

## The anti-metastatic efficacy of $\beta$ -ionone and the possible mechanisms of action in human hepatocarcinoma SK-Hep-1 cells

Chin-Shiu Huang<sup>1\*</sup>, Shih-Chieh Lyu<sup>2</sup>, Jen-Yin Chen<sup>3</sup> and Miao-Lin Hu<sup>2\*</sup>

<sup>1</sup>Department of Health and Nutrition Biotechnology, Asia University, Taichung 41354, Taiwan, ROC

<sup>2</sup>Department of Food Science and Biotechnology, National Chung Hsing University, Taichung 40227, Taiwan, ROC

<sup>3</sup>Department of Anesthesiology, Chi Mei Medical Center, Tainan, Taiwan, ROC

(Received 21 January 2011 – Revised 26 May 2011 – Accepted 26 May 2011 – First published online 26 July 2011)

### Abstract

$\beta$ -Ionone (BI), a precursor for carotenoids, is widely distributed in fruit and vegetables. Recent *in vitro* studies have demonstrated the potential anti-metastatic effects of BI, but the mechanisms underlying such actions are not clear. Because liver cancer is the most endemic cancer in Taiwan and in a large region of the world, we hereby investigate the anti-metastatic effects of BI and its mechanisms of actions in a highly metastatic human hepatocarcinoma SK-Hep-1 cells. We show that incubation of cells with BI (1–50  $\mu$ M) for 24 and 48 h significantly inhibited cell invasion, migration and adhesion. Mechanistically, incubation of cells with BI (1–50  $\mu$ M) for 24 h resulted in the following: (1) significant inhibition of matrix metalloproteinase (MMP)-2, MMP-9 and urokinase-type plasminogen activator activities, (2) up-regulation of protein expression of the tissue inhibitor of matrix metalloproteinase (TIMP)-1, TIMP-2 and plasminogen activator inhibitor-1, (3) down-regulation of the expression of migration-related proteins, including focal adhesion kinase (FAK), phosphorylated form of FAK, Rho, Rac1 and Cdc42 and (4) up-regulation of the expression of nm23-H1 protein ( $P < 0.05$ ). Overall, the results show that BI effectively inhibits the metastasis of SK-Hep-1 cells, and this effect involves the regulation of gene expression and signal pathways related to invasion and migration.

**Key words:**  $\beta$ -Ionone: Metastasis: Nm23-H1: Focal adhesion kinase: Rho GTPase

Hepatocellular carcinoma is the major cause of cancer death in Taiwan and the most endemic cancer in a large region of the world. Tumour metastasis, both intrahepatic and extrahepatic, is a major factor of mortality in hepatocellular carcinoma patients. Tumour cell metastasis is characteristic of tumour progression involving complex processes including the ability to dissolve the basement membrane and the extracellular matrix (ECM) and to migrate through the ECM. The degradative process is mediated largely by matrix metalloproteinases (MMP), cathepsins and plasminogen activator systems<sup>(1)</sup>. MMP-2, MMP-9 and urokinase-type plasminogen activator (uPA) are the most vital proteases for degradation of base membrane and, therefore, are deeply involved in cancer metastasis<sup>(1–3)</sup>. MMP-2 (72 kDa) and MMP-9 (92 kDa) activities are regulated extracellularly, and their regulations are primarily affected by the balance of pro-enzyme activation and inhibition by tissue inhibitors of matrix metalloproteinase (TIMP), TIMP-2 and TIMP-1, respectively<sup>(3–5)</sup>. In addition, serine protease uPA is a protease that cleaves the ECM and activates the conversion of plasminogen to plasmin<sup>(6)</sup>. The conversion of plasminogen

to active plasmin is regulated by two specific and fast-acting plasminogen activator inhibitors (PAI), PAI-1 and PAI-2, with PAI-1 being more important<sup>(7)</sup>. The inhibition of MMP and uPA activity has been adopted as an anti-metastasis therapeutic strategy.

Focal adhesion kinase (FAK) is the most extensively studied focal adhesion protein in hepatocellular carcinoma<sup>(8)</sup>. FAK is a non-receptor tyrosine kinase that is involved in ECM/integrin-mediated signalling pathways, and has been suggested to play an essential role in metastasis through the modulation of tumour cell adhesion, migration and invasion<sup>(9)</sup> probably by the regulation of MMP<sup>(10)</sup>. Inhibition of FAK leads to reduced secretion of MMP-9 in carcinoma cells, and this effect is associated with the selective loss of an invasive cell phenotype<sup>(11)</sup>. FAK has also been shown to regulate cell migration by modulating the assembly and disassembly of the actin cytoskeleton through its effects on the Rho subfamily of small GTPase, a member of the Ras superfamily of small (approximately 21 kDa) GTPase<sup>(9)</sup>. Rho GTPase, which comprises Rho, Cdc42 and Rac1, is involved in various cellular functions

**Abbreviations:** BC,  $\beta$ -carotene; BI,  $\beta$ -ionone; DMEM, Dulbecco's minimal essential medium; ECM, extracellular matrix; FAK, focal adhesion kinase; FAK-p, phosphorylated form of focal adhesion kinase; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; RXR, retinoid X receptor; TIMP, tissue inhibitor of matrix metalloproteinase; uPA, urokinase-type plasminogen activator.

\* **Corresponding authors:** M.-L. Hu, fax +886 4 2281 2363, email mlhuhu@dragon.nchu.edu.tw; C.-S. Huang, fax +886 4 2332 1126, email cshuang@asia.edu.tw

such as cell growth, division, morphology, polarity and migration<sup>(12)</sup>. Furthermore, altered expression of the putative metastasis suppressor gene *nm23-H1* is considered to be an important step during the acquisition of metastatic ability<sup>(13)</sup>.

Epidemiological studies have suggested that elevated intakes of fruit and vegetables are associated with a reduced risk of several types of cancer, and these effects have drawn attention to the possibility that biologically active plant secondary metabolites exert anti-carcinogenic activity<sup>(14)</sup>. Isoprenoids, which are widely distributed in fruits, vegetables and grains, are a class of phytochemicals that encompasses approximately 22 000 individual components<sup>(15)</sup>.  $\beta$ -Ionone (BI), a cyclic isoprenoid, is a precursor for carotenoids, some of which exert anti-carcinogenic and anti-tumour activities *in vitro* and *in vivo* such as induction of cell-cycle arrest in various types of cancer cells<sup>(16–22)</sup>. We have recently shown that  $\beta$ -carotene (BC), which has a similar ionone ring structure to BI, exhibits anti-metastatic effects both *in vitro* and *in vivo*<sup>(2,13)</sup>. Lin *et al.*<sup>(23)</sup> have reported that BI exerts inhibitory effects on the proliferation of SGC-7901 human gastric adenocarcinoma cells and up-regulates TIMP-1 and TIMP-2 mRNA expression. However, it is unclear whether BI may exert anti-metastatic effects in hepatic cancer cells. Therefore, in the present study, we employed a highly invasive human hepatocarcinoma, the SK-Hep-1 cells, to examine the effects of BI on cell invasion, migration and adhesion as well as the possible mechanisms underlying these actions.

