Abstract. A new approach to cancer therapy in recent years has been to target the metastatic process. The anti-metastatic potential of curcusone B, a diterpene isolated from Jatropha curcas Linn. (Euphorbiaceae), a herbal plant that has been used in traditional folk medicine in many tropical countries, was investigated against 4 human cancer cell lines. Treatment with non-cytotoxic doses of curcusone B resulted in a strong reduction of in vitro invasion, motility and secretion of matrix-metalloproteinases (MMP) of the cancer cells, whereas the ability to adhere to a Matrigel-coated surface was variably sensitive to curcusone B treatment. Curcusone B, thus, effectively suppresses the metastatic processes at doses that are non-toxic to cells, which may be of therapeutic benefit for the treatment of metastatic cancers.

Metastasis is a major cause of death in cancer patients. It is a complex, multi-step process, involving detachment of cancer cells from the primary site, induction and invasion into new blood vessels, exiting from the blood circulation and establishment of a new colony at distant sites. Much research effort in recent years has been directed towards disruption of any of the steps of the metastatic process. Preliminary studies have shown that targeting metastatic components has many advantages over traditional cancer therapy, such as high selectivity against cancer cells and lesser potential to develop resistance (1,2). Moreover, animal studies have shown that anti-metastatic therapy can be used in combination with conventional chemotherapy (2).

Many herbal plants are under investigation for their potential as anti-metastatic agents. Curcumin, a non-steroidal polyphenolic compound from Curcuma longa, has been shown to suppress invasion of SK-Hep-1 hepatocellular cells and B16F-10 melanoma cells at non-cytotoxic doses by inhibiting secretion of MMP-2 and MMP-9 (3,4). Similarly, (-)-epigallocatechin, a tea compound, has been shown to cause a marked reduction of HT1080 fibrosarcoma cell invasion by suppressing MMP secretion, with minimal effects on cell viability (5). Other natural products with minimal cytotoxic effects which are under investigation for their anti-metastatic potentials include resveratrol, a phytoalexin found in grapes, and genistein, a flavone compound of soy (6,7). Here, we investigated the anti-metastatic effects of curcusone B, a diterpene isolated from J. curcas, on 4 human cancer cell lines by examining in vitro invasiveness, motility, and the ability to secrete matrix-metalloproteinases and to adhere to matrigel-coated surfaces.

Materials and Methods

Preparation of curcusone B. Curcusone B (Figure 1) was purified from J. curcas as described by Naengchomnong et al. (8). A 100 mM stock solution of curcusone B in DMSO was stored at -20°C, and working concentrations were prepared by diluting the stock solution to the desired concentrations with 0.1% DMSO. For control, 0.1% DMSO without curcusone B was employed.

Cell lines and cell culture. KB (ATCC# CCL-17, human epidermal carcinoma, a derivative of Hela cervical carcinoma cells) and MCF-7 (ATCC# HTB-22, human breast adenocarcinoma) were kindly provided by Dr. Kanyawim Kiratikara, National Science and Technology Development Agency, Thailand. Hep3B (human hepatocellular carcinoma, ATCC# HB-8064) was a generous gift of Dr. Ravat Panvichien, Ramathibodi Hospital, Mahidol University, Thailand. KKU-100 (cholangiocarcinoma) was established from a Thai patient with Opisthorchiasis-associated cholangiocarcinoma (9), and was kindly provided by Dr. Banchob Sripa, Faculty of Medicine, Khon Kaen University, Thailand. KB and MCF-7 cells were maintained in DMEM, while Hep3B was maintained in MEM and KKU-100 was maintained in Ham F-12, supplemented with 10% fetal bovine serum (FBS), L-glutamine and 1X antibiotic-antimycotic solution (Life Technologies, Inc., Rockville, MD, USA). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air.

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In vitro invasion assay. Cancer cell invasiveness was determined using a transwell chamber (Costar, Cambridge, MA, USA) coated with 0.3 μg/μl Matrigel (Collaborative Research Inc., Bedford, MA, USA). Approximately, 1x10^5 cells in culture medium containing 10% FBS and various concentrations of curcusone B were added into the upper and lower compartment of the transwell, respectively, and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 6 h. The non-invading cells on the upper surface of the transwells were removed using cotton swabs. The cells that had invaded into the lower surface were fixed with methanol and stained with 0.5% crystal violet in 25% methanol for 1 h, before rinsing with tap water several times. The number of invaded cells were counted under light microscope in five random 10x microscopic fields and expressed as a percentage of control. The results shown represent mean±s.e.m. of the percentage of invaded cells relative to control from three independent experiments, each carried out in duplicate.

In vitro motility assay. The motility assay was performed in a similar fashion to the invasion assay, except no Matrigel coating was applied to the upper surface of the transwell filters. The number of invaded cells in five random 10x microscopic fields was counted and expressed as percentage of control. The results shown represent mean±s.e.m. of the percentage of invaded cells relative to control from three independent experiments, each carried out in duplicate.

