JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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# Demethoxycurcumin Inhibits Energy Metabolic and Oncogenic Signaling Pathways through AMPK Activation in Triple-Negative Breast Cancer Cells

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**ABSTRACT:** Demethoxycurcumin (DMC), curcumin (Cur), and bisdemethoxycurcumin (BDMC) are major forms of curcuminoids found in the rhizomes of turmeric. This study examined the effects of three curcuminoid analogues on breast cancer cells. The results revealed that DMC demonstrated the most potent cytotoxic effects on breast cancer MDA-MB-231 cells. Compared with estrogen receptor (ER)-positive or HER2-overexpressing breast cancer cells, DMC demonstrated the most efficient cytotoxic effects on triple-negative breast cancer (TNBC) cells. However, nonmalignant MCF-10A cells were unaffected by DMC treatment. The study showed that DMC activated AMPK in TNBC cells. Once activated, AMPK inhibited eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) signaling and mRNA translation via mammalian target of rapamycin (mTOR) and decreased the activity and/or expression of lipogenic enzymes, such as fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC). DMC also targeted multiple AMPK downstream pathways. Among these, the dephosphorylation of Akt is noteworthy because it circumvents the feedback activation of Akt that results from mTOR inhibition. Moreover, DMC suppressed LPS-induced IL-6 production, thereby blocking subsequent Stat3 activation. In addition, DMC also sustained epidermal growth factor receptor (EGFR) activation by suppressing the phosphatases, PP2a and SHP-2. These results suggest that DMC is a potent AMPK activator that acts through a broad spectrum of anti-TNBC activities.

**KEYWORDS:** *demethoxycurcumin, triple-negative breast cancer, AMPK, EGFR*

## INTRODUCTION

Due to modern systemic therapies, breast cancer survival has improved significantly in the past few decades. However, there remain subsets of breast cancer that do poorly; one of these is defined by the so-called triple-negative breast cancer (TNBC), which represents approximately 15−20% of all breast cancers.[1] TNBC is a molecular subtype of breast cancer characterized by the lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) expression. TNBC tends to occur in younger patients; the disease progresses more aggressively and relapses earlier with visceral metastases. There is no targeted agent developed specifically for TNBC at current therapy. The current standard therapy for this subset of patients is limited to chemotherapy, and the data on TNBC therapy issues are insufficient.[2] Hence, there is a clear need to establish new approaches for the therapy of patients with TNBC.

AMP-activated protein kinase (AMPK), a highly conserved energy-sensing serine/threonine kinase, is involved in the regulation of a number of physiological processes including $\beta$-oxidation of fatty acids, lipogenesis, and protein and cholesterol synthesis, as well as cell cycle inhibition and apoptosis. Important changes to these processes are known to occur in cancer due to decreased AMPK activation within cancer cells and in the periphery.[3] A recent study using immunohistochemistry has demonstrated that phosphorylated AMPK was highly expressed in normal breast epithelium, and its expression was significantly reduced in primary breast cancer samples.[4] The loss of phosphorylation of AMPK was also associated with higher histological grade and axillary node metastasis. More-

Received: March 19, 2013
Revised: June 6, 2013
Accepted: June 6, 2013
Published: June 6, 2013





dx.doi.org/10.1021/jf4012455 | J. Agric. Food Chem. 2013, 61, 6366−6375

over, in contrast to normal human tissue, cancer cells display high rates of anabolic metabolism.[3] Recent studies have shown that inactivation of lipogenic enzymes, such as fatty acid synthase (FASN), acetyl CoA carboxylase (ACC), and 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), resulted in either cell death or growth inhibition in tumor cells.[8,9] Drugs that ameliorate the energy metabolic signaling pathway through AMPK activation may be beneficial for breast cancer prevention and treatment.

The AMPK pathway is linked to cancer cell growth and survival in light of its ability to activate tuberous sclerosis complex 2 (TSC2), a tumor suppressor that negatively regulates protein synthesis by inhibiting the mammalian target of rapamycin (mTOR).[5] Therefore, AMPK integrates growth factor signaling with cellular metabolism through the negative regulation of mTOR.[6] The roles of mTOR in mammalian cells are related to the control of mRNA translation by the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1). In its hyperphosphorylation form via mTOR, 4E-BP1 ultimately initiates translation of certain mRNAs, including those needed for cell cycle progression and those involved in cell cycle regulation.[7]

Potential approaches in managing TNBC have included targeting vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), and poly(ADP-ribose) polymerase 1 (PARP1). Subtyping TNBC and biomarker-led understanding will be crucial to better predict benefit from new TNBC targeted drugs. Approximately 60% of basal-like TNBC tumors express EGFR, and higher intratumoral expression of EGFR and CK5/6 is a negative prognostic factor when present, suggesting EGFR is a molecular target for novel therapeutic inhibitors.[10−12]

Curcuminoids are the major natural phenolic compounds found in the rhizome of many *Curcuma* species such as *Curcuma longa* Linn., *Curcuma aromatica*, and *Curcuma phaeocaulis*,[13] and they consist of a mixture of curcumin (Cur, 75−80%), demethoxycurcumin (DMC, 15−20%), and bisdemethoxycurcumin (BDMC, 3−5%). DMC is a structural analogue of curcumin and shows almost the same biological effect as curcumin, such as antioxidative,[14] anti-inflammatory,[15] anticancer,[16] and antiangiogenesis[17] activities. Inhibition by curcuminoids in human breast cancer cells has not been reported, and little is known about their anti-TNBC effects and associated molecular mechanisms.

Recent studies have focused on the potential of targeting metabolic pathways that may be altered during TNBC tumorigenesis and progression. A significant amount of attention has been focused on the inhibition of tumor cell growth by the activation of AMPK. In the current study, we investigated the effects of curcuminoids on the viability of TNBC cells. We obtained evidence that DMC at low micromolar concentrations effectively inhibited TNBC cell proliferation by concurrently blocking multiple oncogenic signaling pathways and energy metabolism through AMPK activation. Our study provides a proof-of-concept that targeting AMPK activation with DMC represents a relevant strategy for TNBC prevention and therapy.

