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The Effect of Bromo Chalcone [1-(4'-bromophenyl)-3-(4-hydroxy-3-methoxyphenyl)-2-propene-1-on] on T47D Breast Cancer Cells

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Abstract. Chalcones have been widely researched its activity as anticancer. The substitution of the functional groups on the chalcones structure affects its anticancer activity. The objective of this research were to investigate the cytotoxic effects of bromo chalcone [1-(4'-bromophenyl)-3-(4-hydroxy-3-methoxyphenyl)-2-propene-1-on] or BHM on T47D breast cancer cells by invitro, and to analyze its effect on cell apoptosis. Cytotoxic activity of BHM on T47D cells was assayed by MTT (3-[4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide] method, while the effect of apoptosis was analyzed using flowcytometer. Furthermore, the apoptotic mechanism was traced by observing the expression of Bcl-2 protein using immunocytochemistry. The results indicated that BHM have cytotoxic effect on T47D cells (IC_{50} = 45 micro molar). The activity occurs because these compounds induce apoptosis by decreasing the expression of Bcl-2. According to the results, BHM could potentially be developed as a new drug candidate for breast cancer therapy.

INTRODUCTION

Breast cancer come under the group of the five most cancer that causing mortality. In the United States the death rate of breast cancer was the second-ranked [1]. In Indonesia, breast cancer is currently the second most frequent one after cervix cancer and has become the major cause of women mortality [2]. There are many cancer therapeutic strategies, one of them is chemotherapy. Some problems are found in the chemotherapy treatment. Among the main problems found are low selectivity and resistance of the drug on the tumor cells [3]. Hence, efforts in developing drug compounds selectively targeting cancer cells are still required.

Chalcones or (E) 1, 3-diphenyl-2-propene-1-one) [4] are abundantly found in edible plants. The compounds are biosynthetic products of shikimate pathway having been reported as precursors of open chain flavonoids group also isoflavonoids. Naturally chalcone products are very limited, but the synthesis of these compounds and their derivatives can be carried out in the laboratory through the reaction of Claisen-Schmidt or cross-aldol condensation between acetophenone and benzaldehyde catalyzed using acids or bases [5]. Chalcones have been an interesting research subject for their various pharmacological used. They show a broad spectrum of pharmacological activities including anti-tumor [6-10]. Most of them are reported able to induce cells to do apoptosis in some types of cancer through various mechanisms. Generally, these compounds decrease the expression of Bcl-2 protein, which can trigger apoptosis in cancer cells [11-18].

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In previous studies we synthesized chalcone derivatives containing bromo substituent (1-(4'-bromophenyl)-3-(4-hydroxy-3-methoxyphenyl)-2-propene-1-on or BHM) by reacting between 4-bromoacetofenon and vanillin through an aldol condensation reaction using an acid catalyst. The compound has a hydroxyl, methoxy and bromo group (Figure 1). BHM inhibits the oxidation process [19] and have cytotoxic activity on HeLa cervical cancer cells. Yet, the action mechanism of the compound on T47D cells has not been reported. This study aimed to investigate the effects of the cytotoxic, apoptotic induction and Bcl-2 expression of BHM on the T47D cells. It was expected that results of this study could benefit for the development of the compound as a novel drug in breast cancer treatment.

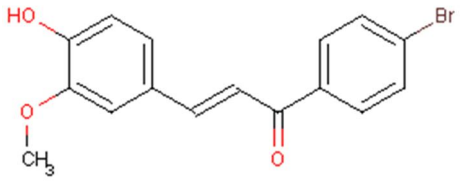

FIGURE 1. BHM structure synthesized from reaction between 4-bromoacetofenon and vanillin

MATERIALS AND METHODS

Materials

BHM was synthesis through a cross aldol condensation reaction between vanillin and 4-bromo acetophenone in an acidic condition. The compound was dissolved in dimethylsulfoxide (DMSO) by preparing a stock of solution with a concentration of 100 mM. DMSO concentration in cell medium was kept to be less than 0.1%.