Survival assay. One thousand to 5,000 cells in 100 μl culture media with 10% FBS and various concentrations of curcusone B were plated into 96-well plates and incubated at 37°C for 6 h in a humidified atmosphere containing 5% CO₂ before the number of live cells was determined using the MTT assay. Briefly, 10 μl of 5 mg/ml MTT solution was added to the culture, incubated for 3 h and the solution was aspirated. The MTT converted into insoluble formazan dye in live cells was then dissolved by addition of 200 μl of DMSO before the absorbance was read at 540 nm using a microtiter plate reader (Multiskan Ex,Thermo Labsystems, Helsinki, Finland) and expressed as percentage of control (OD₅₄₀ of control cells at 6 h in culture was expressed as 100%). The results shown represent mean±s.e.m. of the percentage of OD₅₄₀ of control cells at 6 h in culture was expressed as 100%). The results shown represent mean±s.e.m. of the percentage of OD₅₄₀ relative to control from three independent experiments, each carried out in triplicate.

Adhesion assay. Each well of 96-well plate was coated with 0.3 μg/μl Matrigel overnight and then incubated with 3% BSA for 2 h at room temperature, before addition of ~5,000 cells in culture medium containing 10% FBS and various concentrations of curcusone B. After incubation at 37°C for 6 h, the cells in suspension were aspirated, and those bound to Matrigel were washed twice with PBS before being stained with 0.5% crystal violet in 25% methanol for 15 min. After rinsing with tap water, the stained cells were counted under a microscope and expressed as percentage of control. The results shown represent mean±s.e.m. of the percentage of adherent cells relative to control from three independent experiments, each carried out in triplicate.

Gelatin zymography assay. Cells were starved by culturing in serum-free medium with various concentrations of curcusone B for 6 h before collection of the conditioned medium. The conditioned medium was assayed for gelatinase activity by gelatin zymography assay. Briefly, the conditioned medium was mixed with 2x SDS-sample buffer before separation in a 7.5 % SDS-PAGE containing 1 mg/ml gelatin. After electrophoresis at 200 V for 1 h, the gel was washed in a 2.5% Triton X-100 solution twice, 30 min each time. The gel was then incubated in buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM CaCl₂, 1 μM ZnCl₂, 1% Triton X-100 and 0.02% NaN₃ for 16-18 h in a 37°C water bath, after which the gel was stained with 0.25% Coomasie blue in 45% methanol and 10% acetic acid. After destaining, the clear band of gelatinolytic activity was quantitated using a Biorad GelDoc system (BIO-RAD Laboratories, Hercules, CA, USA).

Statistical analysis. The statistical significance of difference between each group of data was determined by one-way ANOVA. A p-value < 0.01 was considered significant.

Results

The anti-metastatic effects of curcusone B, a diterpene compound isolated from J. curcas, was assessed in vitro in 4 human cancer cell lines. Treatment with curcusone B caused a dose-dependent reduction of invasiveness in all 4 cell lines with similar inhibition profiles (Figures 2-5). The invasiveness of these cell lines was similarly sensitive to curcusone B, showing IC₅₀ values of 7.2±1.6, 10.5±3.7, 5.8±2.0 and 5.7±0.4 μM for KB, MCF-7, Hep3B and KU-100, respectively (Table I). Cell motility was also sensitive to curcusone B, showing IC₅₀ values of 4.9±3.9, 9.3±3.1, 11.0±3.3 and 7.9±0.9 μM for KB, MCF-7, Hep3B and KU-100, respectively (Table I). As in vitro invasiveness and motility were severely impaired by curcusone B treatment, we determined if this was due to the cytotoxic effect of curcusone B by monitoring cell survival during the 6-h treatment by MTT assay. Our results showed that cell survival was significantly less sensitive to curcusone B in this concentration range (p<0.01); the IC₅₀ values of cell survival were 2.60 (MCF-7)- to 4.86 (KB)-fold higher than the IC₅₀ values of cell invasiveness (Table I). The most severe cytotoxic effect was observed in Hep3B, which survived to 20% at 50 μM curcusone B (Figure 3), the concentration at which in vitro invasion and motility were almost completely suppressed.

Figure 1. Chemical structure of curcusone B.
As invasion is a multi-step process, initiated by attachment of cancer cells to the basement membrane, secretion of matrix-degrading enzymes and movement through the basement membrane and the underlying matrix, we began to dissect which of these processes were affected by curcusone B. The ability to adhere to a Matrigel-coated surface was dose-dependently reduced in all 4 cell lines, but with different degrees of severity. The adhesion of MCF-7 and Hep3B cells was markedly reduced by curcusone B (IC\textsubscript{50}=9.7±1.6 and 6.1±3.7 μM, respectively), whereas adhesion of KB and KKU-100 cells was moderately affected (IC\textsubscript{50}=25.9±11.8 and 31.7±12.5 μM, respectively).

By gelatin zymography, KB, MCF-7 and Hep3B cells expressed a very low to undetectable level of gelatinase activity, prohibiting us from further examining the effects of curcusone B in these cells. However, KKU-100 cells displayed a high level of MMP-2 activity at 62 Kd (Figure 6), which was reduced dose-dependently in response to curcusone B, showing IC\textsubscript{50} value of 4.68±0.73 μM.