## Materials and methods

### Materials

The cell line SK-Hep-1 (BCRC 67005) was purchased from the Food Industry Research and Development Institute, Hsin Chu, Taiwan. All chemicals used were of reagent or higher grade. BI (97%; Acros organics, Morris Plains, NJ, USA) was delivered to the cell using ethanol (99%; Sigma, St Louis, MO, USA) solvent. BC was delivered to the cell using tetrahydrofuran (Merck, Germany) as solvent. Dulbecco's minimal essential medium (DMEM), fetal bovine serum, trypsin, penicillin, streptomycin, sodium pyruvate, non-essential amino acid and Giemsa stain were from GIBCO/BRL (Gaithersburg, MD, USA). Transwells were from Costar (Cambridge, MA, USA). Matrigel<sup>®</sup>, anti-human-nm23-H1, anti-Cdc42, anti-Rac1 and anti-Rho mouse monoclonal antibodies were from BD Biosciences (San Diego, CA, USA). Anti-TIMP-2, anti-PAI-1, anti-FAK and anti- $\beta$ -actin monoclonal antibodies and anti-mouse IgG horseradish peroxidase were purchased from Santa Cruz Biotechnology Company (Santa Cruz, CA, USA). The phosphorylated form of FAK (FAK-p, Y397), TIMP-1 rabbit monoclonal antibodies and anti-rabbit IgG horseradish peroxidase were purchased from Epitomics (Burlingame, CA, USA).

### Cell culture and $\beta$ -ionone incorporation

SK-Hep-1 cells were grown in DMEM containing 10% (v/v) fetal bovine serum, 0.37% (w/v) NaHCO<sub>3</sub>, penicillin (100 units/ml) and streptomycin (100 units/ml) in a humidified

incubator under 5% CO<sub>2</sub> and 95% air at 37°C. The cells were harvested at approximately 90% confluence (10<sup>6</sup> cells/dish). The survival rate of cells was always higher than 95% by trypan blue assay<sup>(24)</sup>. A stock BI solution (50 mM) and a BC solution (10 mM) were prepared freshly before each experiment.

### Cell migration assay

Tumour cell migration was assayed in transwell chambers (Costar) according to the methods reported by Repesh<sup>(25)</sup> with some modifications. Briefly, transwell chambers (Costar) with 6.5 mm-polycarbonate filters of 8  $\mu$ m pore size were used. After pre-incubation with BI or BC for 24 and 48 h, SK-Hep-1 cells (5  $\times$  10<sup>5</sup> cells/ml) were finally suspended in DMEM (100  $\mu$ l, serum free) and placed in the upper transwell chamber, and then incubated for 5 h at 37°C. After incubation for 5 h at 37°C, the cells on the upper surface of the filter were completely wiped away with a cotton swab. The cells on the lower surface of the filter were fixed in methanol, stained with Giemsa, and then counted under a microscope. For each replicate, the tumour cells in ten randomly selected fields were determined, and the counts were averaged. The percentage inhibition of invasion was calculated by the following formula: (1 – (treatment/control))  $\times$  100.

### Cell invasion assay

The procedure reported by Repesh<sup>(25)</sup> for the cell invasion assay was similar to cell migration. The invasion of tumour cells was assessed in transwell chambers with a 6.5 mm diameter polyvinylpyrrolidone-free polycarbonate filter of 8  $\mu$ m pore size. Each filter was coated with 100  $\mu$ l of a 1:20 diluted Matrigel<sup>®</sup> in cold DMEM to form a thin continuous film on the top of the filter. After pre-incubation with BI or BC for 24 and 48 h, SK-Hep-1 cells (5  $\times$  10<sup>5</sup> cells/ml) were suspended in DMEM (100  $\mu$ l, serum free) and placed in the upper transwell chamber, and then incubated for 24 h at 37°C. After incubation for 24 h, cells were stained and counted as described earlier, and the number of cells invading the lower side of the filter was measured as the invasive activity. For each replicate, the tumour cells in ten randomly selected fields were determined, and the counts were averaged. The percentage inhibition of invasion was calculated by the aforementioned formula.

### Cell adhesion assay

The procedure reported by Yang *et al.*<sup>(26)</sup> for the cell adhesion was used. The twenty-four-well plates were coated with 100  $\mu$ l of 1:20 diluted Matrigel<sup>®</sup> in cold DMEM to form a thin continuous film and dried in a laminar hood overnight. Cells were adjusted to 5  $\times$  10<sup>4</sup> cells/well in DMEM containing 1–50  $\mu$ M-BI and incubated at 37°C for 24 and 48 h. After the incubation, cells were washed twice in PBS and then incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for 1 h, after which the supernatant was removed and dimethyl sulfoxide was added to dissolve the solid residue

cells. Optical density at 570 nm of each well was then determined by using a microplate reader (FLUOstar OPTIMA; BMG Labtechnologies GmbH, Offenburg, Germany).

### Gelatin zymography

MMP-2 and MMP-9 activities were assayed using gelatin zymography according to the methods described previously<sup>(27)</sup>. The cells ( $5 \times 10^4$  cells/ml) were treated with BI for 24 h in DMEM containing 10% (v/v) fetal bovine serum and incubated for 24 h at 37°C in serum-free DMEM, and then the culture medium was harvested and stored at -20°C until use. For the assay of gelatin zymography, the culture medium of an appropriate volume (adjusted by viable cell number) was used to avoid the possible effect of BI on cell viability, as adopted from the approach reported by Yang *et al.*<sup>(27)</sup>. Then, the gel was electrophoresed in a 10% SDS-PAGE gel containing 0.1% (w/v) gelatin. The gel (MMP-gel) was washed for 30 min at room temperature in a solution containing 2.5% (v/v) Triton X-100 with two changes and subsequently transferred to a reaction buffer for enzymatic reaction containing 1% NaN<sub>3</sub>, 10 mM-CaCl<sub>2</sub> and 40 mM-Tris-HCl (pH 8.0) at 37°C with shaking overnight (for 15 h). Finally, the MMP-gel was stained for 30 min with 0.25% (w/v) Coomassie blue in 10% acetic acid (v/v) and 50% methanol (v/v) and de-stained in 10% acetic acid (v/v) and 50% methanol (v/v). The relative MMP-2 and MMP-9 activities were quantified by Matrox Inspector 2.1 software (Matrox Imaging, Dorval, QC, Canada).

