The anti-metastatic efficacy of β-ionone and the possible mechanisms of action in human hepatocarcinoma SK-Hep-1 cells

Chin-Shiu Huang1*, Shih-Chieh Lyu2, Jen-Yin Chen3 and Miao-Lin Hu2*

1Department of Health and Nutrition Biotechnology, Asia University, Taichung 41354, Taiwan, ROC
2Department of Food Science and Biotechnology, National Chung Hsing University, Taichung 40227, Taiwan, ROC
3Department 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
β-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 μM) for 24 and 48 h significantly inhibited cell invasion, migration and adhesion. Mechanistically, incubation of cells with BI (1–50 μM) 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: β-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. 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 metastasis1–3. 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, respectively4–5. In addition, serine protease uPA is a protease that cleaves the ECM and activates the conversion of plasminogen to plasmin6. 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 important7. 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 carcinoma8. 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 invasion9 probably by the regulation of MMP10. 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 phenotype11. 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(s). Rho GTPase, which comprises Rho, Cdc42 and Rac1, is involved in various cellular functions.

Abbreviations: BC, β-carotene; BI, β-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 2563, email mlhuhu@dragon.nchu.edu.tw; C.-S. Huang, fax +886 4 2352 1126, email cshuang@asia.edu.tw
such as cell growth, division, morphology, polarity and migration[12]. 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[14]. Iso-prenoids, which are widely distributed in fruits, vegetables and grains, are a class of phytochemicals that encompasses approximately 22,000 individual components[15]. β-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[16–22]. We have recently shown that β-carotene (BC), which has a similar ionone ring structure to BI, exhibits anti-metastatic effects both in vitro and in vivo[2,13]. Lin et al.[23] 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 tetrahydrofuran (97%; Acros organics, Morris Plains, NJ, USA) was delivered to the cell using ethanol (99%; Sigma, St Louis, MO, USA) as solvent. Dulbecco’s minimal essential medium (DMEM), fetal bovine serum, trypsin, penicillin, streptomycin, sodium pyruvate, non-essential amino acid and Gibsma stain were from GIBCO/BRL (Gaitherburg, MD, USA). Transwells were from Costar (Cambridge, MA, USA). Matrigel®, anti-human-mm23-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-β-actin monoclonal antibodies and anti-mouse IgG horseradish peroxidase were purchased from Santa Cruz Biotecology 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 β-ionone incorporation

SK-Hep-1 cells were grown in DMEM containing 10% (v/v) fetal bovine serum, 0.37% (w/v) NaHCO3, penicillin (100 units/ml) and streptomycin (100 units/ml) in a humidified incubator under 5% CO2 and 95% air at 37°C. The cells were harvested at approximately 90% confluence (10⁶ cells/dish). The survival rate of cells was always higher than 95% by trypan blue assay[24]. 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[25] with some modifications. Briefly, transwell chambers (Costar) with 6.5-mm-polycarbonate filters of 8 µm pore size were used. After pre-incubation with BI or BC for 24 and 48 h, SK-Hep-1 cells (5 × 10⁵ cells/ml) were finally suspended in DMEM (100 µl, 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)) × 100.