**Discussion**

The present study demonstrates that curcusone B, a diterpene isolated from \textit{J. curcas}, exhibits potent anti-metastatic effects in 4 human cancer cell lines. The anti-metastatic activities encompassed inhibition of \textit{in vitro} invasion, motility, adhesion to matrix and MMP secretion, which occurred dose-dependently at concentrations which were not cytotoxic to the cells. Suppression of \textit{in vitro} invasion was principally due to impairment of cellular motility, as evidenced by the parallel reduction of motility and \textit{in vitro} invasion with similar IC\textsubscript{50} values, and the extent of suppression of both activities being similar in all 4 cell lines. The ability to secrete MMP from KKU-100 cells was also significantly impaired, showing similar IC\textsubscript{50} values to that of \textit{in vitro} invasion (IC\textsubscript{50} values of MMP secretion and \textit{in vitro} invasion were 4.68±0.73 μM and 5.7±0.4 μM, respectively), indicating that the ability to secrete MMP significantly attributes to the invasiveness of KKU-100 cells. Interestingly, the effects of curcusone B on cellular adhesion

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**Figure 2.** Effects of curcusone B on cell survival, cell adhesion, \textit{in vitro} invasion, and motility of KB cells. Data points shown are mean±s.e.m. of results expressed as percentage of control from three independent experiments. * Significantly different from control (p<0.01).

**Figure 3.** Effects of curcusone B on cell survival, cell adhesion, \textit{in vitro} invasion, and motility of Hep3B cells. Data points shown are mean±s.e.m. of results expressed as percentage of control from three independent experiments. * Significantly different from control (p<0.01).

**Figure 4.** Effects of curcusone B on cell survival, cell adhesion, \textit{in vitro} invasion, and motility of MCF-7 cells. Data points shown are mean±s.e.m. of results expressed as percentage of control from three independent experiments. * Significantly different from control (p<0.01).

**Figure 5.** Effects of curcusone B on cell survival, cell adhesion, \textit{in vitro} invasion, and motility of KKU-100 cells. Data points shown are mean±s.e.m. of results expressed as percentage of control from three independent experiments. * Significantly different from control (p<0.01).
Cells were incubated with various concentrations of curcusone B in serum-free media for 6 hours before conditioned media were collected and analyzed for gelatinase activities. Lane 1, protein marker; lane 2, 0 μM; lane 3, 1 μM; lane 4, 5 μM; lane 5, 10 μM; lane 6, 25 μM; lane 7, 50 μM of curcusone B, respectively.

Table I. Effects of curcusone B on survival, adhesion, invasion and motility of the cancer cells expressed as IC50 values.

<table>
<thead>
<tr>
<th>Cancer Cells</th>
<th>KB</th>
<th>Hep3B</th>
<th>MCF-7</th>
<th>KKU-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival</td>
<td>35.0±3.5</td>
<td>22.5±7.7</td>
<td>27.3±5.3</td>
<td>25.1±0.5</td>
</tr>
<tr>
<td>Adhesion</td>
<td>25.9±11.8</td>
<td>6.1±3.7</td>
<td>9.7±1.6</td>
<td>31.7±12.5</td>
</tr>
<tr>
<td>Invasion</td>
<td>7.2±1.6</td>
<td>5.8±2.0</td>
<td>10.5±3.7</td>
<td>5.7±0.4</td>
</tr>
<tr>
<td>Motility</td>
<td>4.9±3.9</td>
<td>11.0±3.3</td>
<td>9.3±3.1</td>
<td>7.9±0.9</td>
</tr>
</tbody>
</table>

Data shown are mean ± s.e.m. of three independent experiments. Each data point of the invasion and motility assays was performed in duplicate, whereas that of the survival and adhesion assays was performed in triplicate.

to matrix were variable among the 4 cell lines, suggesting the differential expression of, and/or dependence on, the molecular targets of this compound in the various cell types.

The cellular targets and molecular mechanism by which curcusone B exerts its inhibitory effects on cancer cells has not been elucidated. However, previous work has shown that the root extract of *J. curcas* demonstrated antidiarrheal and anti-inflammatory activities in animal models which were associated with inhibition of arachidonate metabolism (10,11). The metabolic products of arachidonic acid, either from the cyclooxygenase (COX) pathway or the lipoxygenase (LOX) pathway, have been shown to play important roles in various physiological and pathological processes. In malignant cells, these products have been shown to be overexpressed and involved in multiple steps of carcinogenesis, by modulating xenobiotic metabolism, apoptosis, immune response, angiogenesis, and invasion and metastasis (12-14). Thus, it is possible that the anti-metastatic effects displayed by curcusone B are, at least in part, accounted for by inhibition of arachidonic acid metabolism in the cancer cells. The molecular targets and mechanisms by which curcusone B exerts its anti-invasion/anti-metastasis effects on cancer cells are being further explored.

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References


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