### Casein-plasminogen zymography

The procedure for the casein-plasminogen zymography, which was adopted from that reported by Yoon *et al.*<sup>(28)</sup>, was similar to that of gelatin zymography as described earlier. The culture medium (20 μl) were separated by electrophoresis in 10% SDS-PAGE gel containing 1 mg/ml of casein (Sigma) and 13 μg/ml of human plasminogen (Sigma) under non-reducing conditions. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min, incubated with reaction buffer (1% NaN<sub>3</sub>, 10 mM-CaCl<sub>2</sub> and 40 mM-Tris-HCl, pH 8.0) for 15 h at 37°C, and stained with Coomassie blue G-250. The relative uPA activities were quantified by Matrox Inspector 2.1 software.

### Western blotting

TIMP-1, TIMP-2, PAI-1, FAK, FAK-p, Rho GTPase and nm23-H1 protein levels were assayed using Western blotting as described previously<sup>(27)</sup>. Total cellular proteins were prepared in lysis buffer containing 20% SDS and 1 mM-phenylmethyl sulfonyl fluoride. The lysate was sonicated for 30 s on ice, followed by centrifugation for 30 min at 4°C. The protein concentrations of extracts were determined by the Bio-Rad assay as outlined by the manufacturer (Bio-Rad, Hercules, CA, USA). The relative protein levels were quantified by Matrox Inspector 2.1 software.

### Statistical analysis

Values are expressed as means and standard deviations and analysed by using one-way ANOVA followed by Duncan's multiple range test for comparisons of group means. The statistical analysis was performed using SPSS for Windows, version 10 (SPSS, Inc., Chicago, IL, USA). A *P* value <0.05 was considered statistically significant.

## Results

### β-Ionone inhibits cell invasion, migration and adhesion in vitro

Table 1 shows that incubation of SK-Hep-1 cells with BI (1–50 μM) for 24 and 48 h resulted in the concentration-dependent inhibition of cell invasion, and the longer incubation time (48 h) caused a somewhat stronger inhibition than the shorter incubation time (24 h). No further increase in inhibition was observed, when BI concentrations reached 20 μM, which inhibited cell invasion by 40% (*P*<0.001). Similarly, BI caused a concentration-dependent inhibition of cell migration and adhesion, with a 42% (*P*<0.001) inhibition of cell migration and a 22% (*P*<0.001) inhibition of cell adhesion at 20 μM-BI, but the extent of inhibition was somewhat lower at the incubation time of 48 h than at 24 h. BC (10 μM) also significantly inhibited cell invasion (35 and 49% at 24 and 48 h, respectively), migration (34% at 48 h) and adhesion (30 and 32% at 24 and 48 h, respectively). Based on the time-course experiment, we chose an incubation time of 24 h for BI in the following studies.

### β-Ionone inhibits the activities of matrix metalloproteinase-2, -9 and urokinase-type plasminogen activator

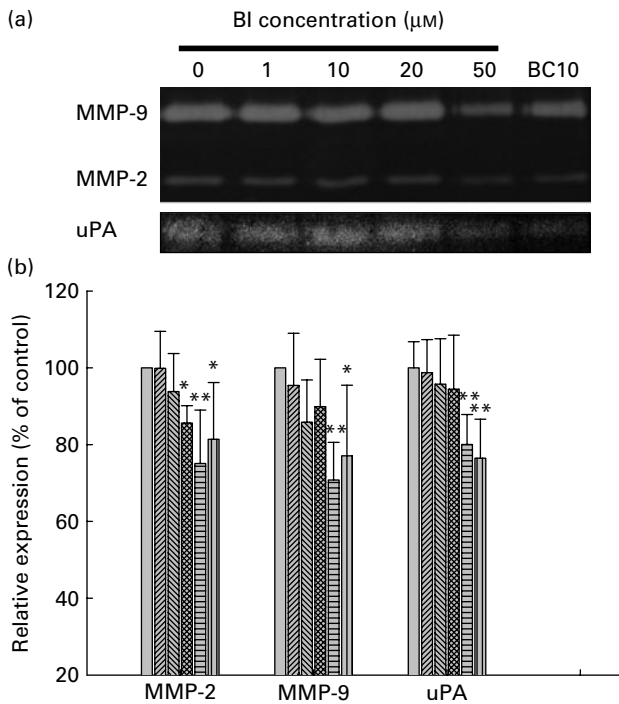
Western blots (Fig. 1(a)) show that BI (1–50 μM) inhibited the activities of MMP-2, MMP-9 and uPA at 24 h incubation.

**Table 1.** Effects of β-ionone (BI) and β-carotene (BC) on cell invasion, migration and adhesion of SK-Hep-1 cells† (Mean values and standard deviations, *n* ≥ 3)

| Treatments  | Inhibition of invasion (%) |    | Inhibition of migration (%) |    | Inhibition of adhesion (%) |    |
|-------------|----------------------------|----|-----------------------------|----|----------------------------|----|
|             | Mean                       | SD | Mean                        | SD | Mean                       | SD |
| <b>24 h</b> |                            |    |                             |    |                            |    |
| Control     | 0                          |    | 0                           |    | 0                          |    |
| BI 1 μM     | 9                          | 4  | 8                           | 15 | 18*                        | 3  |
| BI 10 μM    | 25*                        | 13 | 31*                         | 7  | 19*                        | 4  |
| BI 20 μM    | 40**                       | 16 | 42**                        | 2  | 22**                       | 4  |
| BI 50 μM    | 43**                       | 8  | 49***                       | 1  | 28**                       | 4  |
| BC 10 μM    | 35*                        | 7  | 7                           | 25 | 30**                       | 4  |
| <b>48 h</b> |                            |    |                             |    |                            |    |
| Control     | 0                          |    | 0                           |    | 0                          |    |
| BI 1 μM     | 1                          | 6  | 9                           | 1  | 11                         | 2  |
| BI 10 μM    | 31**                       | 8  | 24*                         | 2  | 12                         | 3  |
| BI 20 μM    | 53***                      | 4  | 37**                        | 6  | 14                         | 1  |
| BI 50 μM    | 54***                      | 8  | 39**                        | 5  | 20*                        | 12 |
| BC 10 μM    | 49***                      | 1  | 34**                        | 15 | 31**                       | 16 |

Mean values were significantly different from those of control: \**P*<0.05; \*\**P*<0.001; \*\*\**P*<0.0001.

† Cells were pre-incubated with BI (1, 10, 20 and 50 μM) or BC (10 μM) for 24 and 48 h. Ethanol (1%) is the solvent for BI.