Cell invasion assay

The procedure reported by Repesh[25] 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 polystyrene filament-free polycarbonate filter of 8 µm pore size. Each filter was coated with 100 µl of a 1:20 diluted Matrigel® 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 × 10⁵ cells/ml) were suspended in DMEM (100 µl, 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.[26] for the cell adhesion was used. The twenty-four-well plates were coated with 100 µl of 1:20 diluted Matrigel® in cold DMEM to form a thin continuous film and dried in a laminar hood overnight. Cells were adjusted to 5 × 10⁴ cells/well in DMEM containing 1–50 µM-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-diphenyldetrazolium bro-mide 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\(^{(27)}\). The cells (5 x 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.\(^{(27)}\). Then, the gel was electrophoresed in a 10% SDS-PAGE gel containing 1 mg/ml of casein (Sigma). The relative protein levels were quantified by Matrox Inspec- tor 2.1 software. The culture medium (20 μl) was separated by electrophoresis for enzymatic reaction containing 1% NaNO\(_3\), 10 mm-CaCl\(_2\) 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% (v/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.\(^{(28)}\), was similar to that of gelatin zymography as described earlier. The culture medium (20 μl) was 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% NaNO\(_3\), 10 mm-CaCl\(_2\) 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\(^{(27)}\). Total cellular proteins were prepared in lysis buffer containing 20% SDS and 1 mm-phenylmethylsulfon 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>
<thead>
<tr>
<th>Treatments</th>
<th>Inhibition of invasion (%)</th>
<th>Inhibition of migration (%)</th>
<th>Inhibition of adhesion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BI 1 μM</td>
<td>9</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>BI 10 μM</td>
<td>25*</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>BI 20 μM</td>
<td>40**</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>BI 50 μM</td>
<td>43**</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>BC 10 μM</td>
<td>35*</td>
<td>7</td>
<td>25</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BI 1 μM</td>
<td>1</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>BI 10 μM</td>
<td>31**</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>BI 20 μM</td>
<td>53**</td>
<td>4</td>
<td>37**</td>
</tr>
<tr>
<td>BI 50 μM</td>
<td>54**</td>
<td>8</td>
<td>39**</td>
</tr>
<tr>
<td>BC 10 μM</td>
<td>49**</td>
<td>1</td>
<td>34**</td>
</tr>
</tbody>
</table>

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.
m-inhibited the protein expression of FAK and FAK-p at 24 h by 19, 23 and 24% respectively. BC (10 µM) also significantly inhibited the activities of MMP-2, MMP-9 and uPA respectively.

expression of TIMP-1, TIMP-2 and PAI-1 by 48, 159 and 100%, P (0.0001) inhibition respectively, at 50 µM-BI (Fig. 4(b)). BC (10 µM) 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 (r² = 0.97, P = 0.002), Rac1 (r² = 0.97, P = 0.002) and Cdc42 (r² = 0.91, P = 0.012) in SK-Hep-1 cells (data not shown).

**β-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 µM) 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 µM-BI (Fig. 4(b)). BC (10 µM) also significantly inhibited the protein expression of FAK-p by 36%.

**β-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 µM) 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 µM-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 µM-BI (Fig. 2(b)). BC (10 µM) also significantly increased the protein expression of TIMP-1, TIMP-2 and PAI-1 by 48, 159 and 100%, respectively.

**β-ionone inhibits the protein expression of focal adhesion kinase and phosphorylated focal adhesion kinase**

Western blots show that BI (20 and 50 µM) 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 µM-BI (Fig. 3(b)). There was no significant difference between 20 and 50 µM-BI. BC (10 µM) also significantly inhibited the protein expression of FAK-p by 36%.

**Fig. 1.** Effects of β-ionone (BI) and β-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 µM) or BC (10 µM) for 24 h. Ethanol (1%) is the solvent for BI. (a) Zymography of MMP-2, MMP-9 and uPA. (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 = 3). Mean values were significantly different from those of control: *P < 0.05; **P < 0.005. BI 0 µM; BI 1 µM; BI 10 µM; BI 20 µM; BI 50 µM; BC 10 µM.

When added at 50 µM, 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 µM) also significantly inhibited the activities of MMP-2, MMP-9 and uPA by 19, 23 and 24%, respectively.

**Fig. 2.** Effects of β-ionone (BI) and β-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 µM) or BC (10 µM) for 24 h. Ethanol (1%) is the solvent for BI. (a) Western blots of TIMP-1, TIMP-2, PAI-1 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 = 3). Mean values were significantly different from those of control: *P < 0.05; **P < 0.005; ***P < 0.0001. BI 0 µM; BI 1 µM; BI 10 µM; BI 20 µM; BI 50 µM; BC 10 µM.
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\(^{(2,27-29)}\). 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\(^{(60)}\). 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\(^{(29)}\).

