

## Report

# Anti-Metastatic Effects of Curcumin Analogues in a Mouse Breast Cancer Model

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**Curcumin is a polyphenol compound derived from the roots of *Curcuma longa*. Although the biological activities of curcumin, such as its anti-inflammatory, anti-oxidant, anti-microbial and anti-cancer effects, have been well applied, its poor chemical stability is a major problem. Pentagamavunon-0 (PGV-0) and Pentagamavunon-1 (PGV-1) were developed as curcumin analogues with higher bioavailability; however, their anti-cancer activity has not yet been assessed. In this study, we evaluated the anti-metastatic activity of PGV-0 and PGV-1 in 4T1 breast cancer cells. Although both curcumin analogues demonstrated similar anti-proliferative effects to curcumin in 4T1 breast cancer cells, they did not inhibit nuclear factor kappa B (NF-κB) activity which is a well-defined molecular target of curcumin for its anti-cancer effects. As PGV-0 and PGV-1 exhibited stronger inhibition of the metastatic capacity in a 4T1 breast cancer model than curcumin, PGV-0 and PGV-1 may be promising curcumin analogues to target cancer metastasis having a distinct molecular mechanism from that of curcumin.**

**Key words** cancer, metastasis, curcumin, nuclear factor kappa B

## INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and the leading cause of death for women in most countries.<sup>1)</sup> Metastasis to distant organs is the major reason for the high mortality rate in breast cancer patients;<sup>2,3)</sup> therefore, the development of new therapeutic options for controlling breast cancer metastasis is required.<sup>4)</sup>

Phytomedicines have been used for medicinal purposes for many centuries and have been playing an important role in supporting human health.<sup>5)</sup> Curcumin is one of the most well-known phytomedicines that has been extensively examined for its medicinal use, including against cancer. However, due to its low chemical stability, the clinical use of curcumin has some limitations and there is a demand to explore new curcumin analogues with modification on its structure for increasing its stability.<sup>6,7)</sup> Recently, we found that Pentagamavunon 0 (PGV-0) and Pentagamavunon 1 (PGV-1) are curcumin analogues with better anti-inflammatory effects than curcumin.<sup>8,9)</sup> In the present study, we evaluated the utility of PGV-0 and PGV-1 based on their anti-cancer effects, particularly their anti-metastatic potential, using a murine 4T1 breast cancer model.

## MATERIALS AND METHODS

**Reagents and Cells** PGV-0 and PGV-1 were synthesized by Ritmaleni, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia.<sup>10)</sup> Curcumin was purchased from Nacalai Tesque (Kyoto, Japan). Each sample was dissolved in DMSO. Antibodies against p65 and β-actin were obtained from Santa Cruz

Biotechnology (Santa Cruz, CA, USA). An antibody against p-p65 was obtained from Cell Signaling Technology (CST Inc., Danvers, MA, USA). The luciferase gene-expressing 4T1 cells (4T1-luc2 or 4T1-NFκB-luc) were established as previously reported<sup>11,12)</sup> and maintained in RPMI-1640 medium (Nissui; Tokyo, Japan) supplemented with 10% bovine serum.

**Cell Viability Analysis** Cell viability was quantified using the WST-1 Cell Counting kit (Wako Pure Chemical Industries) according to the manufacturer's instruction. 4T1-NFκB-luc cells (2 x 10<sup>4</sup> cells/well) were seeded in a 96-well plate and incubated for 24 h prior to the treatment with test compounds. After the incubation with test compounds (24 h), WST-1 reagent was added and the absorbance at 450 nm/620 nm was measured at 2 h using a microplate reader.

**NF-κB Luciferase Assay** The experimental conditions for luciferase activity were similar to the previous conditions for cell viability. After a 24-h incubation with the test compounds, D-luciferin (150 μg/mL Promega; Sunnyvale, CA, USA) was added and the luminescence at 2, 4 and 6 h was measured using *in vivo* imaging system (IVIS Lumina II; Caliper Life Sciences, MA, USA).