**Fig. 1.** Effects of  $\beta$ -ionone (BI) and  $\beta$ -carotene (BC) on the matrix metalloproteinase (MMP)-2, MMP-9 and urokinase-type plasminogen activator (uPA) activity in SK-Hep-1 cells. Cells were pre-incubated with BI (1, 20 and 50  $\mu\text{M}$ ) or BC (10  $\mu\text{M}$ ) for 24 h. Ethanol (1%) is the solvent for BI. (a) Zymography of MMP-2, MMP-9 and uPA. (b) Densitometric analysis of (a). Values are means, with standard deviations represented by vertical bars ( $n \geq 3$ ). Mean values were significantly different from those of control: \* $P < 0.05$ ; \*\* $P < 0.005$ .  $\square$ , BI 0  $\mu\text{M}$ ;  $\square$ , BI 1  $\mu\text{M}$ ;  $\square$ , BI 10  $\mu\text{M}$ ;  $\square$ , BI 20  $\mu\text{M}$ ;  $\square$ , BI 50  $\mu\text{M}$ ;  $\square$ , BC 10  $\mu\text{M}$ .

When added at 50  $\mu\text{M}$ , BI inhibited the activities of MMP-2, MMP-9 and uPA by 25% ( $P < 0.005$ ), 29% ( $P < 0.005$ ) and 20% ( $P < 0.005$ ), respectively (Fig. 1(b)). BC (10  $\mu\text{M}$ ) also significantly inhibited the activities of MMP-2, MMP-9 and uPA by 19, 23 and 24%, respectively.

***$\beta$ -Ionone increases the protein expression of tissue inhibitor of matrix metalloproteinase-1, -2 and plasminogen activator inhibitor-1***

Western blots show (Fig. 2(a)) that incubation of SK-Hep-1 cells with BI (1–50  $\mu\text{M}$ ) for 24 h resulted in concentration-dependent increases in protein levels of TIMP-1, TIMP-2 and PAI-1, the endogenous inhibitors of MMP-9, MMP-2 and uPA. Although the highest increase in TIMP-1, TIMP-2 and PAI-1 levels all occurred at 50  $\mu\text{M}$ -BI (66%,  $P < 0.005$ ; 254%,  $P < 0.0001$ ; 175%,  $P < 0.0001$ ), there were no significant differences in these protein levels between 20 and 50  $\mu\text{M}$ -BI (Fig. 2(b)). BC (10  $\mu\text{M}$ ) also significantly increased the protein expression of TIMP-1, TIMP-2 and PAI-1 by 48, 159 and 100%, respectively.

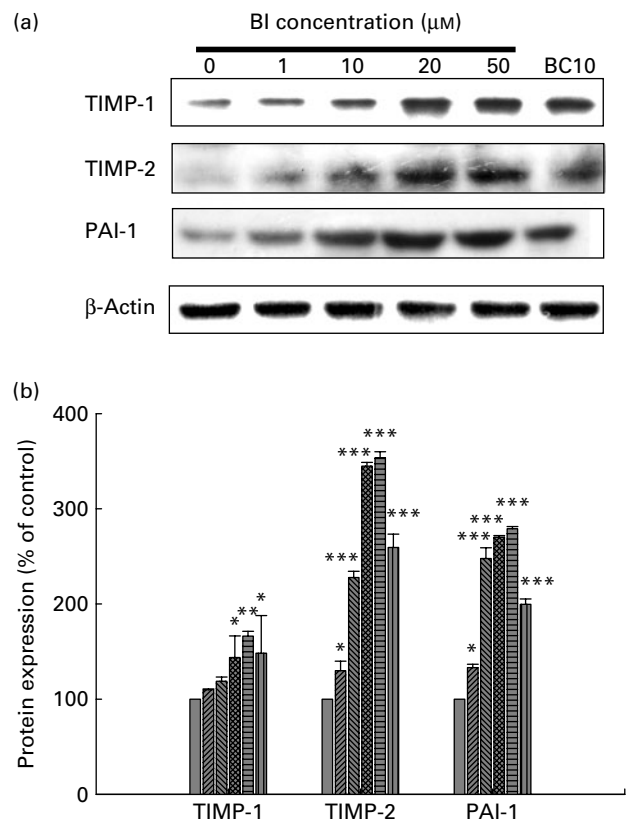
***$\beta$ -Ionone inhibits the protein expression of focal adhesion kinase and phosphorylated focal adhesion kinase***

Western blots show that BI (20 and 50  $\mu\text{M}$ ) significantly inhibited the protein expression of FAK and FAK-p at 24 h

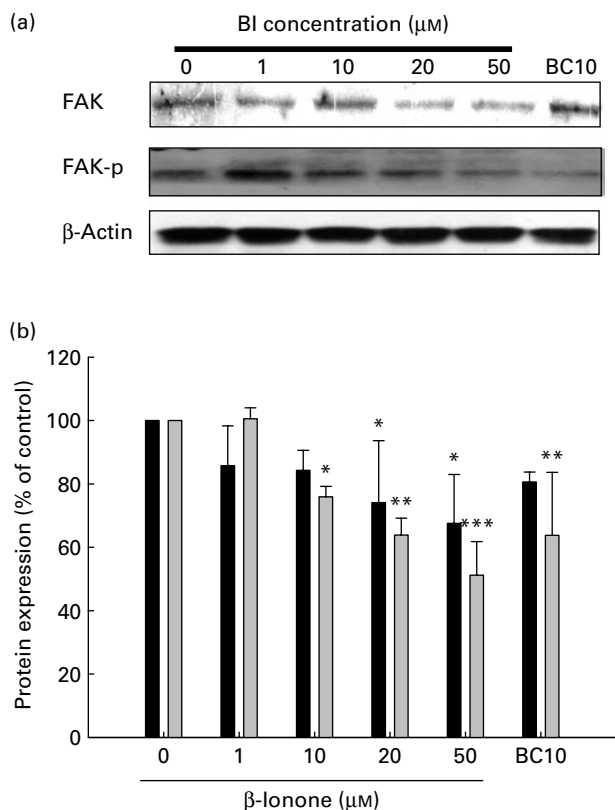
incubation (Fig. 3(a)), with 32% ( $P < 0.05$ ) and 49% ( $P < 0.0001$ ) inhibition, respectively, at 50  $\mu\text{M}$ -BI (Fig. 3(b)). There was no significant difference between 20 and 50  $\mu\text{M}$ -BI. BC (10  $\mu\text{M}$ ) also significantly inhibited the protein expression of FAK-p by 36%.

***$\beta$ -Ionone inhibits the protein expression of Rho GTPase***

To test whether BI inhibits cell migration through the down-regulation of Rho GTPases, we examined the levels of Rho, Rac1 and Cdc42 by Western blotting. We showed that BI (10–50  $\mu\text{M}$ ) significantly inhibited the protein expression of Rho, Rac1 and Cdc42 at 24 h incubation (Fig. 4(a)), with 46% ( $P < 0.001$ ), 42% ( $P < 0.0001$ ) and 36% ( $P < 0.0001$ ) inhibition, respectively, at 50  $\mu\text{M}$ -BI (Fig. 4(b)). BC (10  $\mu\text{M}$ ) also significantly inhibited the protein expression of Rho, Rac1 and Cdc42 by 39, 40 and 13%, respectively. In addition, there were negative correlations between the protein expression and migration for Rho ( $r^2$  0.97,  $P = 0.002$ ), Rac1 ( $r^2$  0.97,  $P = 0.002$ ) and Cdc42 ( $r^2$  0.91,  $P = 0.012$ ) in SK-Hep-1 cells (data not shown).