## MATERIALS AND METHODS

**Reagents and Antibodies.** Compound **c**, lipopolysaccharides (LPS), Oil Red O, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), metformin (1,1-dimethylbiguanide), and antibodies for $\beta$-actin were purchased form Sigma (St. Louis, MO, USA). Antibodies for PARP, FASN, phospho-ACC (Ser79), ACC, EGFR, phospho-EGFR, phospho-4E-BP1 (Thr37/46), 4E-BP1, phospho-ERK1/2, ERK1/2, phospho-mTOR (Ser2448), mTOR, AMPK, phospho-AMPK (Thr172), phospho-glycogen synthase kinase (GSK)3$\beta$ (Ser9), GSK3$\beta$, phospho-Akt (Ser473), Akt, phospho-Stat3 (Tyr705), Stat3, phospho-Jak2 (Tyr1007/1008), and Jak2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody for cyclin D1 was from BD Biosciences. Antibodies for mouse and rabbit conjugated with horseradish peroxidase (HRP) were purchased from Chemicon (Temecula, CA, USA). Western chemiluminescent HRP substrate was from Millipore Corp. (Billerica, MA, USA).

**Curcuminoid Preparation.** The powdered roots of *C. longa* (2.5 kg) were extracted with ethanol (EtOH) at room temperature. The EtOH extract (0.57 kg) was removed in vacuo at 40 °C and extracted successively with *n*-hexane, chloroform ($CHCl_3$), and ethyl acetate (EtOAc) to give four fractions. The EtOAc-soluble fraction (20 g) was dissolved in methanol (MeOH) and then passed through a Sephadex LH-20 column, monitored by Si gel TLC analysis. Subfraction (2 g) was chromatographed on Si gel column using a gradient elution (70 g, 70−230 mesh, 0−10% MeOH in $CHCl_3$) to give three pigments, Cur, DMC, and BDMC (>99% purity). Cur, DMC, and BDMC were dissolved in dimethyl sulfoxide (DMSO) and diluted in medium to different final concentrations in the following experiments.

**Cell Culture.** Cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) (MCF-7, MDA-MB-231, MCF-10A) or DMEM/F12 (BT-20, BT-474, MDA-MB-468) and supplemented with 10% fetal bovine serum (FBS) and 100 units/mL gentamicin at 37 °C and 5% $CO_2$ in 75 $cm^2$ flasks. Cells were passaged by 0.25% trypsin−EDTA when they reached ∼80% confluence.

**Cell Viability.** Cell viability was determined by MTT assay as reported previously.[18] Cells ($1 \times 10^4$) were seeded on the 24-well cell culture cluster overnight. Cells were then treated with different concentrations of agents as indicated in the figure captions and incubated for 48 h. Next, 40 $\mu$L of MTT (stock concentration = 2 mg/mL, Sigma Chemical Co.) was added to each well, the volume of each well was 500 $\mu$L, and incubation was continued for 2 h at 37 °C. MTT−formazan crystals will be formed; then 250 $\mu$L of DMSO was added to dissolve the crystals. Finally, an enzyme-linked immunosorbent assay (ELISA) reader was used to detect absorbance at OD 550 nm.

**Immunoblotting.** Cells ($1 \times 10^6$) were seeded onto a 100 mm tissue culture dish containing 10% FBS. Cells were then treated with various agents as indicated in the figure captions. After treatment, cells were suspended in sodium dodecyl sulfate (SDS) sample buffer, sonicated, and boiled for 10 min. After brief centrifugation, equivalent amounts of proteins from the soluble fractions of cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted as described previously.[19]

**Oil Red O Staining.** Cells ($2 \times 10^5$) were seeded on the 6-well cell culture cluster overnight. After treatment with various agents as indicated in the figure captions, cells were washed with PBS twice and fixed with 10% formaldehyde for 1 h; then they were washed with PBS and 50% isopropanol. Next, Oil Red O staining working buffer was added (stock solution, 3 mg/mL in isopropanol; working solution, 60% Oil Red O stock solution) for 1 h and then washed with PBS and 70% ethanol. Finally, 250 $\mu$L of isopropanol was added to dissolve the Oil Red O, and the absorbance at OD 510 nm was detected by the ELISA reader.

**Statistical Analysis.** The results are expressed as the mean ± SEM. To analyze band intensity, Image Gauge (version 3.12, Fujifilm, Tokyo, Japan) was used. One-way ANOVA was used followed by Newman−Keuls multiple-range test to compare between groups. $P$ values <0.05 were considered to be significant.