**Western Blot Analysis** 4T1-NFκB-luc cells were treated with the test compounds (50 μM) for 5 or 10 min. Treated cells were collected, washed with PBS and lysed in lysis buffer (1 M DTT, 1 M sodium orthovanadate, 1 M β-glycerophosphate, 10 mg/mL of aprotinin, 10 mg/mL of leupeptin, and 0.1 M PMSF). The cell lysates were separated by 10% SDS-PAGE and transferred to PVDF membranes. After blocking with 0.1% Tween<sup>®</sup> 20 in PBS-5% BSA for 1.5 h at room temperature, the membranes were incubated overnight with the prima-

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ry antibodies, and then for 60 min with the secondary antibodies. Primary antibodies were used at a dilution of 1:1,000. The secondary antibodies were used at a dilution of 1:2,000 and visualized using an enhanced chemiluminescence system.

**Experimental Lung Metastasis Model** Female BALB/c mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The protocols and guidelines of the Animal Committee of Toyama University (A2012INM-6) were followed. For the experimental metastasis model, 4T1-luc cells were inoculated intravenously (i.v.,  $5 \times 10^5$ ) with or without pre-treatment with curcumin and its analogues (24 h, 25  $\mu$ M). Four days after tumor inoculation, mice were injected with D-luciferin (Promega; Sunnyvale, CA, USA) and the lungs were removed to measure luminescence using an *in vivo* imaging system (IVIS Lumina II; Caliper Life Sciences, MA, USA) to quantify lung metastasis.

**Statistical Analysis** All data are expressed as the mean  $\pm$  SEM and repeated at least two or three times unless otherwise stated. Significance was analyzed using the Student's t-test.  $P < 0.05$  was considered significant.

## RESULTS

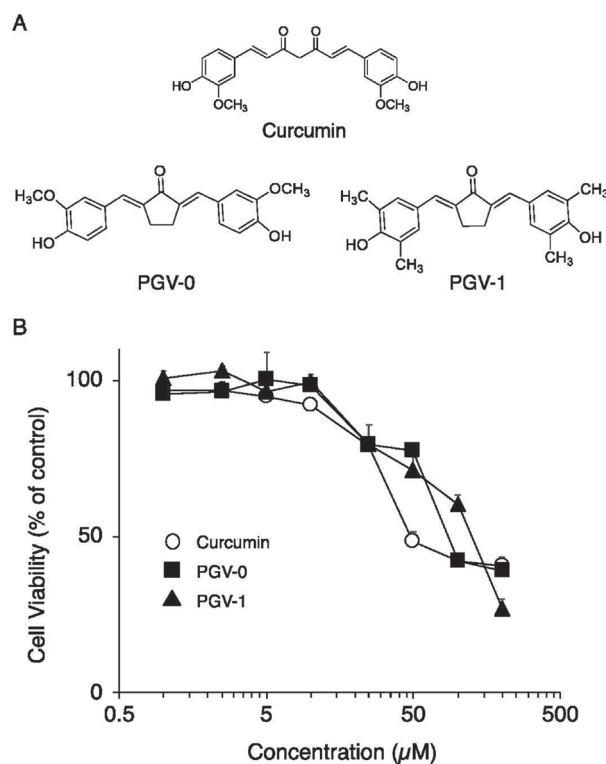
**Anti-Tumor Effects of Curcumin and Its Analogues in 4T1 Breast Cancer Cells** In order to evaluate the anti-tumor effects of curcumin analogues, we first examined the cytotoxic effects of PGV-0 and PGV-1 on murine 4T1 breast cancer cells. Similar to curcumin, PGV-0 and PGV-1 had significant cytotoxic effects on 4T1 cells in a concentration-dependent manner (Fig. 1) with  $IC_{50}$  value 26.1, 61 and 169  $\mu$ M, respectively. This demonstrated that the anti-cancer activity of PGV-0 and PGV-1 is similar to that of curcumin.