T47D cells lines used in this study were obtained from Parasitological Laboratory, Faculty of Medicine, Universitas Gadjah Mada, Indonesia. The medium used to grow the cells are DMEM or the Dulbecco's modified Eagle's Medium (Gibco, USA), a culture medium containing 10% (v/v) FBS or Fetal Bovine Serum (Gibco, USA) and 1% (v/v) penicillin-streptomycin or penstrep (Gibco, USA), kept in a 5%-CO2-incubator at 37^{o}C. Trypsin-EDTA of 0.025% (Gibco) was used to detach cells on the flask.

Methods

Cell culture and cytotoxic assay

A cytotoxic assay was performed using MTT colorimetric method. First, T47D confluent cells were harvested and counted to obtain as many as $5x10^3$ cells per well then the cells were transferred into a 96-well plate, and allowed to attach to the bottom of the plate for 24 h in a humidified incubator at 37^{o}C. Next, the cells were treated with various concentrations of BHM using DMSO as co-solvent and then incubated for 24 h in the 5%-CO2-incubator. After 24 hours, the culture medium was taken with a pipette and 100 micro liters Phosphate Buffered Saline or PBS (Sigma, USA) were added to wash cells. Then the cells were incubated at 37^{o}C with 100 micro litter MTT reagent (Sigma, USA) at a concentration of 0.5 mg/ml in DMEM in each well for 4 h until formazan crystals were formed. After this, 100 micro liter stopper reagent consisting of 10% Sodium dodecyl sulfate (Sigma, USA) in 0.01N HCl (Merck, USA) was added to each well. The plate was then incubated in dark condition for 12 h (overnight) at room temperature. After the 12-h incubation, the absorbance of suspension in each well was measured using ELISA reader (Bio-Rad) at λ 595 nm. The obtained absorbance was converted into a percentage of viable cells

[20]. The IC_{50} value, a concentration causing 50% inhibition of cell growth [21], was calculated according to regression of concentration versus cell viability.

Observation of Apoptosis

As much as 5 x 10^5 of T47D cells were planted per well on a six tissue culture well-plate. After incubation at 37^{o}C in 5%-CO2-incubator for 24 h, the cells were treated using BHM in varied concentrations. The cells were then incubated for 24 h, added trypsin by 0.25% and centrifuged at 2000 rpm for 3 minutes. After incubated, the cells were washed twice using cold PBS, and then each sample was added with apoptotic reagents consisting of 100 micro liter of buffer, 2 micro liter propidium iodide (PI) and 2 micro liters Annexin-V. The mixture was incubated in a light-protected place at room temperature for 10 minutes. Then the cells were observed using FAC-Scan flow-cytometer.

Immunocytochemistry method

T47D cells were plated at a concentration of 5 x 10^4 cells per well and cultured in 24-well plate in cover slips up to 80% of cells have confluent. The media cell was taken with micropipette and BHM was added with various concentrations. Then the plate was placed in 5%-CO2-incubator for 24 h. Next, the treated cells were washed using PBS and added with cold methanol for fixation of the cells and then incubated in freezer at 4°C for 10 min. The cover slip was placed on a respective slide. Cells were washed using PBS and distilled water and then incubated with hydrogen peroxide blocking reagent for 10 min at room temperature. Next, the cells were washed using PBS and then incubated with pre-diluted blocking serum for 10 min. After the successive incubations, the cells were stained with Bcl-2 primary antibody and incubated at room temperature for 1 h. after one hour, cells were washed 3x using PBS and the secondary antibody was applied for 15-20 min. After 15-20 min of antibody application, the cells were washed 3x using PBS and then added with the streptavidin-biotin complex and incubated for 10 min. after 10-min incubation, the cells were washed 3x using PBS again. Next, DAB (3, 3 diaminobenzidine) reagent was added to the slides and then they were incubated for 3-5 min. After being washed in tap water, cells were briefly stained using Mayer-Haematoxylin reagent for 4 min. Next, the cover slips were washed with distilled water, then immersed in absolute ethanol and xylol, and finally added with mounting media. The Bcl-2 expression was then assessed under an optic microscope. Cells with Bcl-2 expression showed a dark brown color, while cells without Bcl-2 expression showed a purple color in their cell membranes.

