Effect of curcumin and paclitaxel on breast carcinogenesis

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Abstract. Global cancer burden increased to 14.1 million new cases in 2012; and breast cancer is the most common cancer in women worldwide, with nearly 1.7 million new cases diagnosed in 2012. Curcumin is the major bioactive ingredient extracted from the rhizome of the plant Curcuma longa (turmeric). Paclitaxel is a microtubule-stabilizing agent originally isolated from the bark of Taxus brevifolia. Curcumin and paclitaxel were evaluated with two human breast cancer cell lines as the luminal MCF-7 and the basal-like MDA-MB-231 that are either positive or negative for hormonal receptors estrogen receptor, progesterone receptor and HER2, respectively. Results indicated that curcumin combined with paclitaxel decreased c-Ha-Ras, Rho-A, p53 and Bcl-xL gene expression in comparison to control and substances alone in MCF-7 cell line. These two substances alone and combined decreased gene expression of Bcl-2 and NF-κB. However, CCND1 increased when both substances were combined in MCF-7 cells. Such substances decreased Bcl-2 and increased Bax protein expression. However, curcumin alone decreased IκBα and Stat-3 gene expression. Paclitaxel alone and combined increased IκBα and Stat-3. Curcumin alone and combined with paclitaxel increased p53, Bid, caspase-3, caspase-8 and Bax gene expression in MDA-MB-231, whereas Bcl-xL decreased such expression in MDA-MB-231 cells. When paclitaxel and curcumin were combined the expression of Bcl-2 protein was decreased. However, either substance alone and combined increased Bax protein expression corroborating the apoptotic effect of these substances. It can be concluded that curcumin may be of considerable value in synergistic therapy of breast cancer reducing the associated toxicity with use of drugs.

Introduction

Global cancer burden was reported as 14.1 million new cases in 2012; breast cancer is the most common cancer in women worldwide, with nearly 1.7 million new cases were diagnosed in 2012. This represents ~12% of all new cancer cases and 25% of all cancers in women (1).

Curcumin is the major bioactive ingredient extracted from the rhizome of the plant Curcuma longa Linn (turmeric). Curcumin has chemopreventive properties against various malignancies, and extensive biological and pharmacological function, for example, anticaner, antioxidant, anti-inflammatory, antibacterial, antispasmodic and anticoagulant, without any major side effects (2,3). As a pharmacologically safe agent, curcumin could be used alone to prevent cancer and in combination with chemotheraphy to treat cancer. Different molecular targets are modulated by curcumin. It induces apoptosis by means of the inhibition of NF-κB activity through downregulation of upstream kinases, IKK-β and IKK-α (4).

Paclitaxel is a microtubule-stabilizing agent originally isolated from the bark of Taxus brevifolia. It is a potent agent which inhibits neoplastic growth in several malignancies including ovarian, breast, non-small cell lung cancer, head and neck cancers (5-7). As with the majority of anticancer agents, paclitaxel causes cancer cell death with signs of apoptosis and also it is supposedly associated with mitotic catastrophe (8,9).

c-Ha-ras is an oncprotein corresponding to a GTP-binding protein (10). DNA transfection studies have suggested that an activated c-Ha-ras oncogene can convert human breast cancer cells to a more aggressive, estrogen-independent phenotype (11). Ras homolog gene family member A (Rho-A) is a small (~22 kDa) G protein/guanosine triphosphatase that is part of the Ras-related C3 botulinum toxin substrate (Rac) subfamily of the Rho family (12). Rho-A can reorganize the cell cytoskeleton and regulate cell migration by activating effector proteins such as Rho-associated coiled-coil kinase (ROCK) (13); such changes are associated with tumor invasion and migration in several types of carcinoma cells (14,15). p53, the tumor suppressor protein plays a key role with respect to apoptosis but also senescence, growth arrest and DNA repair (16,17).

