrated that at μM levels, MG and HK enhanced differentiation in HL-60 cells induced by 1 nM VD_3 or 20 nM ATRA. The toxicities of MG

and HK are relatively low and at <30 μM they were non-toxic to HL-60 cells. Other cells, such as normal neutrophils and peripheral blood mononuclear cells, are even more tolerant to MG and IC_{50} values in these cells are 15-fold higher than in HL-60 cells (Zhong et al., 2003). In animals, HK was found to be non-toxic and well tolerated in therapeutically beneficial doses (Bai et al., 2003).

Our results showed that MG or HK had substantial cytostatic rather than cytotoxic effect on HL-60 cells, which was noted previously by Hirano, Gotoh, & Oka (1994). High concentrations of MG (>50 μM) inhibits colon and liver cancer cell growth in association with G_0/G_1 arrest and an elevated expression of the cyclin-dependent kinase inhibitor p21^{WAF1} (Lin et al., 2002). We observed that HK alone at 20 μM, but not MG, induced G_0/G_1 arrest (Table 1) and also an increased

expression of another cyclin-dependent kinase inhibitor p27^{Kip1} (Fig. 5). Notable increases in G_0/G_1 cells and p27^{Kip1} expression were observed when MG or HK was added to VD$_3$ or ATRA. The p27^{Kip1} protein expression seemed to be associated more with the action of VD$_3$ rather than ATRA (Fig. 5). In this and one of our previous studies, p27^{Kip1}, but not p21^{WAF1}, expression was up-regulated during HL-60 cell differentiation (Zhang, Fong, Wu, Yang, & Cheung, 2003). p21^{WAF1} plays an important role in G_1/S transition and its expression might require the participation of p53 (el-Deiry et al., 1993). It has been suggested that HL-60 is p53-negative (Wolf & Rotter, 1985) and work verifying p53 status in our HL-60 cell line is underway.

MAPK pathways are known to be involved in "non-genomic" differentiation pathway in VD$_3$- and ATRA-treated leukemia cells. Treatment of HL-60 cells with either VD$_3$ or ATRA leads to a rapid and sustained activation of MEK (Marcinkowska, Wiedlocha, & Radzikowski, 1997; Miranda, McGuire, & Johnson, 2002). PD098059 or U0126, which are known inhibitors of MEK, inhibit VD$_3$- and ATRA-induced HL-60 differentiation (Marcinkowska, 2001; Miranda et al., 2002). In line with the above, we showed that PD098059 also inhibited differentiation enhancing effects of MG or HK (Fig. 6A and Fig. 7). However, at exceptionally high concentrations (>80 μM), MG or HK may reduce the phosphorylation levels of MEK (Bai et al., 2003; Yang et al., 2003).

On the contrary, inhibition of p38 MAPK pathway induces granulocytic differentiation of HL-60 cells (Ishii, Sakai, & Honma, 2001; Zhang, Zhuang, Poon, Yang, & Fong, 2003) and also augments VD$_3$- and ATRA-dependent HL-60 cell maturation and differentiation (Alsayed et al., 2001; Wang, Rao, & Studzinski, 2000, and Fig. 6B this study). The p38 MAPK inhibitor SB203580 also up-regulates MEK in HL-60 cells (Ishii et al., 2001; Wang et al., 2000), but curiously this may be partially neutralized by MG and HK. The p38 MAPK pathway is activated by a large number of unique signals including environmental stress and toxins, cellular injury, growth factors, and cytokines (Kumar, Boehm, & Lee, 2003; Obata, Brown, & Yaffe, 2000).

c-Jun N-terminal protein kinase (JNK) signaling is not essential for ATRA-induced differentiation (Battle, Roberson, Zhang, Varvayanis, & Yen, 2001; Yen, Roberson, & Varvayanis, 1999) but may participate in monocytic differentiation of HL-60G cells induced by VD$_3$. The blockade of JNK signaling leads to a marked decrease in differentiation of HL-60 cells (Wang, Wang, & Studzinski, 2003). However, in the present study, the JNK inhibitor SP600125 had no effect on the actions of MG or HK, or on VD$_3$-induced differentiation (Fig. 6C). The cell line HL-60G is particularly selected for its VD$_3$ sensitivity and it is conceivable that additional molecular alterations had taken place which may account for the discrepancy observed.

