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Curcumin Analogs Induce Apoptosis and G2/M Arrest In 4T1 Murine Triple-Negative Breast Cancer Cells

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ABSTRACT

Chemotherapy is the first-line treatment for triple-negative breast cancer (TNBC), yet toxicity and resistance effects have been the current problems. Curcumin,a natural compound, has been reported to exert anti-proliferative effects on various cancer cells, including breast carcinoma cells. However, the β-diketone moiety influences the stability of curcumin. Curcumin analogs, pentagamavunon-0 (PGV-0), and pentagamavunon-1 (PGV-1) were synthesized to improve the stability and activity of curcumin by modified the β-diketone moiety into mono-ketone pentanone. In this study, we evaluated the cytotoxicity, inhibition of cell cycle progression, and induction of apoptosis of curcumin and its analogs (PGV-0 and PGV-1) in murine triple-negative breast cancer 4T1 cell line. The cytotoxic evaluation was done by MTT assay, while apoptosis induction and cell cycle evaluation was performed by annexin V staining and detected by flow cytometry. Curcumin and its analogs, PGV-0, and PGV-1, significantly inhibit the viability of 4T1 breast cancer cells with an IC50 value of 34.34μg/mL, 13.76μg/mL and 38.21μg/mL, respectively. Apoptosis analysis with a dose of 10μg/mL and 15μg/mL in 4T1 breast cancer cells showed that curcumin and its analogs effectively induce apoptotic in a dose-dependent manner. In cell cycle analysis using a dose of 15μg/mL, curcumin inhibited the cell cycle progression in the S phase, whereas PGV-0 and PGV-1 inhibited the cell cycle in the G2/M phase. It could be concluded that curcumin analogs, PGV-0 and PGV-1, have higher potential to be developed as anti-cancer agents by inducing cell cycle arrest and apoptosis in triple-negative breast cancer.

Keywords: Apoptosis; Cell cycle arrest; PGV-0; PGV-1; TNBC

INTRODUCTION

Breast cancer is one of the primary causes of death in women caused by cancer (Bray *et al*. 2018). Triple-negative breast cancer (TNBC), which accounts for 15–25% of breast tumors, has the worst prognosis because of this cancer insensitivity to traditional endocrine therapy and HER2-targeted therapy and has the highest risk of relapse (Kim *et al*. 2013). Chemotherapy is the first line to TNBC therapy; however, its effectiveness is highly restricted due to problems of drug resistance and selectivity (Dm 2018; Isakoff 2010). Therefore, research on a new therapeutic agent that is effective and selective to treat TNBC is critically needed. Preclinical therapeutic studies with cell cycle inhibitors that target adaptive processes for cell proliferation as well as oncogenic signaling that affects the cell-cycle machinery have shown antitumor responses (Cheok, 2012; Wang *et al.,* 2011). Therefore, the development of natural compounds known to have chemotherapeutic properties and specific targets such as cell cycle checkpoints to induce arrest and apoptosis is a powerful strategy to treat cancer (Cheok 2012).

The development of chemotherapeutic treatments for various diseases, such as cancer, is currently being explored (Xiao, Morris-Natschke,

and Lee 2016). Several natural products have played an important role in anti-cancer drug discovery (Khazir *et al*. 2013). Curcumin is a potential natural compound that has several biological activities such as antioxidant activity, anti-inflammatory (Atsumi *et al*. 2005; Motterlini *et al*. 2000), and anti-cancer (Aggarwal *et al*. 2006; Bar-Sela, Epelbaum, and Schaffer 2010; Zhu and Bu 2017). However, the presence of β-diketone moiety influences the stability of curcumin (Vyas *et al*. 2013). Therefore, structural modification is a strategy to improve the stability and solubility of curcumin. The improvement of its stability and solubility is expected to increase the pharmacological effect and decrease the toxicological effect of the compounds (Bevan, Ryder, and Shaw 1995; Guo 2017). PGV-0 and PGV-1 are monoketone curcumin analogs with cyclo-pentanone structure modification (Sardjiman *et al*. 1997). Although several studies of PGV-0, and PGV-1, showed cytotoxic activity on several line cells such as MCF 7 (Meiyanto, 2011) and WiDr (Ikawati and Septisetyani 2018), their activity on 4T1 triple-negative breast cancer cells have not been elucidated. Thus, in this study, we evaluate the effects of curcumin analogs, PGV-0 and PGV-1, focusing on their activity to induce apoptosis and cell cycle arrest in the 4T1 highly metastatic, triple-negative breast cancer cells.