RESULT AND DISCCUSION

The cytotoxic effect of BHM treatment on T47D cells

Graph of cytotoxic activity of BHM obtained in this study was presented in Figure 2. The result indicated that the compound showed an activity inhibiting the growth of T47D cells in a dose-dependent manner. The linear regression of BHM-cytotoxic effect resulted (Fig. 2a) resulted in an IC_{50} of BHM of 45 micro molar ($p<0.05$). BHM treatments had also caused cell morphology changing as presented in Figure 2b-d. Some cells appeared to become rounded and detached from the bottom flask with a massive morphology. The rounded shape of cells indicated the mortality of T47D cells. The changes were more apparent in line with the increased concentrations of BHM.

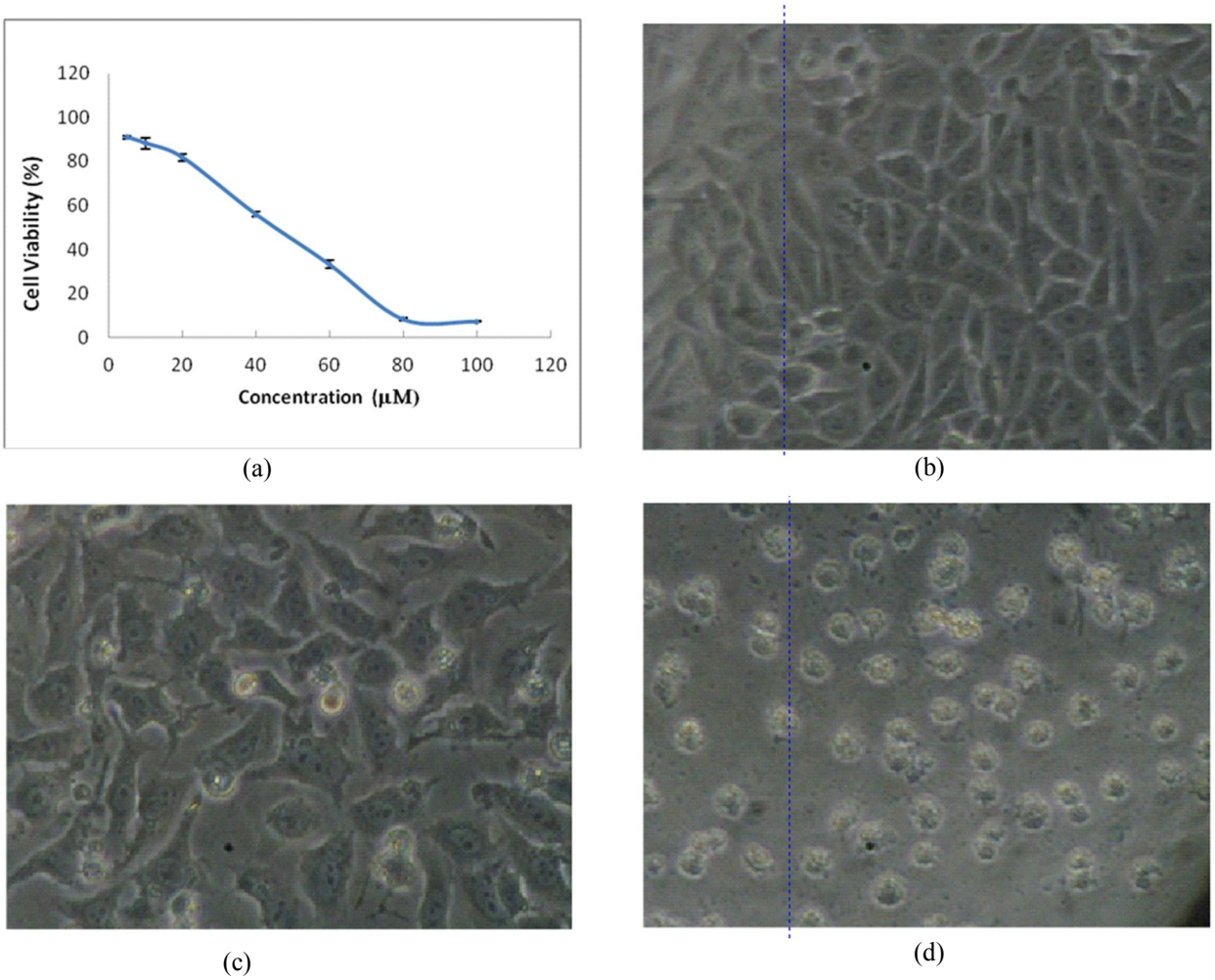

FIGURE 2. The cytotoxic effect of BHM on T47D cells determined using MTT assay. The effect were studied by incubation of $5x10^3$ cells in 96 well plates for 24 h in DMEM medium and treatment using BHM at varied concentrations of: 5, 10, 20, 40, 60, 80, and 100 micro molar. The profile of cell viability is expressed in mean ± SE from three experiments (A). Effect of BHM on morphological changes and cell populations at concentrations of 0 micro molar or control (B) compared to 20 micro molar BHM (C) and 60 micro molar BHM (C). The normal living cells were indicated by bold arrows, whereas the morphology changes were indicated by dashed arrows. This observation is done using an inverted microscope with 100x magnification.