**Fig. 2.** Effects of  $\beta$ -ionone (BI) and  $\beta$ -carotene (BC) on tissue inhibitor of matrix metalloproteinase (TIMP)-1, TIMP-2 and plasminogen activator inhibitor (PAI)-1 protein expression in SK-Hep-1 cells. Cells were incubated with BI (1, 10, 20 and 50  $\mu\text{M}$ ) or BC (10  $\mu\text{M}$ ) for 24 h. Ethanol (1%) is the solvent for BI. (a) Western blots of TIMP-1, TIMP-2, PAI-1 and  $\beta$ -actin. (b) Densitometric analysis of (a). For loading control, expression levels of  $\beta$ -actin were analysed using the same lysate. Values are means, with standard deviations represented by vertical bars ( $n \geq 3$ ). Mean values were significantly different from those of control: \* $P < 0.05$ ; \*\* $P < 0.005$ ; \*\*\* $P < 0.0001$ .  $\square$ , BI 0  $\mu\text{M}$ ;  $\square$ , BI 1  $\mu\text{M}$ ;  $\square$ , BI 10  $\mu\text{M}$ ;  $\square$ , BI 20  $\mu\text{M}$ ;  $\square$ , BI 50  $\mu\text{M}$ ;  $\square$ , BC 10  $\mu\text{M}$ .



**Fig. 3.** Effects of β-ionone (BI) and β-carotene (BC) on focal adhesion kinase (FAK, ■)/phosphorylated form of FAK (FAK-p, □) protein expression in SK-Hep-1 cells. Cells were incubated with BI (1, 10, 20 and 50 μM) or BC (10 μM) for 24 h. Ethanol (1%) is the solvent for BI. (a) Western blots of FAK/FAK-p and β-actin. (b) Densitometric analysis of (a). For loading control, expression levels of β-actin were analysed using the same lysate. Values are means, with standard deviations represented by vertical bars ( $n \geq 3$ ). Mean values were significantly different from those of control: \* $P < 0.05$ ; \*\* $P < 0.005$ ; \*\*\* $P < 0.0001$ .

### β-Ionone increases the protein expression of nm23-H1

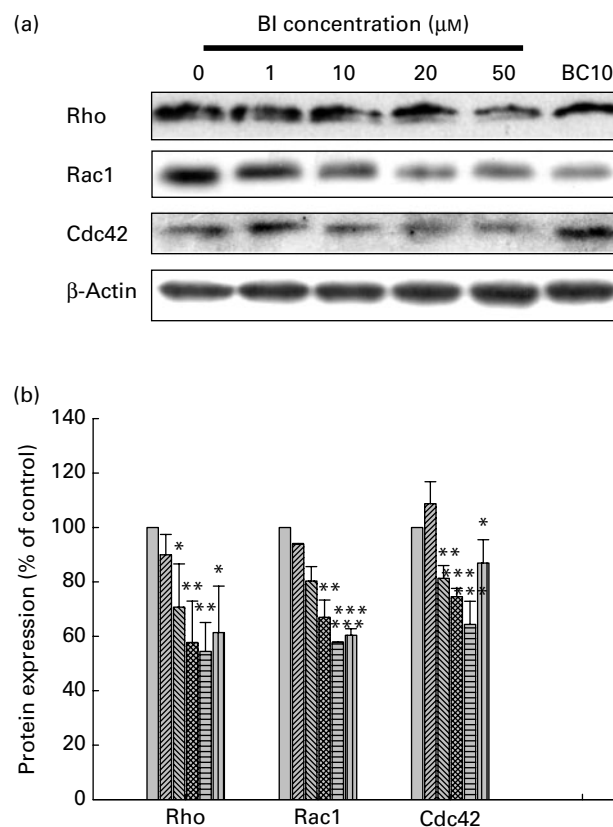
The expression of nm23-H1 protein was significantly increased by BI (10–50 μM; Fig. 5). At 50 μM, BI induced the highest expression of nm23-H1 protein (62%,  $P < 0.005$ ). BC (10 μM) also significantly increased the protein expression of nm23-H1 (55%,  $P < 0.005$ ). In addition, nm23-H1 protein expression was negatively correlated with migration ( $r^2$  0.90,  $P < 0.001$ ) and invasion ( $r^2$  0.89,  $P < 0.005$ ) in SK-Hep-1 cells (data not shown).

### Discussion

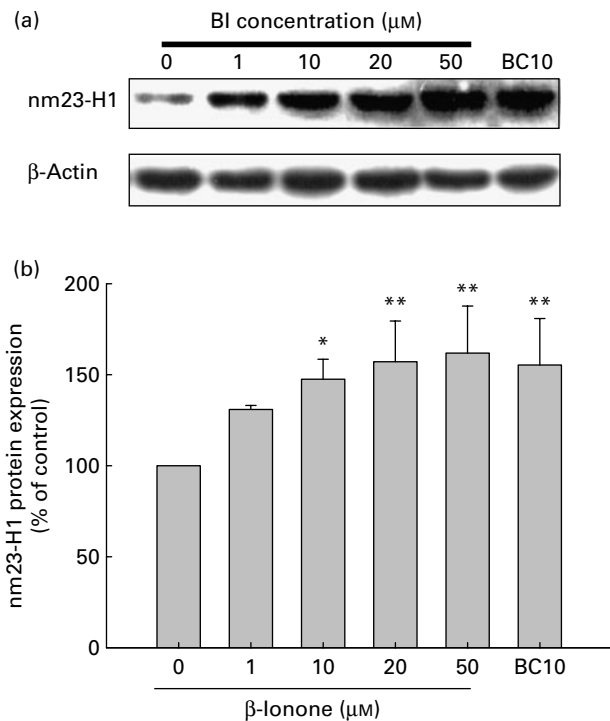
In the present study, we show that BI dose-dependently inhibited the metastasis of SK-Hep-1 cells, as indicated by decreased cell invasion, migration and adhesion. We further show that BI significantly and dose-dependently down-regulated the expression of MMP-2, MMP-9 and uPA, whereas it increased the expression of endogenous protease inhibitors, TIMP-2, TIMP-1 and PAI-1, respectively. These results demonstrate that BI is able to inhibit the *in vitro* metastatic activity of SK-Hep-1 cells.