MATERIALS AND METHODS

Curcumin (Sigma), PGV-0 dan PGV-1 ware synthesized by Curcumin Research Center (CRC), Faculty of Pharmacy, Universitas Gadjah Mada. Each compound was dissolved in DMSO (Sigma). 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazo-leium bromide (MTT) was obtained from Sigma.

Cell line and cell culture

The 4T1 cells were obtained from ATCC. The cells were cultured in RPMI medium supplemented with 10% FBS (Gibco), 150 IU/mL penicillin, and 150μg/mL streptomycin (Gibco). Cultured cells were maintained in a CO_2 incubator at 37°C.

Cytotoxic assay

Cytotoxic evaluation was conducted by using MTT Assay. This assay was carried out triplicate. The 4T1 cells were seeded in a 96-well plate ($1x10^4$ cells/well). A series of concentrations of curcumin, PGV-0, or PGV-1 was diluted in the culture medium. After 24h of incubation, the medium was removed, and cells were washed using phosphate buffer saline (PBS) (Sigma). 5mg/mL of 3-(4,5-Dimethylthiazol-2-yl)- 2,5-Diphenyltetrazolium Bromide (MTT) reagent (Sigma) in PBS was diluted in RPMI medium, and 100 μL of reagent was added to each well. After 3 hours of incubation, the reaction was stopped by the addition of 100 μL of Sodium Dodecyl Sulfate (SDS) 10% in HCl 0.01 N, and incubated overnight at room temperature in a dark place. The absorbance was measured using a microplate reader at λ 595 nm. The percentage of cell viability was defined as (absorbance of treated cells - absorbance of blank)/(absorbance of control - absorbance of blank) x 100%, and were used to calculate IC50 values by a linear regression analysis between cell viability (%, y axis) vs log concentration (μg/mL, x axis) (Doyle and Griffiths 2000).

Apoptosis assay

Apoptosis assay was performed by using Annexin V-FITC/PI staining (BD Bioscience, Catalog no. 556547) flow cytometry. The cells were seeded in a 6-well plate at a density of $5x10^5$ cells/well, respectively, and treated with curcumin and its analogs at 15 μL/mL for 24h. Then, the cells were trypsinized with 0.25% trypsin, centrifuged, and washed with cold PBS and resuspended in Annexin V binding buffer at a concentration of $0.25\text{-}1.0x10^7$cells/mL. Afterward, 100μL of cell suspension was transferred in a test tube followed by the addition of FITC annexin V and 10μL of PI solution. Cells were vortexed and incubated for 10 minutes at room temperature in the dark, then 400 ul annexin V binding buffer was added to each tube. Flow cytometer (BD Bioscience, US) was used to analyze 50,000 cells, and data were analyzed by using the Graphpad Prism 6.0 program.

Cell cycle assay

Cell cycle analysis was performed by using propidium iodide (PI)-staining flowcytometry. Cells ($5x10^4$ cells/well in 6-well plate) were seeded in a 6-well plate and incubate overnight. The media was replaced with the fresh medium that contains curcumin and its analogs at 10μL/mL and 15μL/mL. Treated-cells were washed with cold PBS, fixed with 75% ethanol and then incubated overnight at -20°C. The fixed cells were incubated with propidium iodide (PI) solution in the dark at room temperature for 30min. The samples were immediately measured by (BD Biosciences, US). The data obtained were analyzed by using the Graphpad Prism 6.0 program.