**Effects of Curcumin and Its Analogues on NF- $\kappa$ B Activity in 4T1 Breast Cancer Cells** Curcumin is well-known to exert multiple pharmacological effects, including anti-cancer effects, through the inhibition of NF- $\kappa$ B activation. Therefore, we next examined the effects of curcumin analogues on NF- $\kappa$ B activity in 4T1 breast cancer cells. 4T1-NF $\kappa$ B-luc cells, which stably express the NF- $\kappa$ B-dependent luciferase reporter gene, were treated with PGV-0 or PGV-1 for 2, 4 and 6 h, and luminescence was measured to assess NF- $\kappa$ B activity. Although curcumin demonstrated strong inhibitory effects on NF- $\kappa$ B activity, PGV-0 and PGV-1 did not (Fig. 2). We observed no inhibitory effects of PGV-0 and PGV-1 on NF- $\kappa$ B activity in 4T1 breast cancer cells at all time points after treatment. To further confirm the distinct effects of curcumin analogues on NF- $\kappa$ B activity in 4T1 cells, we examined the effects of PGV-0 and PGV-1 on the phosphorylation of p65 by Western blot analysis. As in the reporter assay, curcumin had inhibitory effects on the phosphorylation of p65, whereas PGV-0 and PGV-1 did not (Fig. 3). These results suggest that PGV-0 and PGV-1 have distinct mechanism of anti-cancer effects from curcumin.

**Anti-Metastatic Effects of Curcumin Analogues** Lastly, we examined the anti-metastatic effects of curcumin analogues using an experimental metastasis model of 4T1 breast cancer cells. As shown in Figure 4, pre-treatment with PGV-0 or PGV-1 at a non-cytotoxic dose (25  $\mu$ M) significantly inhibited the metastatic lung colonization of 4T1 cells. This suggested that both PGV-0 and PGV-1 have significant potential as anti-cancer and anti-metastatic compounds with a distinct molecular mechanism from curcumin.

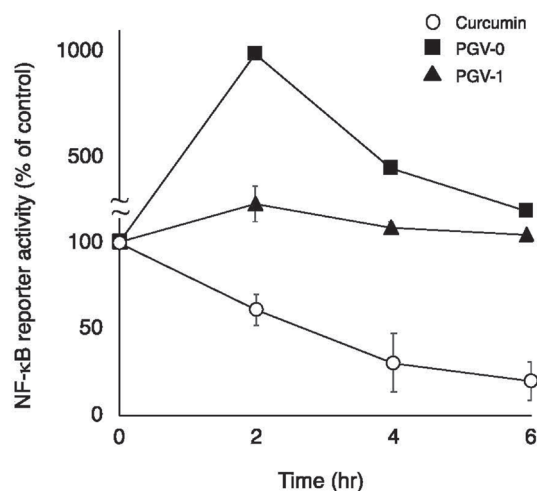
## DISCUSSION

Curcumin is a polyphenol compound derived from the roots of *Curcuma longa*. The biological activity of curcumin, such as its anti-inflammatory, anti-oxidant, anti-microbial, wound



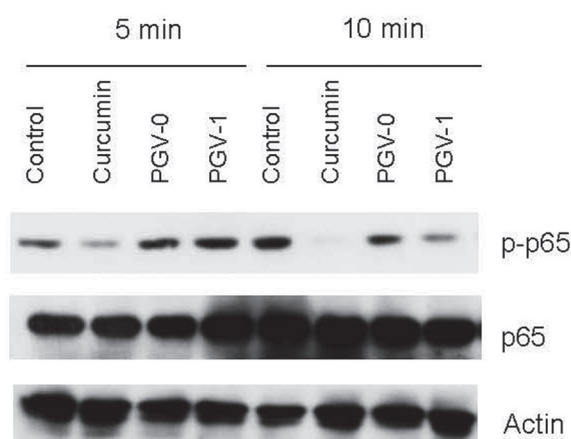
**Fig. 1.** Cytotoxic Effects of Curcumin Analogues on 4T1 Cell Lines

(A) The chemical structures of curcumin, and its analogues, PGV-0 and PGV-1 are shown. (B) Murine 4T1 breast cancer cells were ( $2 \times 10^4$  cells/well) treated with the compounds at the indicated dose. After the 24-h incubation, the WST-1 reagent was added and the absorbance at 450 nm/620 nm was measured. The results are presented as the mean  $\pm$  SEM.