Several probable mechanisms may be involved in the anti-metastatic actions of BI (Fig. 6); one is the inhibition of MMP activity, especially MMP-2 (gelatinase A) and MMP-9 (gelatinase B). Numerous studies have indicated that inhibition of MMP expression or enzyme activity can be used as early targets for preventing cancer metastasis<sup>(2,27,29)</sup>. The present study demonstrates that BI significantly inhibited the invasion, migration and adhesion of SK-Hep-1 cells and suppressed the activities of MMP-2, MMP-9 and uPA; the latter (uPA) is an upstream enzyme of MMP and an extremely specific serine protease that catalyses plasminogen degradation to plasmin<sup>(6)</sup>. Indeed, it has been suggested that the inhibition of MMP is of great promise with inhibitors as anti-tumour (anti-angiogenic, anti-proliferative and anti-metastatic) agents in preclinical models<sup>(29)</sup>.

Another possible anti-metastatic mechanism of BI is through increased protein expression of TIMP-1, TIMP-2 and PAI-1, as TIMP and PAI-1 have been shown to play an important role in the invasion and metastasis of cancerous cells<sup>(4,5,7)</sup>. The activities of MMP are inhibited by TIMP, which are specific inhibitors of MMP, and the imbalance between MMP and TIMP may promote degradation of the ECM<sup>(3–5)</sup>. Indeed, it has



**Fig. 4.** Effects of β-ionone (BI) and β-carotene (BC) on Rho GTPase protein expression in SK-Hep-1 cells. Cells were incubated with BI (1, 10, 20 and 50 μM) or BC (10 μM) for 24 h. Ethanol (1%) is the solvent for BI. (a) Western blots of Rho, Rac1, Cdc42 and β-actin. (b) Densitometric analysis of (a). For loading control, expression levels of β-actin were analysed using the same lysate. Values are means, with standard deviations represented by vertical bars ( $n \geq 3$ ). Mean values were significantly different from those of control: \* $P < 0.05$ ; \*\* $P < 0.001$ ; \*\*\* $P < 0.0001$ . □, BI 0 μM; ▤, BI 1 μM; ▥, BI 10 μM; ▦, BI 20 μM; ▧, BI 50 μM; ▨, BC 10 μM.



**Fig. 5.** Effects of  $\beta$ -ionone (BI) and  $\beta$ -carotene (BC) on nm23-H1 protein expression in SK-Hep-1 cells. Cells were incubated with BI (1, 10, 20 and 50  $\mu$ M) or BC (10  $\mu$ M) for 24 h. Ethanol (1%) is the solvent for BI. (a) Western blots of nm23-H1 and  $\beta$ -actin. (b) Densitometric analysis of (a). For loading control, expression levels of  $\beta$ -actin were analysed using the same lysate. Values are means, with standard deviations represented by vertical bars ( $n \geq 3$ ). Mean values were significantly different from those of control: \* $P < 0.05$ ; \*\* $P < 0.005$ .

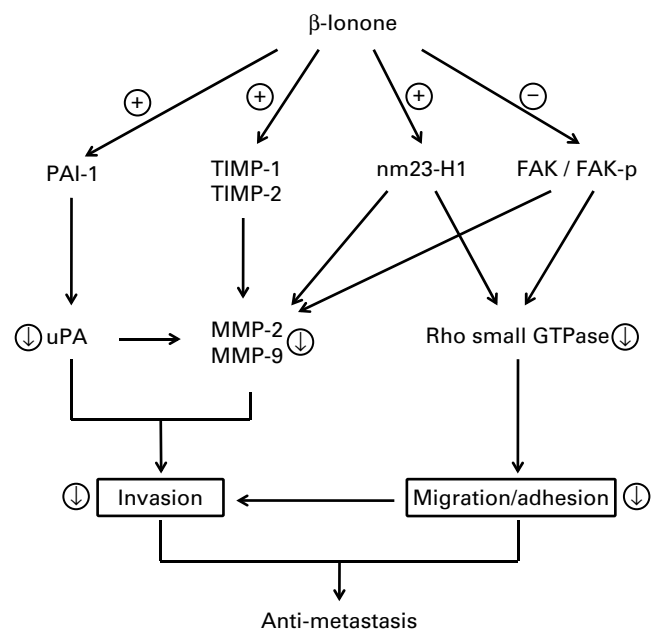
been shown that the imbalance between MMP and TIMP produced by tumour tissue may be a major determinant of the progression in hepatocarcinoma<sup>(30)</sup>. The transfection of TIMP-1 cDNA into HepG2 cells was shown to result in the suppression of metastasis potential of proliferation and invasion<sup>(31)</sup>. For instance, overexpression of TIMP-1 and TIMP-2 has been shown to inhibit the pulmonary metastasis in a rat model of bladder carcinoma<sup>(32)</sup>. The present findings demonstrate that the significantly increased TIMP-1 and TIMP-2 protein expression in SK-Hep-1 cells is probably an important anti-metastatic feature of BI.

Still another possible anti-metastatic mechanism of BI is the inhibition of cancer cell migration, as we demonstrated that BI significantly decreased the FAK signalling pathway, including down-regulation of FAK, FAK-p and Rho GTPase expression. FAK is an attractive therapeutic target because it is a key convergence point for many growth factor pathways required for survival and metastatic functions of cancer cells<sup>(8,9)</sup>. A well-understood regulation of FAK is phosphorylation, particularly tyrosine phosphorylation<sup>(9)</sup>. Phosphorylation of FAK on the Y397 site promotes the Src homology domain 2-dependent binding of Src family tyrosine kinases and the formation of an activated FAK(Y397)-Src complex<sup>(33)</sup>. Activated FAK(Y397)/Src signal transduction through multiple downstream targets, such as PI3K/AKT and Ras/ERK1/2 cascades, and the promotion of MMP-9 and Rho A signalling

mechanisms in cancer cells result in cellular invasion and cytoskeletal rearrangements, cellular adhesion and migration, respectively<sup>(34)</sup>. In addition to anti-metastasis, BI has been shown to down-regulate extracellular signal-regulated kinase and mitogen-activated protein kinase/ERK kinase expression, leading to reduced cancer cell proliferation<sup>(35)</sup>.

Rho GTPases are overexpressed in human tumours and are involved in a variety of cellular processes such as organisation of the actin cytoskeleton, cell-cell contact and malignant transformation<sup>(36,37)</sup>. Both Cdc42 and Rac1 promote actin polymerisation at the leading edge, and thereby the formation of filopodia and lamellipodia is required for carcinoma migration and invasion<sup>(38)</sup>. Rho induces the assembly and contraction of the actomyosin fibres, which contributes to pulling the trailing edge forwards during migration<sup>(39)</sup>. Here, we show that BI significantly decreased the expression of Rho, Cdc42 and Rac1, and these actions of BI were highly correlated with migration ( $r^2$  0.99,  $P < 0.0001$ ;  $r^2$  0.91,  $P = 0.012$ ;  $r^2$  0.97,  $P = 0.002$ , respectively) in SK-Hep-1 cells.