The BHM effect on apoptosis

The effect of BHM treatment on apoptosis of T47D cells was determined using the flow cytometric method. The results showed that the BHM treatment could induce cell at early and late apoptosis as shown in Fig. 3. The increasing concentration of BHM will increase apoptosis induction.

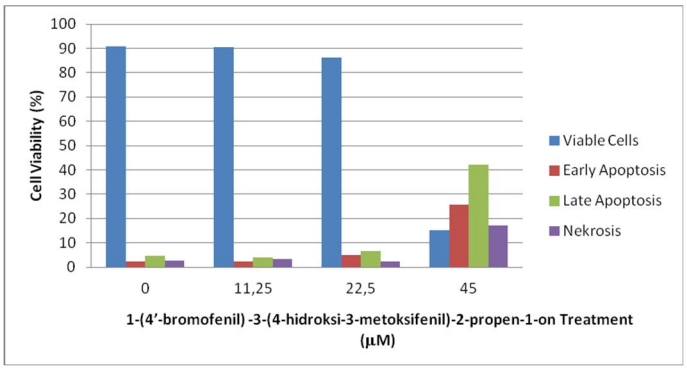

FIGURE 3. The apoptotic induction effects of BHM treatment on T47D cells. The varied concentrations of BHM were 0, ¼ IC_{50}, ½ IC_{50}, and IC_{50}. After 24 h incubation, the cells were harvested and added with AnnexinV and PI reagents, then subjected to FACS flow cytometer

The effect of BHM treatment on Bcl-2 expression

To confirm the apoptosis mechanism of BHM, the expression of Bcl-2 was examined using immunocytochemistry method. The presence of Bcl-2 protein is characterized by the existence of brown color. Bcl-2 is one of the proteins that play a role in apoptosis. The protein suppresses cells not to do apoptosis. The results of this study are very interesting, because the expression of Bcl-2 protein in T47D cells added with BHM was lower compare to that of untreated cells (Fig. 4). These results showed that BHM has the potential to be apoptotic induction via decreasing the level expression of Bcl-2 protein.