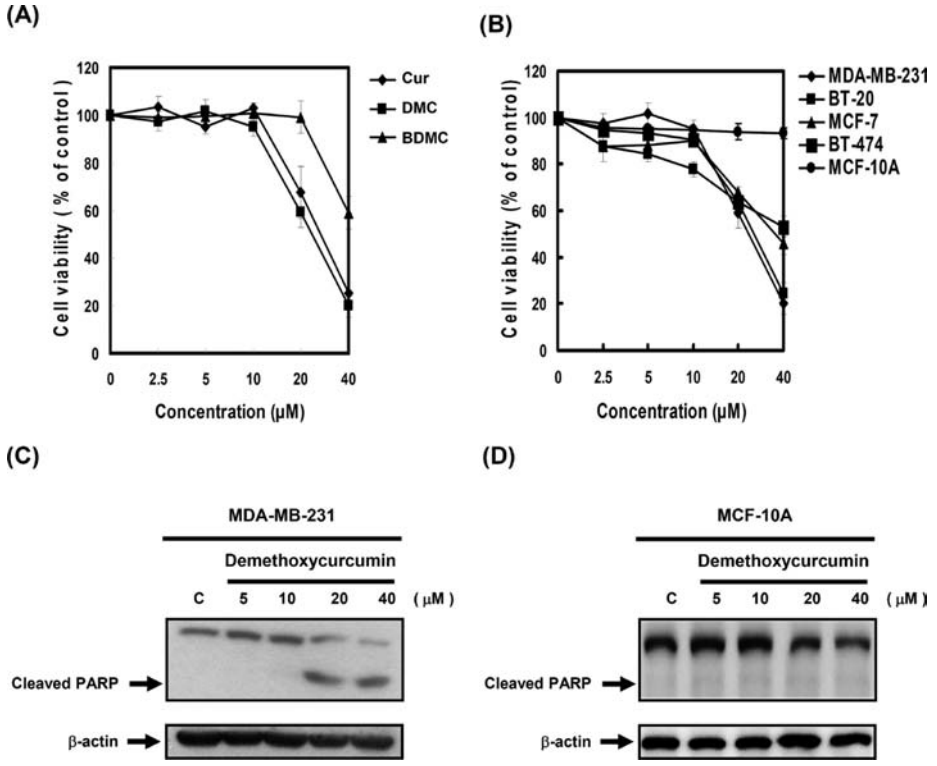


**Figure 1.** Proliferation-inhibitory effect of curcuminoids on TNBC cells. (A) MDA-MB-231 cells were treated with various concentrations of Cur, DMC, and BDMC at 37 °C for 48 h. (B) MDA-MB-231, BT-20, MCF-7, BT-474, and MCF-10A cells were treated with various concentrations of DMC at 37 °C for 48 h. The effect on cell growth was examined by MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without DMC as 100%. This experiment was repeated three times. Bar represents the SEM. (C) MDA-MB-231 cells were treated with DMSO (control) or different concentrations of DMC for 48 h. Cell lysates were analyzed by Western blot with anti-PARP and anti-$\beta$-actin antibodies. (D) MCF-10A cells were treated with DMSO (control) or different concentrations of DMC for 48 h. Cell lysates were analyzed by Western blot with anti-PARP and anti-$\beta$-actin antibodies. Western blot data presented are representative of those obtained in at least three separate experiments.

## RESULTS

**DMC Exhibits High Potency in Inhibiting Cell Proliferation in TNBC Cells.** The antitumor effect of curcuminoids has been successfully demonstrated in a wide range of human malignant cell lines.[20,21] To evaluate the antitumor effect of curcuminoids against TNBC cells, MDA-MB-231 cells were treated with various concentrations of Cur, DMC, and BDMC (Figure 1A) for 48 h and examined for cell viability by MTT assay. Among these curcuminoids, DMC exhibited the most efficient inhibitory effects on TNBC MDA-MB-231 cells. To further confirm the specificity of DMC on TNBC cells, we treated estrogen receptor (ER)-positive (MCF-7), HER2-overexpressing (BT-474), and TNBC (MDA-MB-231 and BT-20) cells with various concentrations of DMC for 48 h and examined for cell viability by MTT assay. Compared with ER-positive or HER2-overexpressing breast cancer cells, DMC demonstrated the most efficient inhibitory effects on TNBC (MDA-MB-231 and BT-20) cells (Figure 1B). Our results also showed that upon treatment with various concentrations of DMC for 24 h, DMC exhibited the most efficient inhibitory effects on TNBC (data not show). Notably, DMC had no apparent effect on the viability of nonmalignant MCF-10A cells over the same concentration range (Figure 1B), indicating the selectivity of DMC for malignant cells. The suppressive effect of DMC on cancer cell viability was, at least in part, attributable to apoptosis, as evidenced by PARP cleavage (Figure 1C). In contrast, MCF-10A cells were not susceptible to the effect of the drug on apoptosis, as indicated by the lack of PARP cleavage (Figure 1D). These results showed that the inhibitory effect of DMC was more remarkable in TNBC cells than in other cells and that there was no apparent effect on nonmalignant MCF-10A cells.

**DMC Decreases mRNA Translation in TNBC Cells via the Up-regulation of AMPK Activity in TNBC Cells.** AMPK, as a tumor suppressor system, has gained more interest because of its character as a negative regulator of tumor proliferation.[3] The level of AMPK phosphorylation in DMC-treated MDA-MB-231 cells was examined to determine if DMC was involved in the regulation of AMPK. Western blot analysis indicated that DMC stimulated AMPK phosphorylation in a dose- and time-dependent manner (Figure 2A). AMPK activation was associated with decreased phosphorylation of mTOR and 4E-BP1 in a dose- and time-dependent manner (Figure 2B). To determine whether DMC suppressed the protein synthesis by activating AMPK to inhibit mTOR pathway, we added compound **c**, an AMPK inhibitor, in the absence or presence of DMC. The AMPK activity was suppressed by compound **c** in the presence of DMC and the mTOR activity was recovered (Figure 2C). A similar effect has been found when cells were treated with metformin (1,1-dimethylbiguanide hydrochloride), a biguanide commonly used in the treatment of type 2 diabetes mellitus (Figure 2C). We hypothesized that DMC, by up-regulating AMPK activity, would inhibit mTOR activation and downstream signaling events. To examine whether the effects of DMC on AMPK activation were general in TNBC cells, we examined the