Apoptosis is a process of programmed cell death that occurs in response to environmental stimuli and appropriated strategy for prevention and treatment of cancer (18). Gene and protein expression of Bax in breast cancer cells increase sensitivity to apoptotic stimuli and decrease tumor growth. Apoptosis is a phenomenon usually linked to the presence of functional p53 (19). Bcl-2 protein family plays an important role in the survival or death of a cell and it is a target of many
antitumor drugs (20,21). The Bcl-2 protein family, of which Bax is a member, plays a critical role in determining either cell death or survival (22,23). Bax is a pro-apoptotic protein that acts as an enhancer of apoptosis while Bcl-2 has anti-apoptotic properties as Bcl-xL that inhibits apoptosis and stimulates the progress of breast cancer (20,24). In particular, the balance between Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) protein levels is important for the regulation of apoptosis (25). Overexpression of Bax leads to apoptosis in the absence of any stimulus, suggesting that tight regulation of Bax, from transcription to post-translational, is necessary for cell survival (26). Anti-apoptotic Bcl-2 family members are overexpressed in a variety of cancers through genetic alterations, such as chromosomal translocation (Bcl-2) or amplification (Bcl-xL and Mcl-1) (27-29).

The nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) is a pro-inflammatory and pro-survival transcription factor and it is known to be highly involved in the initiation and progression of breast cancer (30). NF-κB deregulation of activity alters the expression of cell death-regulating genes, leading to the upregulation of anti-apoptotic and pro-survival genes, such as members of the Bcl-2 family (31). Protein inhibitors of κB (IκB) suppress activation of NF-κB including IκBα, IκBβ, IκBe and IκBε (32-40). The aim of this study is to demonstrate the effect of curcumin and paclitaxel on gene and protein expression related to apoptosis in human breast cancer cell lines to provide new therapeutic potential of curcumin combined with paclitaxel on human breast cancer and to establish whether paclitaxel acquired chemoresistance in breast cancer cells.

Materials and methods

Cell culture conditions. Human breast carcinoma cell lines MDA-MB-231 (ATCC® HTB26™) and MCF-7 (ATCC® HTB22™) were maintained in a culture medium at 37°C in a humidified atmosphere of 5% CO₂ in air. The culture medium consisted of basic medium supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA). The basic medium was RPMI-1640 for MDA-MB-231 and minimum essential media (Life Technologies) for MCF-7 with 10 µg/ml of human insulin (Sigma-Aldrich, St. Louis, MO, USA), both supplemented with 1% penicillin-streptomycin (Life Technologies). Curcumin and paclitaxel were obtained from Sigma-Aldrich. Both substances were dissolved in dimethylsulfoxide (0.1%). Cells were seeded after 24 h prior to treatment with curcumin (15 and 30 µM), paclitaxel (10-240 nM) and combination of these two drugs. All the treatments were performed at 37°C. Cells were harvested and seeded at 4x10⁴ cells/ml of culture medium into 24-well plates (Corning Costar, Corning, NY, USA). After a 24-h pre-incubation period allowing cells to attach, the culture medium was replaced by either culture medium without drugs or medium with one of tested treatments of curcumin and/or paclitaxel at desired concentrations for 48 h and it was replaced by culture medium without drugs for 96 h. Cells were stained with trypan blue (Sigma-Aldrich) with a 1:9 ratio of trypan blue for cell suspension. Cells were counted in TC20™ Automated Cell Counter (Bio-Rad Laboratories, Hercules, CA, USA). Dark blue cells were evaluated as dead cells.

Doubling time (DT). Cell growth was observed and counted at 0, 24, 48 and 96 h to determine doubling time (h). Upon treatment, both control and treated cells were replated as described above. Their growth was measured by determining the total number of doublings by applying the formula: Total doublings: \[ TD = \ln N_t / \ln N_0 \] where \( N_t \) and \( N_0 \) are the number of cells at times \( t \) and \( t_0 \), \( N_t \) is the total number of cells counted at confluence and \( N_0 \) is the initial plating density. Total doublings were evaluated over multiple passages. The number of doublings was determined by dividing the total number of doublings by the number of days which each particular sample was followed. Based upon these values, the time required for the total cell number to double from any reference point, or doubling time, was calculated (41).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), and the concentration and purity of RNA were determined using a UV spectrophotometer (Thermo Scientific, Rochester, NY, USA). Total RNA was reverse transcribed into cDNA using high capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) and 10 U of RNase inhibitor (Applied Biosystems) according to the manufacturer’s protocol. A CFX 96 Touch Real-Time PCR Detection systems (Bio-Rad Laboratories) was used with an aliquot of cDNA (2 µl) in 20 µl qPCR reaction containing SYBR Green PCR Master Mix (Agilent, La Jolla, CA, USA) for measurement of target genes such as c-Ha-ras, Rho-A, p53, Bax, Bcl-xL, CCND1, NF-κB, IκBα, IκBβ, IκBe, Stat-3, Bid, caspase-3 and caspase-8, and β-actin as reference to obtain the relative fold change for target genes using the comparative Ct method and using Bio-Rad CFX Manager 2.1 software. Table I shows the primers for the genes selected to develop cDNA probes. Relative expression was normalized to the average of controls.