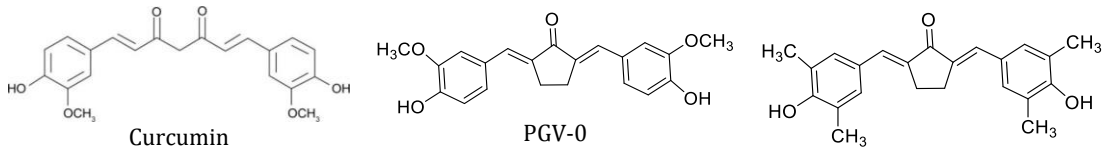

Figure 1. Structure of Curcumin and its analog

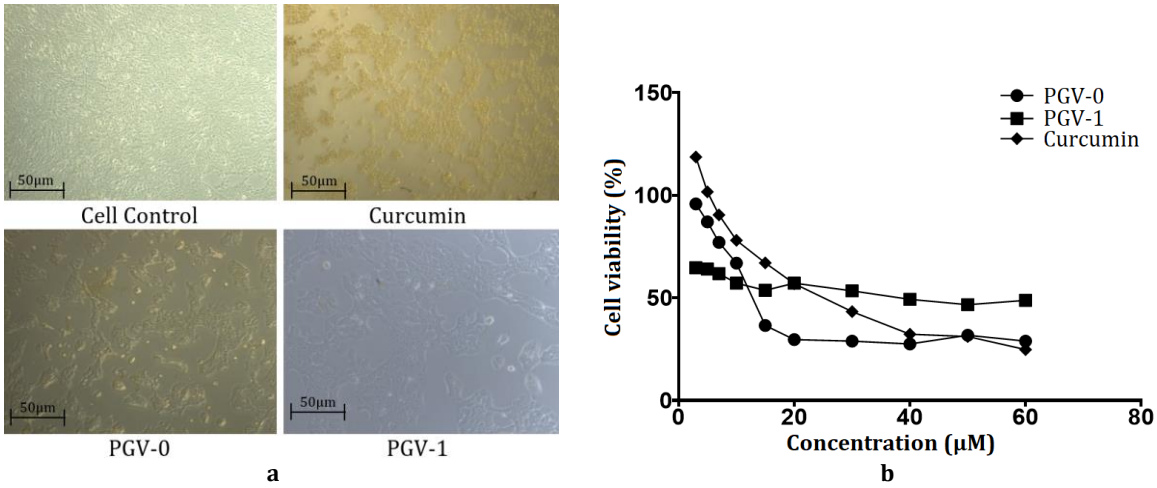

Figure 2. Cytotoxic effect of curcumin and its analog on 4T1 cells. An in vitro study was initiated by treating 4T1 breast cancer cell with increasing doses of curcumin, PGV-0 and PGV-1 for 24h. (A) The viability of the curcumin and its analog-treated cell was measuring using MTT assay. (B) The histograms of viability cells. Vertical bars represent the standard deviation of means (SD) (n= 3).

RESULTS AND DISCUSSION

Curcumin and its analogs inhibit cancer cell viability on the 4T1 breast cancer cells.

The effect of curcumin and its analogs (Figure 1) on the viability of the 4T1 breast cancer cell was measured by MTT assay. Data illustrates that the treatment of curcumin, PGV-0, and PGV-1 significantly inhibits 4T1 breast cancer cell viability in a dose-dependent manner (Figure 2). The IC_{50} value of curcumin, PGV-0, and PGV-1 for 24h in the 4T1 breast cancer cells are 34.34µg/mL, 13.76µg/mL, and 38.21µg/mL, respectively.

Exposure to curcumin and its analogs stimulates cell apoptosis of 4T1 breast cancer cells.

Annexin V-FITC/PI double staining was performed to confirm whether the effects of curcumin and its analog on the cytotoxicity of 4T1 cells were related to apoptosis. Curcumin, PGV-0, and PGV-1 treatments at 10µg/mL for 24h show that the apoptotic cell population increase from 0.23% to 0.94%, 1%, and 2.38%, respectively, compared with the untreated cell population (Figure 4). From this result, it shows that PGV-1 can significantly induce apoptosis. However, the percentage of the late apoptotic cell at 10µg/mL was relatively low at all treatment but increased to 5.19%, 6.79%, and 4.81%, respectively, at 15µg/mL. This result shows that curcumin, PGV-0, and PGV-1 can significantly increase apoptosis with dosage 15µg/mL. From this result, we can conclude that curcumin and its analogs effectively induce apoptotic occurrence in a dose-dependent manner. PGV-0 and PGV-1 show higher apoptotic effects in 4T1 breast cancer cells compared to curcumin. PGV-1 appears better in inducing the early apoptosis phase than curcumin. In contrast, PGV-0 shows more significant effect in the late apoptosis phase than curcumin.

Curcumin and its analog induce cell cycle arrest on the 4T1 breast cancer cells.

