The in vivo anti-tumor effect of curcumin derivative (2E,6E)-2,6-bis(4-hydroxy-3-methoxybenzylidene)cyclohexanone (BHMC) on 4T1 breast cancer cells

Nursyamirah Abd Razak,a M. Nadeem Akhtar,b Nadiah Abu,c Wan Yong Ho,d Sheau Wei Tan,a Seema Zareen,b Saiful Nizam bin Taj-ud-din,b Kamariah Long,e Noorjahan Banu Alitheen#f and Swee Keong Yeap*g

Curcumin is one of the promising natural products extracted from the rhizomes of curcuma longa and has been extensively investigated by researchers to explore its potential as a chemopreventive and therapeutic agent against several chronic diseases. To further enhance the cytotoxic potential of curcumin, its derivative (2E,6E)-2,6-bis(4-hydroxy-3-methoxybenzylidene)cyclohexanone (BHMC) has been synthesized and investigated, and its antitumor effect on tested on 4T1 challenged mice. BHMC was recorded with in vitro cytotoxicity on murine 4T1 breast cancer cells with IC50 value 13.66 μM, which was 2 times lower than curcumin after 72 hours of treatment. An in vivo study indicated that BHMC possessed antitumor effect on the 4T1 cells of the challenged mice by induction of apoptosis, antiproliferation, anti-inflammation and antimetastasis. This effect is better compared to curcumin treatment at the same evaluated concentration. Thus, BHMC is a potential antitumor agent against breast cancer.

1. Introduction

Cancer is still among the leading causes of death worldwide with breast cancer as the primary cancer incidence for women.1 Triple negative breast cancer (TNBC) is the subtype of breast cancer with absence of estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (HER2). TNBC is highly aggressive associated with high event of relapse, metastasis and poor survival. Chemotherapy is the common strategy among the limited treatment options for TNBC. However, TNBC may possess resistance against standard chemotherapeutic drugs such as anthracycline and taxane if they have been previously used as adjuvant or neoadjuvant. Thus, there is a need to discover potential treatment for TNBC.2 Natural compound has been proposed as safe and potential agent against TNBC.3 Among the natural compounds, curcumin that present in the spice turmeric has been reported with antitumor effect on TNBC.4,5 Resolving cancer challenged by natural product hold better potential with the introduction of chemical synthesis, which resolve the availability of the compounds of interest.6 On the other hand, synthetic compounds also help to improve the drug sensitivity and specificity while reduce the toxicity via chemical modification.7 Curcumin (Fig. 1A) is one of the classical natural product extracted from the rhizomes of Curcuma longa (turmeric) and named it curcumin.8 Curcumin mainly identified as

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**Fig. 1** The chemical structures of (A) curcumin and (B) (2E,6E)-2,6-bis(4-hydroxy-3-methoxybenzylidene)cyclohexanone (BHMC).
antioxidant, anti-inflammatory,3–9 anti-cancer10–17 and anti-
acetylcholinesterase38 activities. Several biological activities
including the hepato- and nephro-protective,18–22 thrombosis
suppressing,23 myocardial infarction protective,24,25 curcumin
has proved to be potential candidate for future drug discovery.
In spite of several pharmacological properties, curcumin that
was classified under “pans-assay interference compounds” and
“invalid metabolic panaceas” still facing several liabilities
including instability, poor solubility, poor selectivity and
multiple modes of assay interference particularly to the in vitro
based assays.26,27 Thus, more efforts to improve efficacy, selectivity
of curcumin are needed. In recent years, chemical modi-
fication of curcuminoids has been increasingly investigated. For
example, various curcumin analogues have been reported to
enhance antitumor effect than curcumin.28 A curcumin deriva-
tive, 2,6-bis(4-hydroxy-3-methoxybenzylidene)cyclohexanone
(BHMC) (Fig. 1B) has been synthesised with diketone moiety
tive, 2,6-bis(4-hydroxy-3-methoxybenzylidene)cyclohexanone
potential curcumin analog.