Western blot analysis. Cells were lysed with 1 ml lysis buffer (pH 7.2) [Tris Base (50 mM), NaCl (100 mM), EDTA (1 mM), Ortovanadate (1 mM), PMSF (1 mM), Triton X-100 (0.1%) and centrifuged (13,200 rpm x 15 min)]. The supernatant with cellular proteins was dissolved in SDS-PAGE sample solution (60 mM) Tris, pH 6.5, 10% (w/v) glycerol, 5% (w/v) β-mercaptoethanol, 20% (w/v) SDS, and 0.025% (w/v) bromophenol blue and denatured by boiling (2x5 min), and vortex mixing (2x30 sec). The total amount of protein was 30 µg in each lane with standard protein markers (Bio-Rad Laboratories). After fractionation by SDS-PAGE on gels (7x14 cm), proteins were electro-blotted onto PVDF membrane (Amersham, Biosciences, Amersham, UK) using a blotting apparatus (Bio-Rad Laboratories). Prestained SDS-PAGE (Standards) blots were blocked for 2 h in 10% defatted dry milk-TBS-0.1% Tween and then incubated for 2 h at room temperature with corresponding primary antibodies [1:200, Bcl-2 (sc-7382), Bax (sc-7480), and β-actin (Sigma A-5316) followed by incubation with secondary peroxidase-conjugated mouse IgG (1:5,000) (Cell Signaling, CA, USA) in 5% defatted dry milk-TBS-0.1% Tween]. All steps were performed at room temperature, and blots were rinsed between incubation steps with TBS-0.1% Tween. Cell blots were probed with mouse anti-β-actin antibody as control. Immunoreactive bands were visualized by using the ECL™ Western Blotting Detection Reagent
detection method (Amersham, Dübendorf, Switzerland) and exposure of the membrane to X-ray film (Kodak, Rochester, NY, USA). Protein determination was performed using the Bicinchoninic Acid Method (Bio-Rad Laboratories) and BSA as the standards. Experiments were performed in triplicates.

**Statistical analysis.** Data were expressed as the average ± standard error of the mean (SEM). Comparisons of multiple groups were performed between treated groups and controls carried out by ANOVA and Dunnet's test with GraphPad Prism 5 software. A P<0.05 was considered to be significant. Assays were performed at least three times independently.

### Results

**Effect of curcumin and paclitaxel on DT of tumor cell lines.**

The DT of curcumin and paclitaxel was evaluated with two cell lines that are either positive or negative for hormonal receptors (ER⁺ PR⁺ and HER2⁺) as the luminal MCF-7 and the basal-like MDA-MB-231, respectively. Fig. 1 indicates that curcumin at 15 µM and paclitaxel at 120 nM had significantly (P<0.05) longer DT than control and substances alone with other combinations in MCF-7 cell line. On the other hand, these results showed that MDA-MB-231 cell line had significantly (P<0.05) longer DT with curcumin at 15 µM combined with paclitaxel at 10, 20 and 240 nM as well as curcumin at 30 µM with paclitaxel at 10 nM than control and curcumin at 15 µM alone. Therefore, these combinations were used for determining several important gene targets in malignancy and apoptosis. All the doses used significantly decreased the rate of growth of cells above its controls as indicated by the DT after several passages.