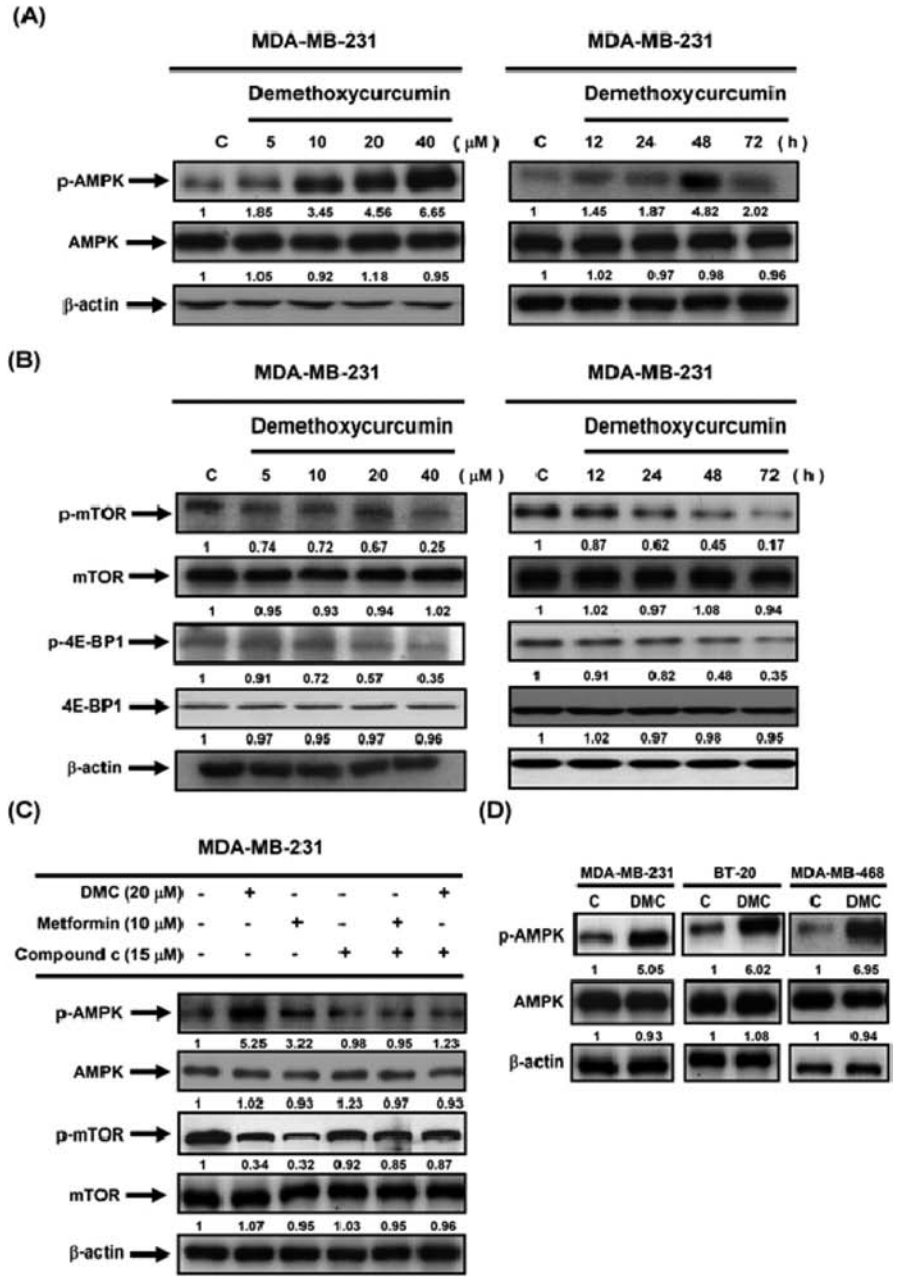


**Figure 2.** DMC activates AMPK and decreases general mRNA translation in TNBC cells. (A) MDA-MB-231 cells were treated with DMSO (control) or different concentrations of DMC for 48 h (left panel) or 20 $\mu$M DMC for different durations (right panel). Cell lysates were analyzed by Western blot with antiphosphorylated AMPK (Thr 172), anti-AMPK, and anti-$\beta$-actin antibodies. (B) MDA-MB-231 cells were treated with DMSO (control) or different concentrations of DMC for 48 h (left panel) or 20 $\mu$M DMC for different durations (right panel). Cell lysates were analyzed by Western blot with antiphosphorylated mTOR (Ser 2448), anti-mTOR, antiphosphorylated 4E-BP1 (Thr 37/46), anti-4E-BP1, and $\beta$-actin antibodies. (C) MDA-MB-231 cells were treated with 20 $\mu$M DMC or 10 $\mu$M metformin in the absence or presence of compound **c** for 48 h. Cell lysates were analyzed by Western blot with antiphosphorylated AMPK (Thr 172), anti-AMPK, antiphosphorylated mTOR (Ser 2448), anti-mTOR, and $\beta$-actin antibodies. (D) MDA-MB-231, BT-20, MDA-MB-468 cells were treated with DMSO (control) or 20 $\mu$M DMC for 48 h. Cell lysates were analyzed by Western blot with antiphospho-AMPK (Thr 172), anti-AMPK, and anti-$\beta$-actin antibodies. Western blot data presented are representative of those obtained in at least three separate experiments. Immunoblots were quantified, and relative expression to control is indicated.

phosphorylation of AMPK in several TNBC cells. Western blot analysis indicated that DMC stimulated AMPK phosphorylation in several TNBC cells (MDA-MB-231, BT-20, and MDA-MB-468) (Figure 2D). These results suggest that the phosphorylation of AMPK is required for DMC inhibited cell proliferation in TNBC cells.

**DMC Decreases Lipid Synthesis by Decreasing FASN Expression and Inhibiting ACC Activity via the Up-regulation of AMPK Activity in TNBC Cells.** The activities of FASN and ACC were known to be negatively regulated by AMPK.[8] We next examined whether DMC decreased lipid synthesis by decreasing FASN expression and inhibiting ACC activity. MDA-MB-231 cells were treated with different concentrations of DMC, and Western blot analysis indicated that DMC decreased the protein levels of FASN and increased ACC phosphorylation in a dose-dependent manner (Figure