To investigate whether the increased of apoptosis is related to cell cycle arrest, treated cells were analyzed by flow cytometry at the different phases of the cell cycle (sub-G1, G1, S, and G2/M). Curcumin treatment showed that the percentage of 4T1 breast cancer cells in the S phase was increased (Figure 3 (B)).

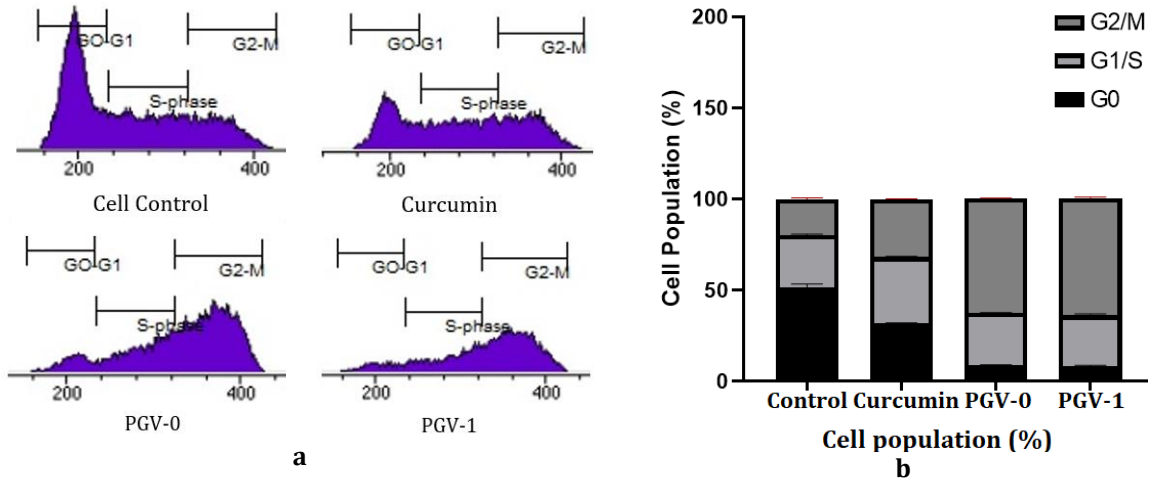

Figure 3. Effects of curcumin, PGV-0, and PGV-1 treatment on cell cycle in 4T1 cells. (A) The 4T1 breast cancer cell treated with 15μg/mL concentration of curcumin and it analog for 24h and stained with PI to analyze cell distribution by flow cytometry. (B) The histograms of the cell cycle. Vertical bars represent the standard deviation of means (SD) (n= 3).