MG and HK have been reported to trigger a variety of cell signaling events involving protein kinase B/Akt (Bai et al., 2003), Src tyrosine kinase (Bai et al., 2003), MAP kinases (Bai et al., 2003; Yang et al., 2003), protein kinase C (Wang, Lin, Hsu, & Chen, 1998), caspases activation (Chen, Wu, Hsiao, & Yen, 2003; Yang et al., 2002, 2003; Zhong et al., 2003), calcium response (Lin et al., 2001; Wang & Chen, 1998; Yamahara, Miki, Matsuda, & Fujimura, 1986; Zhai, Nakade, Mitsumoto, & Fukuyama, 2003) and oxidative stress (Liou, Shen, Chen, Tsao, & Tsai, 2003a,b; Park et al., 2003; Shen, Sung, & Chen, 1998). At the present time, it is only possible to assume that some of these molecular signals may be involved in the differentiation enhancing effect of MG and HK. Further work to clarify this is underway.

One mechanism to be considered is that MG and HK might be able to enhance the sensitivity of HL-60 cells to receptor mediated genomic pathway. The first candidate in this category are nuclear receptors for Vitamin D (VDR) and 9-*cis* RA (RXR) which are able to form homo- (VDR–VDR) or heterodimeric (VDR–RXR) complexes that activate or repress target gene expression related to cell differentiation (reviewed in Banerjee & Chatterjee, 2003). Another nuclear factor to be considered is nuclear factor-kappa B (NF-κB), an essential transcription factor in the control of expression of the cytokine-induced genes in immune and inflammatory responses (reviewed in Li & Stark, 2002). NF-κB may be involved in the transcription activity of Vitamin D response element (VDRE) and over-expression of NF-κB subunits may suppressed VD$_3$-stimulated transcription (Farmer, He, Schmitz, Rubin, & Nanes, 2000). Phosphorothioate antisense oligonucleotide to the p65 subunit of NF-κB enhances the HL-60 cell differentiation induced by VD$_3$ (Sokoloski, Narayanan, & Sartorelli, 1998). NF-κB is constitutively activated

(A)

(B)