dx.doi.org/10.1021/jf4012455 | J. Agric. Food Chem. 2013, 61, 6366−6375

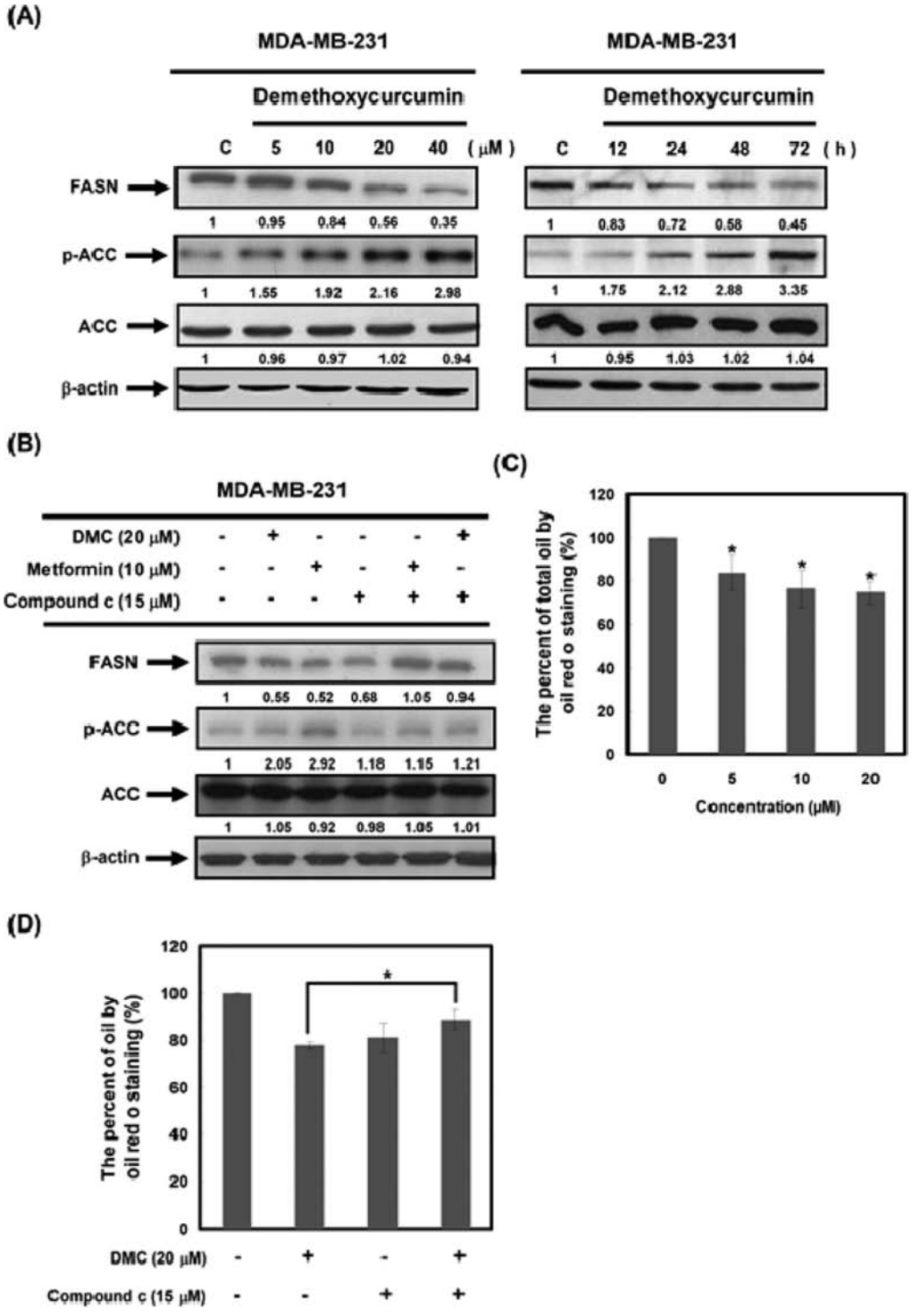


**Figure 3.** DMC activates AMPK and its downstream target, ACC and FASN. (A) MDA-MB-231 cells were treated with DMSO (control) or different concentrations of DMC for 48 h (left panel) or 20 $\mu$M DMC for indicated duration (right panel). Cell lysates were analyzed by Western blot with anti-FASN, antiphospho-ACC, anti-ACC, and $\beta$-actin antibodies. (B) MDA-MB-231 cells were treated with 20 $\mu$M DMC or 10 $\mu$M Metformin in the absence or presence of compound **c** for 48 h. Cell lysates were analyzed by Western blot with anti-FASN, antiphospho-ACC, anti-ACC, and $\beta$-actin antibodies. Western blot data presented are representative of those obtained in at least three separate experiments. Immunoblots were quantified, and relative expression to control is indicated. (C) MDA-MB-231 cells were treated with DMSO (control) or different concentrations of DMC for 48 h. The total lipids were measured by Oil Red O staining, and the percentage of total lipids was calculated by defining the absorption of cells without DMC as 100%. (D) DMA-MB-231 cells were treated with 15 $\mu$M compound **c** in the absence or presence of 20 $\mu$M DMC for 48 h. The total lipids were measured by Oil Red O staining, and the percentage of total lipids was calculated by defining the absorption of cells without DMC as 100%. This experiment was repeated three times. Bar represents the SEM. An asterisk indicates that the values are significantly different from the control ($*$, $p < 0.05$).