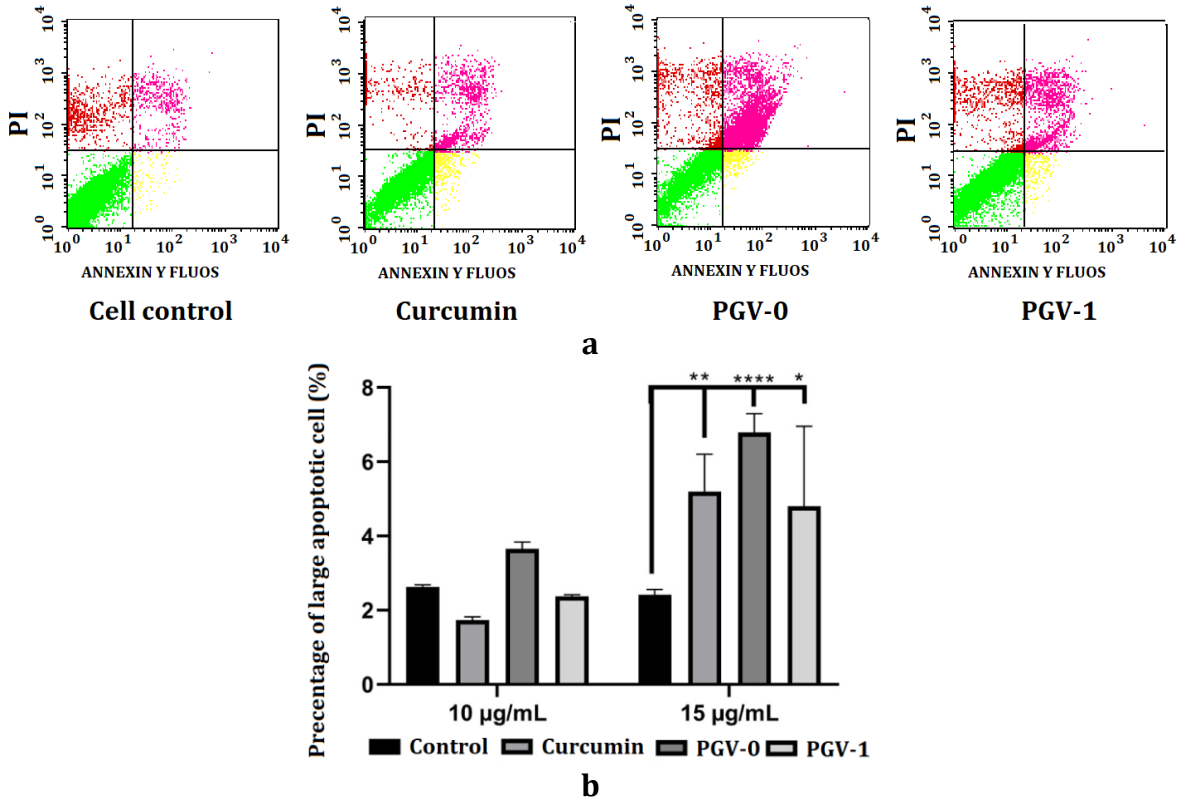

Figure 4. Effects of curcumin, PGV-0, and PGV-1 treatment on apoptosis in 4T1 cells. (A) The cells were treated with 10 μg/ml and 15 μg/ml curcumin, PGV-0, and PGV-1, stained with Annexin V, PI. After staining, flow cytometry was performed to determine apoptosis. (B) The histograms of the apoptotic cells. Total apoptotic cells significantly increased following exposure to 15 μg/ml, compared with 10 μg/ml after 24 hours. Vertical bars represent the standard deviation of means (SD) (n= 3). *$p < 0.05$.

On the contrary, PGV-0 and PGV-1 treatment increased cell accumulation in the G2/M phase. These results suggest that curcumin and its analogs inhibit the cell cycle of 4T1 breast cancer cells through different mechanisms. Curcumin induces cell cycle arrest at the S phase. In addition, PGV-0 and PGV-1 induce G2/M phase cell cycle arrest.

Several studies on mono carbonyl curcumin analogs reported that they have been more potent than curcumin in MDA-MB231, DU145, Hela, K562 dan MCF7 cell (Adams *et al.,* 2005; Zamrus *et al.,* 2018; Zhang *et al.,* 2015). These results could be due to the chelating and enolization effects of hydrogen bonding that caused a strong binding with the receptor. Moreover, mono-carbonyl curcumin analog has a bis-enone conjugated system that more selective to bind with the targeted nucleophile (Zamrus *et al*. 2018b). On the other hand, modification of β-diketone into mono-ketone could increase the stability, solubility, and biodistribution of curcumin (Xia *et al*. 2014). From Figure 2, we can see that PGV-0 has higher cytotoxic activity than curcumin and PGV-1 in 4T1 cells. In this respect, the curcumin analog with mono-carbonyl structure could be potential to be developed as a new anti-cancer drug and considered as a remarkable approach for improvement of curcumin's bioavailability problems.

The inhibitory effect of curcumin and its analogs on the cancer cell line were due to its ability to induce cell cycle arrest (Lee *et al.,* 2009; Meiyanto, 2011). Cell cycle checkpoint is the surveillance mechanism of controlling abnormal cell conditions, including cancer. In the cell cycle phase, an abnormal cell could repair by cell cycle arrest or apoptosis. During the early apoptosis, phosphatidylserine was translocated from the plasma membrane to the outer side. Curcumin induces cell cycle arrest at S phase. In addition, PGV-0 and PGV-1 induce G2/M phase cell cycle arrest. The G2 checkpoint prevents cells from entering mitosis when DNA is damaged, providing an opportunity for repair and stopping the proliferation of damaged cells (Khodjakov and Rieder 2009). The eukaryotic cell cycle progression is regulated by the coordinated activity of cyclin-dependent kinase (Cdk) and cyclin complexes. It is known that the G2/M transition is dependent mainly on the cyclin B/Cdk-1 activity (Graña and Reddy 1995). Literature shows that p53 is one of the most important regulators in mediating growth arrest and apoptosis induced by chemotherapeutic compounds. The active p53 can transcriptionally increase the expression levels of its target genes, especially p21. This protein, in turn, stops the cell cycle progression, by blocking the function of cyclin-Cdk complexes, to repair damages induced by various stresses. Once the damages are unable to be repaired, p53 activates the transcription of various pro-apoptotic genes, including Bax, and suppresses transcription of various anti-apoptotic genes, such as Bcl-2 (Robbins and Zhao 2012).