(C)
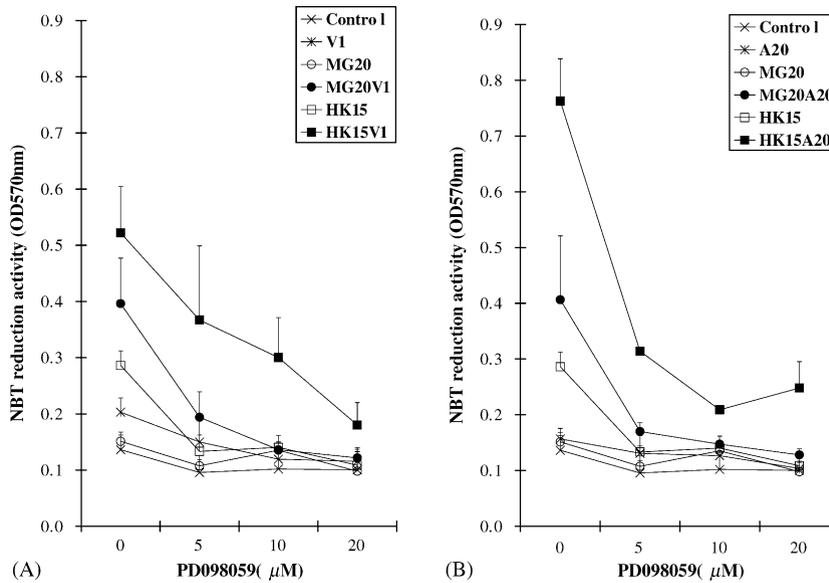
Fig. 7. Dose-dependent effects of the MEK inhibitor PD098059 on VD$_3$- or ATRA-induced differentiation of HL-60 cells and the effects of MG and HK. HL-60 cells were treated for 72 h with (A) VD$_3$ (1 nM) or (B) ATRA (20 nM) with MG (20 μM), HK (15 μM), and various concentrations (as indicated) of the MEK inhibitor PD098059. NBT reducing activity was determined and results are mean ± standard derivation of triplicate determinations.
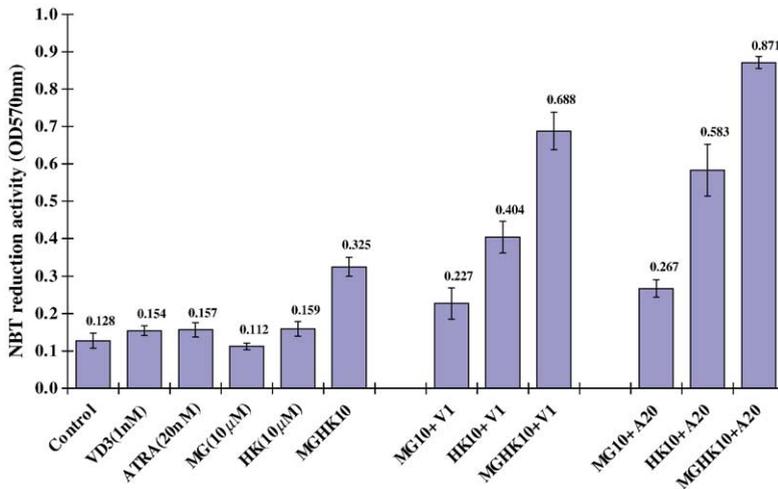
Fig. 8. Magnolol and honokiol cooperatively enhance cell differentiation induced by VD$_3$ and ATRA. HL-60 cells were incubated for 72 h with various concentrations of MG, HK, MGHK (MG and HK in combination) with 1 nM VD$_3$ or 20 nM ATRA. NBT reducing activity was determined and results are mean ± standard derivation of triplicate determinations and the mean values are shown *above each bar*.

Fig. 6. Effects of MAP kinase inhibitors on VD$_3$- or ATRA-induced differentiation of HL-60 cells and the effects of MG and HK. HL-60 cells were treated for 72 h with solvent control, VD$_3$ (1 nM), ATRA (20 nM), MG (20 μM), HK (15 μM), (A) the MEK inhibitor PD098059 (20 μM), (B) the p38 MAPK inhibitor SB203580 (10 μM), and (C) the JNK inhibitor SP600125 (10 μM). NBT reducing activity was determined and results are mean ± standard derivation of triplicate determinations. Statistically significant from the corresponding group treated with various MAP kinase inhibitors: $^*p < 0.01$ and $^{**}p < 0.001$.

in human myelogenous leukemia cells including HL-60 (Baumgartner et al., 2002; Frankenberger et al., 1994; Griffin, 2001; Guzman et al., 2001). In HL-60 cells, many compounds such as curcumin (Sokoloski, Shyam, et al., 1997), vitamin E and other antioxidants (Sokoloski, Hodnick, et al., 1997), costunolide (Kim, Kang, Kim, & Kim, 2002) and parthenolide (Kang et al., 2002) have been shown to enhance VD$_3$-induced differentiation with a concomitant inhibition of intrinsic NF-κB activity. MG at a concentration of 5 μM was shown to inhibit tumor necrosis factor-α induced NF-κB activation in human aortic endothelial cells (Chen, Lin, Chen, Ku, & Chen, 2002). Our most recent data show that both MG and HK, at doses leading to differentiation enhancement, inhibited the intrinsic NF-κB activities in VD$_3$-treated HL-60 cells (data not shown).

In summary, the results presented here show that MG and HK enhance VD$_3$- and ATRA-induced HL-60 cell differentiation via MEK pathway. These results suggest a possible clinical application of MG and HK in differentiation-inducing therapy for acute promyelocytic leukemia.

Acknowledgements

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