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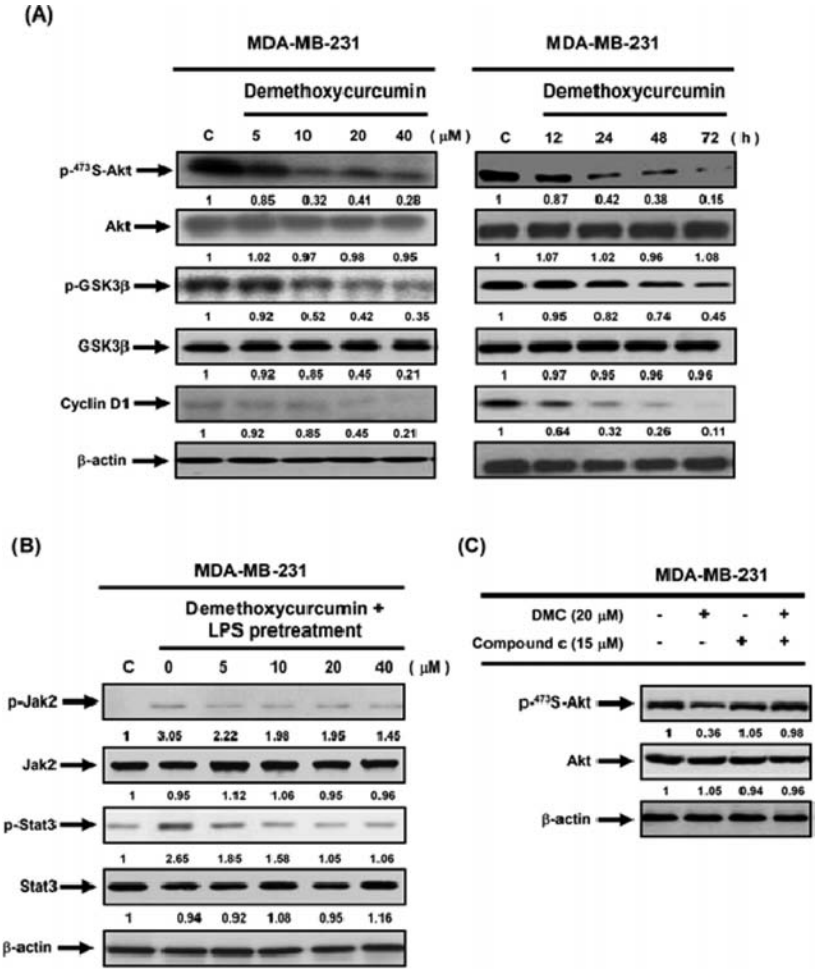


**Figure 4.** Effect of DMC on Akt and IL-6 signaling pathways. (A) MDA-MB-231 cells were treated with DMSO (control) or different concentrations of DMC for 48 h (left panel) or 20 $\mu$M DMC for indicated duration (right panel). Cell lysates were analyzed by Western blot with antiphospho-Akt, anti-Akt, antiphospho-GSK3$\beta$, anti-GSK3$\beta$, anti-Cyclin D1, and anti-$\beta$-actin antibodies. (B) MDA-MB-231 cells were treated with different concentrations of DMC after 6 h of pretreatment with LPS for 24 h. Cell lysates were analyzed by Western blot with antiphospho-AMPK (Thr 172), antiphospho-Jak2 (Tyr 1007/1008), anti-Jak2, Tyr(P)705-Stat3, Stat3, and $\beta$-actin antibodies. (C) MDA-MB-231 cells were treated with 20 $\mu$M DMC in the absence or presence of compound **c** for 48 h. Cell lysates were analyzed by Western blot with antiphospho-Akt, anti-Akt, and $\beta$-actin antibodies. Western blot data presented are representative of those obtained in at least three separate experiments. Immunoblots were quantified, and relative expression to control is indicated.

3A). To address this time-dependent effect, we treated MDA-MB-231 cells with 20 $\mu$M DMC for various periods of time. Western blot analysis indicated that DMC decreased the protein levels of FASN and increased ACC phosphorylation in a time-dependent manner (Figure 3A). To further study the effect of AMPK in regulating the activity of fatty acid synthesis enzymes, we added compound **c** in the absence or presence of DMC. After the treatment of DMC, the protein levels of FASN were decreased and phospho-ACC was increased. However, the activities of enzymes of fatty acid synthesis were restored in the presence of compound **c** in MDA-MB-231 (Figure 3B) cells. We also tested for the effect of treatment with metformin (10 $\mu$M), an AMPK activator, in regulating the activity of fatty acid synthesis enzymes. In the presence of metformin, the protein levels of FASN were decreased and phospho-ACC was increased. The activities of enzymes of fatty acid synthesis were restored in the presence of compound **c** in MDA-MB-231 (Figure 3B) cells. To examine whether DMC influenced total fatty acid content, we treated MDA-MB-231 cells with different concentrations of DMC for 48 h and examined the total lipids by Oil Red O staining. Oil Red O staining analysis indicated that DMC decreased the total fatty acid content in a dose-dependent manner (Figure 3C). This implied that intracellular lipid content could be reduced significantly by treatment with DMC. To further study the effect of AMPK in regulating the total fatty acid content, compound **c**, an AMPK inhibitor, was added in the absence or presence of DMC. Results showed that the lipid content was decreased in MDA-MB-231 cells treated with DMC, but the total fatty acid content was restored when compound **c** was added (Figure 3D). These results might suggest that DMC decreased total lipid content in TNBC cells by activating AMPK.

**DMC Inhibits Oncogenic Signaling Pathway in TNBC Cells.** It has been reported previously that adiponectin-induced AMPK activation facilitated the dephosphorylation of Akt.[22] The level of Akt phosphorylation in DMC-treated MDA-MB-231 cells was examined to determine if DMC-induced AMPK activation facilitated the dephosphorylation of Akt. Western blot analysis indicated that DMC decreased the phosphorylation of Akt at Ser473 in a dose- and time-dependent manner (Figure 4A). This reduction in p-Akt levels was accompanied by parallel decreases in GSK3$\beta$ phosphorylation and cyclin D1