2. Materials and methods

2.1. Synthesis of BHMC

Curcumin (C7727) was purchased from Sigma-Aldrich (USA). BHMC
was chemically synthesized from 4-hydroxy-3-methoxybenzaldehyde (cyclohexanone). Briefly, a mixture of 3-
methoxy-4-hydroxy (vanillin) (40 mmol, 2 equiv.) and cyclohex-
anone (20 mmol, 1 equiv.) was dissolved in 25 mL of absolute ethanol. The mixture was heated at 50 °C for half hour and conc.
HCl (2.0 mL) was added drop wise over 5 min to the stirred mixture. The mixture was further stirred for one hours and left
over night in refrigerator. The mixture was dissolved in ice water
(500 mL) and transferred into separating funnel. A yellow viscous product was floating on the surface of water, which was
extracted with ethyl acetate (250 mL × 3 times). The ethyl acetate layer was collected, dried over rotary evaporator and
then pass to sodium sulphate anhydrous. The crude product
was subjected to further purification by silica gel column
chromatography. The column was eluted with ethyl acetate and
hexane (40 : 60%). Yield 76%; yellow powder, UV (CHCl3): λmax
302, 339 nm; IR (CHCl3 cm−1): vmax 3393 (OH), 2928 (Ar–C–H)
1618 (C=O), 1527 (Ar C=–C str.); 1H-NMR (600 MHz, CDCl3):
δ in ppm: 7.78 (bs, 2H, –C=C–H), 7.12 (dd, 2H, J = 8.34, 1.92 Hz,
H-6', H-6”), 7.02 (d, 2H, J = 1.92 Hz, H-2', H-2”), 6.90 (d, 2H, J =
7.90 Hz, J = 8.34, H-5’, H-5”), 3.91 (s, 6H, 2 × OCH3, C-3’, C-3”),
2.90 (m, 4H, H-3, H-5), 1.82 (m, 2H, H-4). 13C-NMR (150 MHz, CDCl3) δ in ppm: 190.41, (C=O), 151.89 (C-4’, C-4’’), 149.71 (C-
3’, 3”), 136.83, (–C=C–H), 134.80 (C-2, 6), 132.23 (C-1’, 1”) 128.36, (C-6’, 6”, 2’, 2”), 115.34, (C-5’, 5”), (56.07, OCH3), 28.74,
(C-3, C-5), (23.42, C-4), MS: m/z = 366 (100%) [M]+, EI-MS m/z
(rel. int.) 351 (56), 335 (68), 321 (17), 309 (12), 161 (24), 131 (15).

The purity of compound was determined by using JASCO-
HPLC attached with ChromNAV-software (JASCO Corporation,
Tokyo, Japan). The column used XBridge RP-18 (5 μm particle
size, 4.6 × 150 mm i.d.; Waters Corporation, Wexford, Ireland)
kept at ambient temperature. Injection volume of 20 μL, flow
rate at 1 mL min−1, and detector wavelength was adjusted at 250
and 366 nm, mobile phase H2O and MeOH (40 : 60), retention
time (tR) 11.66 min. The percentage of purity was determined by
calculating the peak purity method automatically.36

2.2. In vitro cell viability assay

Murine 4T1 TNBC cells were purchased from American Type
Culture Collection and cultured in RPMI-1640 medium (Sigma-
Aldrich, USA) supplemented with 10% Fetal Bovine Serum (FBS)
(Gibco, Thermo Fisher Scientific, USA) at 37 °C, 5% CO2. Cell
viability was determined by MTT assay where 4T1 cells were
seeded (0.8 × 105 cells per mL) in 96 well plate overnight. Then,
BHMC and curcumin (Sigma-Aldrich, USA) were added to the cells
at concentration ranging within 30–0.47 μg mL−1 by two fold
serial dilution and further incubated at 37 °C, 5% CO2 for
24, 48 or 72 hours. After the incubation time, MTT solution
(5 mg mL−1) was added into all wells and further incubated for 4
hours. The purple formazan was formed when dissolved by 100
μL of DMSO (Sigma-Aldrich, USA) and the absorbance was
measured at 570 nm using μQuant plate reader (Bio-Tek
Instrument, USA). Cell viability was calculated as below:

Cell viability (%) = (absorbance of treated well)/
(absorbance of control well) × 100%

IC50 (concentration that reduce 50% of cell viability
comparing to untreated cells) was obtained from the graph of
cell viability vs. concentration.

2.3. In vivo antitumor effect of BHMC

Female mice (n = 18, 6 weeks old) were purchased from
Comparative Medicine and Technology Unit (COMET), Institute
of Bioscience, Universiti Putra Malaysia. The mice were fed with
distilled water and standard pellets ad libitum at room
temperature and 12 hours of day/dark light cycles. This study
was conducted according to the guidelines and was approved by
Institution of Animal Care and Use Committee (IACUC), University Putra Malaysia (UPM/IACUC/AUP-009/2015). All animal experiments were carried out in accordance with the Malaysia Animals Act 1953 (Revised-2006) under Malaysian Veterinary Council (MVC) for animal experiments.