A recent study reports that curcumin induced G1/S and G2/M cell cycle arrest in human osteosarcoma (HOS) cell (Lee, Lee, and Kim 2009b). Moreover, curcumin induces G2/M arrest in human pancreatic cell and induces G0/G1 arrest in mesothelioma cell (Mayol *et al*. 2015). PGV-1 inhibits cell growth of breast cancer MCF7 and T47D cell line by inducing G2/M arrest, while PGV-0 induces G2/M arrest in MCF7 cell line (Meiyanto 2011b; Meiyanto *et al*. 2018). Furthermore, curcumin analog of A501 and WZ35 induces G2/M cell cycle arrest in non-small lung cancer cells, and prostate cancer cells (Xia *et al.,* 2014; Zhang *et al.,* 2015) and EF24 also induce G2/M arrest in MAD-MB cell line by increasing level of ROS, while simultaneously decrease the cellular level of GSH (Adams *et al*. 2005b). Results of this present study was supported by a previous study that demonstrated G2/M cell cycle arrest upon PGV-1 treatment (2 and 4 μM) in 4T1 cells (Meiyanto et al. 2019). Moreover, the same author showed S phase arrest upon curcumin treatment in concentration of 25 μM in 4T1 cells. Taken together, the mechanism of curcumin and PGV-1 in cell cycle arrest is occurred in concentration independent manner.

Cell cycle checkpoint is the surveillance mechanism of controlling abnormal cell conditions, including cancer. In the cell cycle phase, an abnormal cell could repair by cell cycle arrest or apoptosis. Our results suggest that curcumin and its analogs effectively induce apoptosis in a dose-dependent manner. PGV-0 and PGV-1 show a higher apoptotic effect in 4T1 breast cancer cells compared to curcumin. PGV-1 appears better to inducing apoptosis in an early stage. It defines by changes in nuclear morphology follow a little later, as does as cell shrinkage. In contrast, PGV-0 shows a more significant effect in inducing late apoptosis and causes cells to undergo necrosis. However, the detailed mechanism of action of PGV-0 and PGV-1 is not clearly elucidated. Several recent studies show that curcumin induces apoptosis in several adenocarcinoma cell lines by decreasing the anti-apoptosis like inhibitor of apoptotic protein (IAP),

X-chromosome-linked IAP, Bcl-2, Bcl-xL, and Bfl-1/A1. Curcumin also inhibits NF-kB activation and NF-kB-regulated gene expression through inhibition of IKK and Akt activation (Aggarwal 2005). Furthermore, curcumin decreases methylglyoxal-induced ROS formation, which would cause apoptosis in human hepatoma G2 cells (Chan, Wu, and Hsuuw 2005). Another study revealed that PGV-0 and PGV-1 induce apoptosis in MCF7 cell line (Meiyanto 2011b), and T47D breast cancer cell line through induction Caspase 3 (Meiyanto et al. 2018). Therefore, PGV-0 and PGV-1 might induce apoptosis through the inhibition of NF-kB activation and regulation of BCL protein in a triple-negative breast cancer cell. Some limitations should be noted. We used only one cell line, 4T1 murine cell line. So, experiments on normal cells and human TNBC cells have not been conducted. Further exploration is needed to elucidate their specific mechanism of actions.

CONCLUSION

Curcumin analogs, PGV-0 and PGV-1, have the potency to be developed as chemotherapeutic agents for triple-negative breast cancer through inhibition of tumor growth by inhibit the cell cycle progression and induce apoptosis. Hence, their molecular mechanism of cell cycle arrest and apoptotic induction in triple-negative breast cancer needs to be further explored.

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