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\(^{(4,5,7)}\). 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\(^{(3-5)}\). Indeed, it has 

**\(\beta\)-ionone increases the protein expression of nm23-H1**

The expression of nm23-H1 protein was significantly increased by BI (10–50 \(\mu\)M; Fig. 5). At 50 \(\mu\)M, BI induced the highest expression of nm23-H1 protein (62%, \(P<0.005\)). BC (10 \(\mu\)M) 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.

**\(\beta\)-ionone inhibits experimental metastasis**

635

\[\text{British Journal of Nutrition}\]

![Image](https://www.cambridge.org/core/content/image/fig1a.png)

**Fig. 3.** Effects of \(\beta\)-ionone (BI) and \(\beta\)-carotene (BC) on focal adhesion kinase (FAK, \(\beta\)-phosphorylated form of FAK (FAK-p, \(\Box\)) 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 FAK/FAK-p 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: *\(P<0.05\); **\(P<0.005\); ***\(P<0.0001\).

![Image](https://www.cambridge.org/core/content/image/fig2a.png)

**Fig. 4.** Effects of \(\beta\)-ionone (BI) and \(\beta\)-carotene (BC) on Rho GTPase 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 Rho, Rac1, Cdc42 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: *\(P<0.05\); **\(P<0.005\); ***\(P<0.0001\). 

\[\text{British Journal of Nutrition}\]
been shown that the imbalance between MMP and TIMP produced by tumour tissue may be a major determinant of the progression in hepatocarcinomu\textsuperscript{(30)}. The transfection of TIMP-1 cDNA into HepG2 cells was shown to result in the suppression of metastasis potential of proliferation and invasion\textsuperscript{(31)}. For instance, overexpression of TIMP-1 and the suppression of metastasis potential of proliferation and TIMP-1 cDNA into HepG2 cells was shown to result in formation of an activated FAK(Y397)–Src complex\textsuperscript{(33)}. Activation of the actin cytoskeleton, cell–cell contact and malignant transformation\textsuperscript{(36,37)}. 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\textsuperscript{(38)}. Rho induces the assembly and contraction of the actomyosin fibres, which contributes to pulling the trailing edge forwards during migration\textsuperscript{(39)}. 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\textsuperscript{(40,41)}. In addition, nm23-H1 has been reported to inhibit the pulmonary metastasis of Cdc42 and other Rho family members\textsuperscript{(42). Miyamoto et al.\textsuperscript{(43)} have suggested that the binding of nm23-H1 to Tiam1 and Db1 activates Rac and Cdc42 small GTPase, respectively. The same authors\textsuperscript{(43)} have also suggested that nm23 functions in cancer cells result in cellular invasion and cytoskeletal rearrangements, cellular adhesion and migration, respectively\textsuperscript{(34)}. 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\textsuperscript{(35)}.

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\textsuperscript{(36,37)}. 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\textsuperscript{(38)}. Rho induces the assembly and contraction of the actomyosin fibres, which contributes to pulling the trailing edge forwards during migration\textsuperscript{(39)}. 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\textsuperscript{(40,41)}. In addition, nm23-H1 has been reported to inhibit the pulmonary metastasis of Cdc42 and other Rho family members\textsuperscript{(42). Miyamoto et al.\textsuperscript{(43)} have suggested that the binding of nm23-H1 to Tiam1 and Db1 activates Rac and Cdc42 small GTPase, respectively. The same authors\textsuperscript{(43)} have also suggested that nm23 functions in cancer cells result in cellular invasion and cytoskeletal rearrangements, cellular adhesion and migration, respectively\textsuperscript{(34)}. 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\textsuperscript{(35)}.

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\textsuperscript{(36,37)}. 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\textsuperscript{(38)}. Rho induces the assembly and contraction of the actomyosin fibres, which contributes to pulling the trailing edge forwards during migration\textsuperscript{(39)}. 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.
β-Ionone inhibits experimental metastasis

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)\(^{140}\). It is well established that regulation of RXR-α 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.\(^{20}\) found that BI up-regulates the expression of RXR-α 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-α protein levels in target tumours may be effective therapies for cancers\(^{45}\). 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