dx.doi.org/10.1021/jf4012455 | J. Agric. Food Chem. 2013, 61, 6366−6375

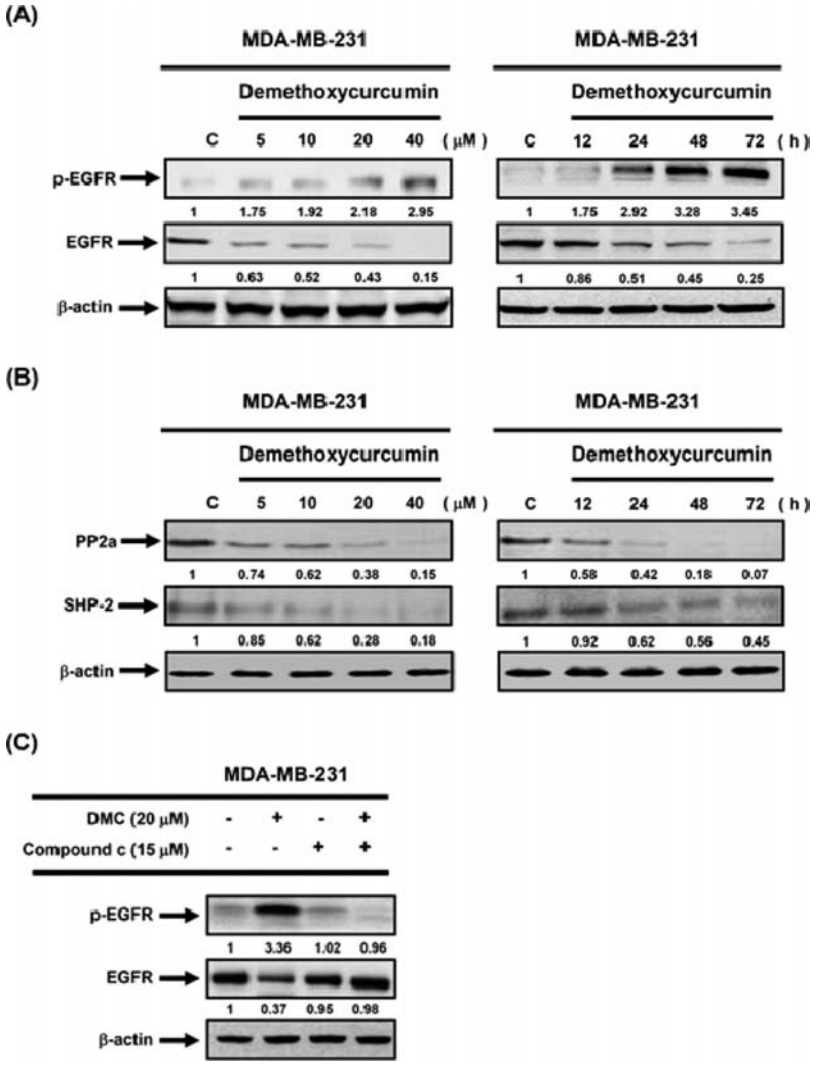


**Figure 5.** DMC down-regulates EGFR and activates EGFR phosphorylation. (A) MDA-MB-231 cells were stimulated with DMSO (control) or different concentrations of DMC for 48 h. Cell lysates were analyzed by Western blot with anti-EGFR, anti-EGFR phosphorylation, and $\beta$-actin antibodies. (B) MDA-MB-231 cells were exposed to 20 $\mu$M DMC for 48 h. Cell lysates were analyzed by Western blot with anti-PP2a and SHP-2 antibodies. An anti-$\beta$-actin antibody was used as the protein loading control. (C) MDA-MB-231 cells were treated with 20 $\mu$M DMC in the absence or presence of compound **c** for 48 h. Cell lysates were analyzed by Western blot with anti-EGFR, antiphospho-EGFR, and $\beta$-actin antibodies. Western blot data presented are representative of those obtained in at least three separate experiments. Immunoblots were quantified, and relative expression to control is indicated.

expression, indicative of the blockade of the Akt signaling cascade (Figure 4A). To determine whether DMC decreased the phosphorylation of Akt by activating AMPK, we added compound **c** in the absence or presence of DMC. AMPK activity was suppressed by compound **c** in the presence of DMC, and the phosphorylation of Akt was recovered (Figure 4C). Moreover, relative to MDA-MB-231 cells, MCF-10A cells exhibited very low phosphorylation levels of Akt and GSK3$\beta$ and were thus not susceptible to the suppressive effects of DMC on Akt signaling, as manifested by a lack of appreciable changes in cyclin D1 expression, a marker of GSK3$\beta$ activation status (data not shown).

AMPK activation has been shown to suppress the expression of IL-6,[23−25] which plays a key role in promoting breast cancer progression via Jak2/Stat3 signaling.[26] We next examined whether DMC was effective in blunting LPS-mediated activation of the IL-6/Jak2/Stat3 pathway through AMPK activation. As shown, relative to DMSO control, LPS stimulated the phosphorylation of Jak2 and Stat3, which, however, could be blocked by DMC in a concentration-dependent manner (Figure 4B). This suppressive effect of DMC on LPS-induced IL-6 production, and the subsequent activation of Jak2/Stat3 signaling was attributable to AMPK activation (Figure 4B).

**DMC Down-regulates EGFR Expression.** EGFR is known to be essential for the growth and maintenance of TNBC.[10−12] To determine if the expression of EGFR was regulated by AMPK, EGFR levels in DMC-treated MDA-MB-231 cells were analyzed by Western blotting. EGFR expression was significantly decreased by treatment with DMC in a dose- and time-dependent manner (Figure 5A). The effect of DMC on EGFR phosphorylation was also examined by Western blotting, which showed a significant increase in EGFR phosphorylation levels in a dose- and time-dependent manner (Figure 5A). To determine whether DMC down-regulated EGFR expression by activating AMPK, we added compound **c** in the absence or presence of DMC. AMPK activity was suppressed by compound **c** in the presence of DMC, and suppressed EGFR expression and increased EGFR phosphor-


dx.doi.org/10.1021/jf4012455 | J. Agric. Food Chem. 2013, 61, 6366−6375

ylation were recovered (Figure 5C). These results demonstrated that DMC, an AMPK activator, suppressed EGFR expression and increased EGFR phosphorylation in MDA-MB-231 cells. To determine if the phosphorylation of EGFR was correlated with phosphatase activity, the expression of PP2a and SHP-2 was examined in DMC-treated cells for 48 h. The expression of these phosphatases was significantly decreased by treatment with DMC compared to the controls (Figure 5B). These results suggest that DMC up-regulated EGFR phosphorylation through phosphatases down-regulation.

## DISCUSSION

In this study, we investigated the effects of three natural curcuminoids on the proliferation of TNBC cells. DMC exhibited greater antiproliferative potency than Cur, and BDMC, more importantly, directly activated AMPK. Recent studies reported that DMC showed the highest antitumor bioactivity in three different curcuminoids.[16,27] Consistent with these studies, we observed that DMC exhibited the most antiproliferative potency in TNBC cells.