After 2 weeks of acclimatization of mice (body weight ~22 g), mice were separated into groups and challenged with $1 \times 10^5$ 4T1 cells per mouse subcutaneously. BHMC and curcumin were dissolved in olive oil. Untreated mice ($n = 6$) were fed with olive oil, curcumin 50 mice were orally fed with 50 mg kg$^{-1}$ body weight (BW) of curcumin (Sigma-Aldrich, USA) and BHMC 50 mice were orally fed with 50 mg kg$^{-1}$ BW of BHMC for 28 days. Throughout the experiment period, tumors were measured using a caliper and the tumor volumes were calculated by the following formula:

$$
\text{Tumor volume} = \frac{d^2 \times a}{2}
$$

$d$ = tumor measurement at the widest point
$a$ = tumor dimension at the longest point.

After the 28 days of treatment, mice were amnestied with isoflurane, and euthanised by cervical dislocation. Serum, lung and tumor were collected and subjected to the following assays. Tumor per body weight ratio was calculated.

2.4. Hematoxylin and eosin (H&E) histology analysis of tumors

Harvested tumor was fixed in 10% formalin, embedded in paraffin, section and stained with H&E. Histology of the tumor was viewed under bright-field microscope (Nikon, Japan). Even of mitotic cells were counted from five random fields of the slides.

2.5. Lung clonogenic assay

Lung was harvested, washed with ice-cold PBS, minced, treated with 2 mg mL$^{-1}$ collagenase type IV for 1 hour at 37 °C and filtered through 70 μm strainer. Then, the filtrate was pelleted at 2000 rpm for 5 min. The lung pellet was cultured in 10 mL of RPMI-1640 supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Thermo Fisher Scientific, USA) at 37 °C, 5% CO$_2$ for 10 days. After the incubation period, all wells were fixed with methanol for 1 hour and stained with 0.5% crystal violet for 2 hours. Number of colonies formed per organ was counted for untreated, curcumin treated and BHMC treated groups.

2.6. Expression of MMP9, TNF-α, IL-1β, IL-4, G-CSF and NF-κB in tumor by quantitative reverse transcription PCR (RT-qPCR)

Total RNA from the tumor was extracted using RNaseasy mini kit (Qiagen, USA) according to the manufacturer’s protocol. cDNA was synthesized from 1 μg of total RNA using NEXscript cDNA synthesis kit (NEX Diagnostics, Korea) according to the manufacturer’s protocols. Primers for target genes MMP9, TNF-α, IL-1β, IL-4, G-CSF, NF-κB and house-keeping gene β-actin were listed in Table 2. Evaluation of primer efficiency and expression of the targeted genes were performed using NEXpro qPCR EvaGreen Master Mix (NEX Diagnostics, Korea) using Eco Real Time PCR system (Illumina, USA) by the following steps: 95 °C for 2 min, 40 cycles of 95 °C for 10 s, 60 °C for 45 s and acquisition of fluorescent signal. Expression of the targeted genes in the samples were normalized by β-actin and the fold change in the expression of each target gene was calculated by the Eco 48 Software (Illumina, USA) using the efficiency-corrected method.

2.7. Serum IL-1β and TNF-α levels

IL-1β and TNF-α cytokines level in the serum were measured by ELISA kits (R&D system, USA) according to the manufacturer’s protocol.

2.8. Secondary tumor regeneration

Tumor was harvested and dissociated using accutase (Innovative Cell Tech, USA). Mice ($n = 10$, 8 weeks old) were separated into two groups. All mice were injected subcutaneously with $1 \times 10^6$ cells harvested from untreated tumor in the lower left abdomen. On the other hand, mice in group 1 and 2 were injected subcutaneously with $1 \times 10^6$ cells harvested from the tumor of curcumin 50 mg kg$^{-1}$ BW mice and BHMC 50 mg kg$^{-1}$ BW tumor in the lower right abdomen, respectively. Tumor progression was then observed for 21 days.

3. Results

3.1. In vitro cytotoxicity of BHMC on 4T1 cells

BHMC was prepared as previously described. The in vitro cytotoxicity of BHMC was compared with curcumin on 4T1 cells by MTT assay. As shown in Table 1, both BHMC and curcumin were cytotoxic to 4T1 cells in time dependent manner where the IC$_{50}$ value of both treatment reduced from 24 to 72 hours. More interestingly, IC$_{50}$ value of BHMC at 48 and 72 hours were approximately 2 folds lower than curcumin on 4T1 cells.