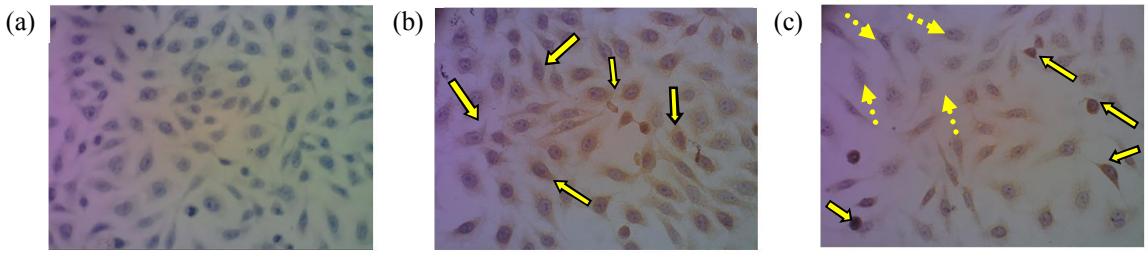

FIGURE 4. The level expression of Bcl-2 of T47D cells as observed by immunocytochemistry method. The control cells without antibody (A) compared to the untreated cells with Bcl-2 Ab (B), and cell treated with 11.25 micro molar BHM (C). This experiment used DAB chromogen. Positive results of the Bcl-2 expression were indicated by the presence of brown color in the cytoplasm of cells and mitochondrial membranes (bold arrow). While the negative results were indicated by purple color (dashed arrows).

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From the research, the potential of BHM as anticancer could be demonstrated. BHM gave a toxic effect on T47D cells as indicated by the low of IC_{50} values. The IC_{50} of BHM was 45micro molar (less than 100 micro molar) indicated that BHM potential to be developed as medicine to cancer treatment [22]. Cytotoxic effects on T47D cells were further investigated by observing the ability of BHM to stimulate apoptosis.

Apoptosis is the cell death program marked by the changes in morphology, membrane blebbing and chromatin [23]. As observed in this study, the characteristics of changing in cell morphology, such as nuclear fragmentation, cell shrinking, and apoptotic cell appeared to be more extensive after treated with BHM than untreated (Figure 1). The flowcytometry analysis showed that treatment with 22.5 micro molar BHM increased the cell apoptosis from 6.67% to 11.44%. While the treatment with 45 micro molar BHM increased apoptosis of T47D cell up to 67.97%.

These data showed that BHM have activity to induce apoptosis. Compounds that have the ability to induce apoptosis in cancer cells are very prospective to be developed as anticancer drugs because the character of cancer cells is generally able to avoid apoptosis.

Apoptosis is one of the cell death programs that can occur with various mechanisms, one of which involves the NF-kB protein. The protein inhibits apoptosis by increasing of Bcl-2 transcription process. This condition will prevent Bax to release the cytochrome c in mitochondria. Also, inhibition of NF-kB activation will decrease Bcl-XL expressions. Bcl-2 and Bcl-XL are anti-apoptotic proteins. The study showed that BHM treatment is able to reduce the level of Bcl-2, which could further trigger apoptosis in T47D cells.

Several previous studies had reported that the core structure of chalcone have a chemo preventive effect on MCF-7 and MDA-MB-231 breast cancer cells [13] as well as on T24 and HT-1376 bladder cancer cells [14]. Chalcone had shown abilities to inhibit the proliferation of bladder cancer cells by spurred apoptosis and arrest cell cycle progress at G2/M phase. Cell cycles inhibition is influenced by the expression of p21 and p27 proteins. The increasing expression of these proteins will decrease the expression of cyclin B1, cyclin A and Cdc2 which contribute to cell cycle inhibition. Chalcone is also reported to increase the expression of Bax and Bak, and can reduce levels of Bcl-2 and Bcl-XL. This can trigger apoptosis through the mitochondrial pathway by releasing cytochrome c and activating caspase-9 and caspase-3. Chalcone has been also shown to inhibit NF-kB activity. NF-kB is a transcription factor that plays a major role in cancer cells because it regulates many genes involved in inflammation, cell survival, cell proliferation, invasion, angiogenesis, apoptosis, cell cycle, and metastasis [24-26]
It is suspected that BHM, one of chalcone derivatives, which have hydroxyl, bromo and methoxy substituent, would also have the same mechanism in triggering apoptosis. In this study demonstrated that the increasing of apoptosis induction of T47D cells by BHM through suppression of the Bcl-2 expression. This indicates that BHM may have potential effects on the NF-kB. However, the detail molecular mechanism of the apoptotic induction still needs to be explored.

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