The objective of our study was to determine if the viability of TNBC cells was directly regulated by DMC and, if so, what molecules and signaling pathways were involved. Our findings show that DMC is a potent antitumor agent that exhibits in vitro efficacy in suppressing TNBC cell proliferation via diverse AMPK-dependent mechanisms. Decreased AMPK activation is implicated in human metabolic disorders associated with increased cancer risk.[3] Drugs that ameliorate metabolic syndrome conditions through AMPK activation may be beneficial for TNBC cancer prevention and treatment. The association of AMPK with diminished tumor cell viability raises several questions regarding the mechanism by which DMC can suppress tumor growth. In addition to the AMPK-induced down-regulation of mTOR signaling and lipogenesis, DMC also modulates a series of pathways downstream of the AMPK cascade that govern survival and cytokine production.

The mTOR pathway plays a critical role in energy metabolism, growth, and proliferation and has been evaluated as a target for therapy in various malignancies.[28] A recent study showed that PI3K/mTOR inhibitor BEZ235 was more potent and effective than agents that target active receptor tyrosine kinase (RTKs) in TNBC, suggesting that targeting PI3K/mTOR could be important in TNBC. Moreover, knockdown of mTOR profoundly compromised the proliferation of TNBC cell lines.[29] Those results confirm the important role of the mTOR kinase routes in the control of proliferation of TNBC cells. Approaches to block the pathway are being actively pursued in many laboratories and pharmaceutical companies. There are a number of target proteins for which intervention would be predicted to lower mTOR activity and have an impact on cancer cells. Activation of AMPK results in decreased mTOR signaling and in turn inhibition of protein synthesis. The data presented here show the inhibition of protein translation via AMPK-mTOR pathway by DMC.

Differences in energy metabolism between normal and cancer cells are one of the hallmarks of cancer. The general principles of metabolic control analysis can be effective for cancer management as abnormal energy metabolism and biological disorder are characteristics of tumors.[30] The effects of DMC on energy metabolism in TNBC cells, which parallel those reported for metformin in skeletal muscle and neuronal cells,[31] were characterized by the blocked activation or expression of key enzymes involved in fatty acid biosynthesis (ACC and FASN). FASN and ACC are responsible for the synthesis of precursors of cholesterol, and their role in the pathogenesis and progression of breast cancer is well established.[32] Such changes in lipid metabolism have been shown experimentally using fatty-acid synthase inhibitor C75 to induce apoptotic death in breast cancer cells.[33] Jointly, these effects on energy homeostasis can contribute to the antiproliferative activity of DMC by inhibiting fatty acid synthesis and shifting cellular metabolism toward oxidation.

The suppressive effect of DMC on Akt phosphorylation is particularly noteworthy, because it circumvents the feedback activation of Akt that results from mTOR inhibition, a drawback to the use of early-generation mTOR inhibitors. The ability of DMC to concurrently block signaling through both kinases, reminiscent of that of the second generation mTOR inhibitors and dual PI3K-mTOR inhibitors,[34] provides therapeutic advantages over rapamycin. The ability of DMC to suppress IL-6 production is clinically relevant in light of the major role of this cytokine in driving Stat3 activation in breast cancer.[35] Stat3 represents an important therapeutic target in breast cancer because constitutively activated Stat3 occurs in >50% of primary breast tumors, is associated with a poor prognosis, and endows tumor cells with chemoresistance and angiogenic potential.[36]

A subclass of TNBC overexpresses EGFR, and exhaustive gene expression profiling identified several EGFR-associated poor prognostic signatures.[37] Contrary to other breast cancer subtypes, for which therapy targeting biological drivers proved to be successful, no molecular targeted agents are found for TNBC. Therefore, down-regulation of EGFR plays a fundamental role in the phenotypic changes of TNBC.[38] In this study, we found that DMC decreased the expression of the phosphatases, PP2a and SHP-2, whereas phosphorylation of EGFR was elevated. These results indicated that AMPK induced EGFR degradation by regulating phosphatase expression in TNBC cells.

Recent studies have shown that Cur, a member of the curcuminoids, has potent antitumor effects on breast cancer.[39] However, Cur can be easily degraded both in vitro and in vivo.[40] The antitumor activity of Cur will be enormously reduced, and this instability property of Cur limits its clinical use in cancer treatment. It is feasible to develop new stable compounds that are structurally similar to Cur but without the loss of antitumor activity. DMC, an analogue of Cur, is one such compounds. In comparison with Cur, the structure of DMC lacks one methoxy group directly linking to the benzene ring. Although the structure difference between Cur and DMC is slight, the chemical characteristics of DMC are more stable. Moreover, despite this broad spectrum of antitumor activities, nonmalignant MCF-10A mammary epithelial cells were unaffected by DMC. These results provided the translational potential of DMC, a novel AMPK activator, as a therapeutic agent for TNBC.

In conclusion, these results show that DMC is a novel AMPK activator with a distinct mode of action that inhibits TNBC by modulating multiple signaling pathways associated with cancer cell survival, metabolism, and progression. Future studies will focus on elucidating the activity of DMC in other preclinical models of cancer.

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### Funding

This study was supported by the National Science Council of the Republic of China, Grant NSC 101-2320-B-039-031-MY3. Thanks are also due to support (in part) by grants from the China Medical University (CMU100-N2-06 and CMU99-COL-29-2).


### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS USED

4E-BP1, eukaryotic initiation factor 4E-binding protein-1; ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; BDMC, bisdemethoxycurcumin; Cur, curcumin; DMEM, Dulbecco's modified Eagle's medium; DMC, demethoxycurcumin; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; ER, estrogen receptor; FASN, fatty acid synthase; FBS, fetal bovine serum; HER2, epidermal growth factor receptor-2; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase; LPS, lipopolysaccharides; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; TNBC, triple-negative breast cancer

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