3.2. In vivo antitumor effect of BHMC on 4T1 challenged mice

To evaluate the antitumor effect of BHMC on breast cancer, mice challenged with 4T1 breast cancer cells were subjected to 50 mg kg$^{-1}$ BW of curcumin or BHMC treatment. Tumor start to observe at day 5 in untreated mice post-inoculation of 4T1 cells. Both curcumin and BHMC treated mice were observed with 5 and 9 days delay of tumor formation comparing to the untreated mice (Fig. 2A). After 28 days of treatment, without changes of the body weight were observed in all the mice (Fig. 2B), tumor burden of curcumin and BHMC treated mice were 1.86 and 3.10 folds smaller than the untreated mice (Fig. 2C and D). Based on the histology analysis, tumor

<table>
<thead>
<tr>
<th>Table 1</th>
<th>IC$_{50}$ value (μM) of BHMC and curcumin on murine 4T1 breast cancer cells</th>
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<tbody>
<tr>
<td></td>
<td>24 h</td>
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<tr>
<td>BHMC [μM]</td>
<td>54.64 ± 2.33</td>
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<tr>
<td>Curcumin [μM]</td>
<td>81.44 ± 2.44</td>
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harvested from untreated mice was observed with highest number mitotic cells. On the other hand, curcumin and BHMC treated mice were recorded with 1.67 and 3.75 folds less number of mitotic cells in the tumor (Fig. 3), which indicated that lower tumor burden in the curcumin and BHMC treated mice maybe contributed by anti-proliferation effect. In addition, clonogenic assay has shown that high number of 4T1 breast cancer cells have metastasized to lung in the untreated mice. On the other hand, mice treated with curcumin and BHMC were observed with lower event of 4T1 metastasis into lung indicating that both treatment possess anti-metastasis effect (Fig. 4). Overall, antitumor effect of BHMC was better comparing to curcumin as the BHMC treated mice was recorded with lower tumor burden (Fig. 2), mitotic cells in the tumor (Fig. 3) and lung metastasis (Fig. 4) comparing to the curcumin treated mice.

To understand the regulation of inflammation and metastasis related genes by both curcumin and BHMC treatment, which contribute to the delay of tumor progression, expression of MMP9, TNF-α, IL-1β, IL-4, G-CSF and NF-kB genes in the tumor of untreated, curcumin 50 and BHMC 50 treated mice were evaluated by RT-qPCR (Fig. 5). BHMC treatment was able to suppress expression of MMP9, TNF-α, IL-1β, IL-4, G-CSF and NF-kB genes comparing to the untreated mice. On the other hand, curcumin treated was able to suppress expression of TNF-α, IL-1β, IL-4, G-CSF and NF-kB genes comparing to the untreated mice. However, expression of MMP9 was not significantly regulated by curcumin treatment (Fig. 5). Level of IL-1β and TNF-α were further validated by checking serum IL-1β and TNF-α by ELISA. Both curcumin and BHMC treatment were able to reduce the serum level of IL-1β and TNF-α comparing to the untreated mice (Fig. 6).

Furthermore, regeneration capacity of the tumor harvested from untreated and curcumin or BHMC treated mice were evaluated by transplanting 1 million cells to healthy mice. When injected with tumor cells harvested from untreated and BHMC treated 4T1 challenged mice, tumor/BW ratio generated

Fig. 2 (A) Tumor volume change; (B) body weight change; (C) representative image of tumor harvested (D) tumor/BW ratio of untreated, curcumin 50 mg kg⁻¹ BW mice and BHMC 50 mg kg⁻¹ BW 4T1 challenged mice. Significant values were calculated against untreated group (*P < 0.05).

Fig. 3 (A) Histological staining of the tumor. Red circles indicate mitotic cells. (B) Numbers of mitotic cells per groups. Significant values were calculated against untreated group (*P < 0.05).
Significantly, only the untreated control. Fold changes >2 were indicated as significant.

Unrelated tumor cells was approximate 2.8 fold higher than the tumor generated from BHMC group. On the other hand, tumor generated from curcumin treated mice was only 1.25 fold lower than tumor generated from the untreated control (Fig. 7).