The decrease in Rho protein expression and MMP activity by BI may be mediated in part by its up-regulation of nm23-H1 protein expression, a tumour metastasis suppressor gene. The expression of MMP, including MMP-9 and MMP-2, has been shown to be down-regulated by nm23-H1 protein<sup>(40,41)</sup>. In addition, nm23-H1 has been reported to negatively regulate cell migration and tumour metastasis by modulating the activity of Cdc42 and other Rho family members<sup>(42)</sup>. Miyamoto *et al.*<sup>(43)</sup> have suggested that the binding of nm23-H1 to Tiam1 and Db1 activates Rac and Cdc42 small GTPase, respectively. The same authors<sup>(43)</sup> have also suggested that nm23 functions



**Fig. 6.** Proposed anti-metastatic mechanisms of  $\beta$ -ionone in SK-Hep-1 cells. ⊕, Promoted by  $\beta$ -ionone; ⊖, inhibited by  $\beta$ -ionone; ⊕, dependent inhibition as a result of activation or inactivation of upstream signalling molecules by  $\beta$ -ionone. PAI, plasminogen activator inhibitor; TIMP, tissue inhibitor of matrix metalloproteinase; FAK, focal adhesion kinase; FAK-p, phosphorylated form of focal adhesion kinase; uPA, urokinase-type plasminogen activator; MMP, matrix metalloproteinase.

as a negative regulator for cell motility and migration by binding to Rho-type specific guanine-nucleotide exchange factors and suppressing Rho GTPase.

An interesting observation of the present study is that the anti-metastatic actions and mechanisms of BI were similar to those of BC. It appears that the anti-metastatic effects of BI and BC may be related to their common chemical structure, i.e. an ionone ring. The chemical structure of BI is similar to that of 9-*cis*-retinoic acid, vitamin A and BC. Retinoids, which have significant anti-cancer effects, regulate gene transcription through two families of nuclear receptor, i.e. retinoic acid receptors and retinoid X receptors (RXR)<sup>(44)</sup>. It is well established that regulation of RXR- $\alpha$  may lead to several molecular/cellular changes, which in turn lead to reduced proliferation, migration and invasion and to enhanced apoptosis in cancer cells. Janakiram *et al.*<sup>(20)</sup> found that BI up-regulates the expression of RXR- $\alpha$  dose-dependently in human colon cancer cells, indicating that BI may act as an RXR agonist. It has been suggested that the use of RXR agonists in conjunction with pharmacological or genetic approaches to elevating RXR- $\alpha$  protein levels in target tumours may be effective therapies for cancers<sup>(45)</sup>. Further studies are needed to prove that BI may exert anti-metastatic effects through the RXR pathway.

In summary, we have demonstrated that BI effectively inhibits the metastasis of SK-Hep-1 cells *in vitro*, and that this effect involves the regulation of gene expression and signal pathways related to invasion and migration. The anti-metastatic potential of BI warrants further studies *in vivo*.

### Acknowledgements

The present study was funded by grants from the National Science Council, Taiwan, ROC (NSC98-2320-B-005-005-MY3 and NSC99-2320-B-468-001-MY3). There is no conflict of interest for any of the authors. C.-S. H. and M.-L. H. contributed equally in the study design, supervision of experimental execution, writing and revising of the manuscript; S.-C. L. performed the study; J.-Y. C. contributed to the statistical analysis and data interpretation. All authors read and approved the final manuscript.

### References

1. Ulisse S, Baldini E, Sorrenti S, *et al.* (2009) The urokinase plasminogen activator system: a target for anti-cancer therapy. *Curr Cancer Drug Targets* **9**, 32–71.
2. Huang CS, Liao JW & Hu ML (2008) Lycopene inhibits experimental metastasis of human hepatoma SK-Hep-1 cells in athymic nude mice. *J Nutr* **138**, 538–543.
3. Yu YM, Lin HC & Chang WC (2008) Carnosic acid prevents the migration of human aortic smooth muscle cells by inhibiting the activation and expression of matrix metalloproteinase-9. *Br J Nutr* **100**, 731–738.
4. Cairns RA, Khokha R & Hill RP (2003) Molecular mechanisms of tumor invasion and metastasis: an integrated view. *Curr Mol Med* **3**, 659–671.
5. Park SY, Lim SS, Kim JK, *et al.* (2010) Hexane-ethanol extract of *Glycyrrhiza uralensis* containing licoricidin inhibits the metastatic capacity of DU145 human prostate cancer cells. *Br J Nutr* **104**, 1272–1282.
6. Blasi F (1997) uPA, uPAR, PAI-1: key intersection of proteolytic, adhesive and chemotactic highways? *Immunol Today* **18**, 415–417.
7. Itoh T, Hayashi Y, Kanamaru T, *et al.* (2000) Clinical significance of urokinase-type plasminogen activator activity in hepatocellular carcinoma. *J Gastroenterol Hepatol* **15**, 422–430.
8. Yam JW, Tse EY & Ng IO (2009) Role and significance of focal adhesion proteins in hepatocellular carcinoma. *J Gastroenterol Hepatol* **24**, 520–530.
9. Zhao J & Guan JL (2009) Signal transduction by focal adhesion kinase in cancer. *Cancer Metastasis Rev* **28**, 35–49.
10. Mon NN, Ito S, Senga T, *et al.* (2006) FAK signaling in neoplastic disorders: a linkage between inflammation and cancer. *Ann N Y Acad Sci* **1086**, 199–212.
11. Mon NN, Hasegawa H, Thant AA, *et al.* (2006) A role for focal adhesion kinase signaling in tumor necrosis factor- $\alpha$ -dependent matrix metalloproteinase-9 production in a cholangiocarcinoma cell line, CCKS1. *Cancer Res* **66**, 6778–6784.
12. Etienne-Manneville S & Hall A (2002) Rho GTPases in cell biology. *Nature* **420**, 629–635.
13. Huang CS, Shih MK, Chuang CH, *et al.* (2005) Lycopene inhibits cell migration and invasion and upregulates Nm23-H1 in a highly invasive hepatocarcinoma, SK-Hep-1 cells. *J Nutr* **135**, 2119–2123.
14. Johnson IT (2007) Phytochemicals and cancer. *Proc Nutr Soc* **66**, 207–215.
15. Bach TJ (1995) Some new aspects of isoprenoid biosynthesis in plants – a review. *Lipids* **30**, 191–202.
16. Kim MO, Moon DO, Kang CH, *et al.* (2010) beta-Ionone enhances TRAIL-induced apoptosis in hepatocellular carcinoma cells through Sp1-dependent upregulation of DR5 and downregulation of NF- $\kappa$ B activity. *Mol Cancer Ther* **9**, 833–843.
17. Cardozo MT, de Conti A, Ong TP, *et al.* (2011) Chemopreventive effects of  $\beta$ -ionone and geraniol during rat hepatocarcinogenesis promotion: distinct actions on cell proliferation, apoptosis, HMGCoA reductase, and RhoA. *J Nutr Biochem* **22**, 130–135.
18. Mo H & Elson CE (1999) Apoptosis and cell-cycle arrest in human and murine tumor cells are initiated by isoprenoids. *J Nutr* **129**, 804–813.
19. Liu JR, Sun XR, Dong HW, *et al.* (2008) beta-Ionone suppresses mammary carcinogenesis, proliferative activity and induces apoptosis in the mammary gland of the Sprague–Dawley rat. *Int J Cancer* **122**, 2689–2698.
20. Janakiram NB, Cooma I, Mohammed A, *et al.* (2008) Beta-ionone inhibits colonic aberrant crypt foci formation in rats, suppresses cell growth, and induces retinoid X receptor- $\alpha$  in human colon cancer cells. *Mol Cancer Ther* **7**, 181–190.
21. Zhu J, Zhang L, Jin X, *et al.* (2010) beta-Ionone-induced apoptosis in human osteosarcoma (U2os) cells occurs via a p53-dependent signaling pathway. *Mol Biol Rep* **37**, 2653–2663.
22. Sun X, Liu J, Sun W, *et al.* (2007) Beta-ionone induced apoptosis in SGC-7901 cells. *Wei Sheng Yan Jiu* **36**, 667–670.
23. Lin JR, Chen BQ, Yang YM, *et al.* (2005) Inhibitory effect of potential metastasis in SGC-7901 cells induced by beta-ionone. *Wei Sheng Yan Jiu* **34**, 435–438.
24. Phillips HJ (1973) Dye exclusion tests for cell viability. In *Tissue Culture in Method and Applications*, pp. 406–408. London: Academic Press.
25. Repesh LA (1989) A new *in vitro* assay for quantitating tumor cell invasion. *Invas Metast* **9**, 192–208.