**Fig. 2.** Effects of Curcumin and Its Analogues on NF- $\kappa$ B Activity in 4T1 Cells

4T1-NF $\kappa$ B-luc cells were placed at  $2 \times 10^4$  cells/well in 96-well plates and incubated for 24 h. After the 24-h incubation, cells were treated with the compounds (50  $\mu$ M) and D-luciferin (150  $\mu$ g/mL) was added. The luminescence at 2, 4 and 6 h after the luciferin treatment was measured using an *in vivo* imaging system. The results are presented as the mean  $\pm$  SEM.



**Fig. 3.** Effects of Curcumin, PGV-0, and PGV-1 on Phosphorylated p65

4T1-NFκB-luc cells were treated with the indicated compounds (50 μM) for 5 or 10 min. Treated cells were collected and subjected to Western blotting.

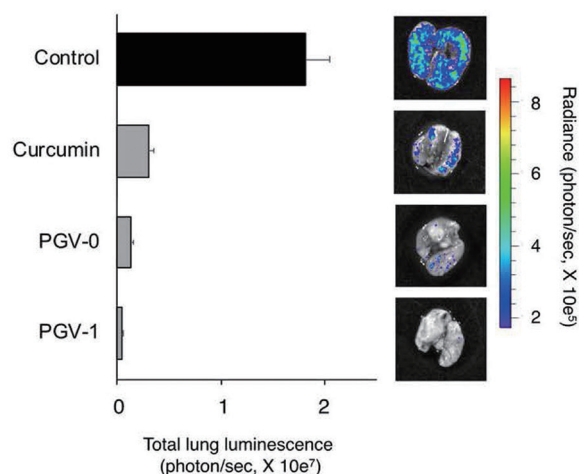
healing and anti-cancer effects is well known.<sup>13,14</sup> Curcumin is also known for having the ability to regulate the tumor cell cycle, suppressing the growth of cancer cells, and preventing invasion and metastasis.<sup>15-17</sup> Several studies reported that curcumin prevents NF-κB activation by inhibiting the phosphorylation and degradation of IκBα, and activation of p65 in ER-negative breast cancer cells.<sup>18,19</sup> NF-κB is one of the primary transcription factors that regulate expression of genes involved in cell proliferation and survival.<sup>20</sup> Although the activation of NF-κB is known to function in carcinogenesis and the growth of cancer cells by suppressing apoptosis, it is also essential for cancer cell invasion and the metastatic process.<sup>12</sup> Therefore, targeting of NF-κB pathway in cancer cells is an important strategy to control distant metastasis. In this regard, curcumin has been recognized as a promising natural compound to target NF-κB activation causing cancer progression and metastasis.<sup>12</sup>

In contrast to curcumin, its analogues PGV-0 and PGV-1 did not affect on NF-κB activity in 4T1 breast cancer cells even though both analogues demonstrated significant anti-cancer and anti-metastatic effects. We previously reported that PGV-0 and PGV-1 are more stable than curcumin,<sup>21-25</sup> which may be responsible for their more potent anti-metastatic effects. Although further studies are required to determine the exact mechanism of the anti-metastatic activity of PGV-0 and PGV-1, our present study confirmed that PGV-0 and PGV-1 have anti-metastatic potential in breast cancer cells.

**Conflict of interest** There is no conflict of interest to disclose. This study was partly supported by a Grant-in-Aid for Scientific Research on Innovative Areas (17H06398), The Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (Y.H.), and the Cooperative Research Project from the Institute of Natural Medicine, University of Toyama (S.U. and Y.H.).

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**Fig. 4.** Anti-Metastatic Effects of Curcumin and Its Analogues in an Experimental Lung Metastasis Model

Mice were intravenously injected with  $5 \times 10^5$  4T1-luc cells pre-incubated with 25 μM curcumin, PGV-0 or PGV-1 for 24 h. Four days after tumor inoculation, mice were sacrificed and lung metastases were measured by the IVIS system. The images are representative of each group and the results are presented as the mean luminescence  $\pm$  SEM. \*P<0.05.

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