4. Discussion

Prognosis of TNBC remains poor in spite of advance in understanding of breast cancer pathologic features and molecular characteristics.37 TNBC are more difficult to treat as they carry fewer targets and generally develop resistant to conventional chemotherapeutic agents.38 Thus, there is a need to continue the discovery of novel effective cytotoxic agents against TNBC with minimum or no toxic side effect. Curcumin is a polyphenol presents in the turmeric Curcuma longa. It belongs to the 1,3-
dicarboxyl or β-diketone class of compounds, which possess several biological activities including important building blocks for the synthesis of core heterocycles such as pyrazole, isoxazole, and triazole in medicinal chemistry.39 Curcumin has been identified as strong anti-inflammatory agents that has broad spectrum of antitumor activity.40 Structure activity relationship of curcumin and its analogous is well established in the literatures.40,41 Curcumin was reported as potential agents targeting TNBC as recent study has reported that it was more sensitive to human TNBC MDA-MB-231 cells than human estrogen dependent MCF-7 breast cancer cells.42 A part of interesting biological activities, still curcumin not recommended for therapeutic potential because of low bioavailability.26,27 Therefore, there is need to modify its structure to improve the bioavailability, stability and selectivity. Recent study further support the idea that synthetic chemistry help to enhance the efficacy and safety of known natural metabolites.43 For examples, curcumin analogues were reported with enhance cytoxicity on various types of cancer cells.28,44 In this study, BHMC that is the analog of curcumin was synthesized. Previously, the antinociceptive activity31 and anti-inflammatory effect29,30 of BHMC were evaluated. Unlike curcumin that contain enol moiety, BHMC possessing α,β-unsaturated bis-enone system which is an essential for the several biological activities including anticancer effect as in chalcones and bischalcones.41 Such compounds with conjugated enones or enone-like compounds exhibited potential interaction with Michael acceptor can react selectively with target nucleophiles.41 This structure may contributed to the greater cytotoxic effect of BHMC on 4T1 cells in vitro, which was similar to the previous in vitro studies on human TBNC MDA-MB-231 cells.44

In addition, in vivo antitumor effect of BHMC was compared with curcumin using mouse 4T1 model. Mice challenged with 4T1 has been used as in vivo model to evaluate antitumor and antimetastasis effect of chemicals on TNBC.45,46 In this study, untreated mice was recorded with tumor development on day 3 post challenged. In addition, high tumor mitotic event and lung metastasis were observed too. On the other hand, both curcumin and BHMC treatment were found with delayed tumor formation. Curcumin was reported to suppress cell metabolism...
proliferation of TNBC \textit{in vitro}. Lower number of mitotic cells observed in the tumor of curcumin and BHMC treated mice supported the report on the \textit{in vitro} antiproliferation effect and given the idea that lower tumor burden in the treated mice maybe contributed by the inhibition of 4T1 cells proliferation. The antiproliferative effect of BHMC has been correlated to its interactions with nuclear type II sites.

TNBC is a type of highly metastatic cancer. Inflammation was found to promote tumor cell proliferation and expression of MMP9 that support the cancer metastasis. Curcumin has been reported with \textit{in vitro} antimetastatic effect on breast cancer via suppression of MMP-9 (ref. 50) and suppression of inflammation. In this study, both curcumin and BHMC were found with lower lung metastasis comparing to untreated mice. This antimitastatic effect maybe contributed by the suppression of inflammatory and MMP9 as observed in the gene expression study. Although both curcumin and BHMC show similar inhibition on inflammatory related genes, significant suppression of MMP9 expression by BHMC may contribute to its better antimitastatic effect than curcumin. Previous study has shown that curcumin possessed substantial antitumor and antimetastasis when treated at 800 mg kg\(^{-1}\) BW. Encapsulation of curcumin with dendrosome help to reduce the effective dosage to 40 mg kg\(^{-1}\) BW. In addition, TNBC was also known with high rate of recurrence. In this study, recurrence of untreated tumor with tumor harvested from curcumin or BHMC treatment were compared by injected them in the secondary healthy mice. After 21 days of secondary challenge, tumor burden originated from BHMC treated mice were remain lowest comparing those from untreated or curcumin treated mice. This result indicate that BHMC may even help to delay recurrence of TNBC even better than curcumin.

5. Conclusions
This study reported that the synthesis BHMC, which is an analogue of curcumin showed better \textit{in vitro} cytotoxicity and \textit{in vivo} antitumor effect on 4T1 TNBC model than curcumin \textit{via} suppression of inflammation, cancer cells proliferation and metastasis.

Conflict of interest
All authors in this article declare no conflict of interest.
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