26. Yang CM, Liu YZ, Liao JW, *et al.* (2010) The *in vitro* and *in vivo* anti-metastatic efficacy of oxythiamine and the possible mechanisms of action. *Clin Exp Metastasis* **27**, 341–349.
27. Yang HL, Kuo YH, Tsai CT, *et al.* (2011) Anti-metastatic activities of *Antrodia campborata* against human breast cancer cells mediated through suppression of the MAPK signaling pathway. *Food Chem Toxicol* **49**, 290–298.
28. Yoon WH, Song IS, Lee BH, *et al.* (2004) Differential regulation of vimentin mRNA by 12-*O*-tetradecanoylphorbol 13-acetate and all-*trans*-retinoic acid correlates with motility of Hep 3B human hepatocellular carcinoma cells. *Cancer Lett* **203**, 99–105.
29. Libra M, Scalisi A, Vella N, *et al.* (2009) Uterine cervical carcinoma: role of matrix metalloproteinases (review). *Int J Oncol* **34**, 897–903.
30. Altadill A, Rodríguez M, González LO, *et al.* (2009) Liver expression of matrix metalloproteinases and their inhibitors in hepatocellular carcinoma. *Dig Liver Dis* **41**, 740–748.
31. Xia D, Yan LN, Xie JG, *et al.* (2006) Overexpression of TIMP-1 mediated by recombinant adenovirus in hepatocellular carcinoma cells inhibits proliferation and invasion *in vitro*. *Hepatobiliary Pancreat Dis Int* **5**, 409–415.
32. Kawamata H, Kawai K, Kameyama S, *et al.* (1995) Overexpression of tissue inhibitor of matrix metalloproteinases (TIMP1 and TIMP2) suppresses extravasation of pulmonary metastasis of a rat bladder carcinoma. *Int J Cancer* **63**, 680–687.
33. Schlaepfer DD, Hanks SK, Hunter T, *et al.* (1994) Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* **372**, 786–791.
34. Meng XN, Jin Y, Yu Y, *et al.* (2009) Characterisation of fibronectin-mediated FAK signalling pathways in lung cancer cell migration and invasion. *Br J Cancer* **101**, 327–334.
35. Liu JR, Yang YM, Dong HW, *et al.* (2005) Effect of beta-ionone in human mammary cancer cells (Er – ) by MAPK pathway. *Wei Sheng Yan Jiu* **34**, 706–709.
36. Grise F, Bidaud A & Moreau V (2009) Rho GTPases in hepatocellular carcinoma. *Biochim Biophys Acta* **1795**, 137–151.
37. Kim TY, Vigil D, Der CJ, *et al.* (2009) Role of DLC-1, a tumor suppressor protein with RhoGAP activity, in regulation of the cytoskeleton and cell motility. *Cancer Metastasis Rev* **28**, 77–83.
38. Keely PJ, Westwick JK, Whitehead IP, *et al.* (1997) Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* **390**, 632–636.
39. Guo W & Giancotti FG (2004) Integrin signalling during tumour progression. *Nat Rev Mol Cell Biol* **5**, 816–826.
40. Cheng S, Alfonso-Jaume MA, Mertens PR, *et al.* (2002) Tumour metastasis suppressor, nm23-beta, inhibits gelatinase A transcription by interference with transactivator Y-box protein-1 (YB-1). *Biochem J* **366**, 807–816.
41. Ohba K, Miyata Y, Koga S, *et al.* (2005) Expression of nm23-H1 gene product in sarcomatous cancer cells of renal cell carcinoma: correlation with tumor stage and expression of matrix metalloproteinase-2, matrix metalloproteinase-9, sialyl Lewis X, and c-erbB-2. *Urology* **65**, 1029–1034.
42. Murakami M, Meneses PI, Lan K, *et al.* (2008) The suppressor of metastasis Nm23-H1 interacts with the Cdc42 Rho family member and the pleckstrin homology domain of oncoprotein Dbl-1 to suppress cell migration. *Cancer Biol Ther* **7**, 677–688.
43. Miyamoto M, Iwashita S, Yamaguchi S, *et al.* (2009) Role of nm23 in the regulation of cell shape and migration via Rho family GTPase signals. *Mol Cell Biochem* **329**, 175–179.
44. Altucci L & Gronemeyer H (2001) The promise of retinoids to fight against cancer. *Nat Rev Cancer* **1**, 181–193.
45. Xiao JH, Ghosn C, Hinchman C, *et al.* (2003) Adenomatous polyposis coli (APC)-independent regulation of beta-catenin degradation via a retinoid X receptor-mediated pathway. *J Biol Chem* **278**, 29954–29962.