**Gene expression in MCF-7 breast cancer cells.** Gene expression analyzed by RT-qPCR indicated that curcumin combined (15 µM) with paclitaxel

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Product length (bp)</th>
<th>Primer sequencea</th>
<th>Primer sequenceb</th>
</tr>
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<tbody>
<tr>
<td>c-Ha-ras</td>
<td>112</td>
<td>F: CACCAGTACAGGGAGCAGAT</td>
<td>R: GAGCCTGCGAGATATCCACA</td>
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<tr>
<td>Rho-A</td>
<td>140</td>
<td>F: CCATCATCTCGTTGGGAAT</td>
<td>R: CATGTACCACAAAGCGCCA</td>
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<tr>
<td>p53</td>
<td>128</td>
<td>F: CCTCAGCATCTTATCCGAGTGG</td>
<td>R: TGGAATGGTGTCACAGTCAGC</td>
</tr>
<tr>
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<td>143</td>
<td>F: GCGAGTGTCTCAAGCGCATC</td>
<td>R: CCAGTTGAGGTGGCAGTCAGA</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>211</td>
<td>F: CTTGACCTACTATGAGAAC</td>
<td>R: TGGAATGGTGTCACAGTCAG</td>
</tr>
<tr>
<td>CCND1</td>
<td>60</td>
<td>F: GAGGCCTCCTAAGTAGGAAGGA</td>
<td>R: GGTGTAGATGCACAGCTTCT</td>
</tr>
<tr>
<td>NF-κB</td>
<td>114</td>
<td>F: ATCTGCCGAGTGAAACCGAAAATC</td>
<td>R: CCAGCCTCCTGGCAGTGAA</td>
</tr>
<tr>
<td>IkBa</td>
<td>135</td>
<td>F: CTCCGAGACTTCAGAGAGAAC</td>
<td>R: GCCATTTGATGTGAGCTTCA</td>
</tr>
<tr>
<td>Stat-3</td>
<td>163</td>
<td>F: GGTGGACATGACACCGACTAT</td>
<td>R: AGGGCAGACTCAAACTTTCAG</td>
</tr>
<tr>
<td>Bid</td>
<td>199</td>
<td>F: GCTTCCAGTGTAGACGGAGC</td>
<td>R: GTGCAGATCTCATGTGAGT</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>192</td>
<td>F: CAGAACTGGACTGTGGCATTG</td>
<td>R: GCTTGCAGATGTTCAG</td>
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<tr>
<td>Caspase-8</td>
<td>128</td>
<td>F: CATCCAGTCTATTTGGGAG</td>
<td>R: GCATCTGGTTTCCATGTT</td>
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<tr>
<td>β-actin</td>
<td>569</td>
<td>F: ACTACCTCATGGAAGATCTTCT</td>
<td>R: TAGAAGCATTTGCGGATGAGG</td>
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Table I. Selected primers for target genes to develop cDNA probes.

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Detection and exposure of the membrane to X-ray film (Kodak, Rochester, NY, USA). Protein determination was performed using the Bicinchoninic Acid Method (Bio-Rad Laboratories) and BSA as the standards. Experiments were performed in triplicates.

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Table I. Selected primers for target genes to develop cDNA probes.

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1. Length of cDNA product amplified by gene-specific RT-qPCR analysis.
2. PCR primer sequences used to generate a product of the indicated size, listed in 5'-3' orientation. F, forward; R, reverse.
Figure 1. Doubling time of (A) MCF-7 and (B) MDA-MB-231 cell lines exposed to different doses of curcumin (0, 15 and 30 µM) and paclitaxel (0, 10, 20, 120 and 240 nM). Bars represent the mean ± SEM of three independent experiments.

Figure 2. Effect of curcumin (15 µM) and paclitaxel (120 nM) on gene expression analyzed by RT-qPCR on (A) c-Ha-Ras, (B) Rho-A, (C) p53, (D) Bax and (E) Bcl-xL gene expression in MCF-7 cell line. Bars represent the mean ± SEM of three independent experiments (*P<0.05).
Figure 3. Effect of curcumin (15 µM) and paclitaxel (120 nM) on gene expression analyzed by RT-qPCR on (A) CCND1, (B) NF-κB, (C) IκBα, (D) Stat-3 gene expression in MCF-7 cell line. Bars represent the mean ± SEM of three independent experiments (*P<0.05 versus control cells).

Figure 4. Western blot analysis of (A) Bcl-2 and (C) Bax protein expression in MCF-7 cell line treated with paclitaxel (120 nM) and curcumin (30 µM). β-actin was used as control for loading. Western blotting results were quantified with Adobe Photoshop program and were expressed as average density to β-actin. (B and D) Bars represent the mean ± SEM of three independent experiments (*P<0.05).
Figure 5. Effect of curcumin (30 µM) and paclitaxel (10 nM) on gene expression analyzed by RT-qPCR on (A) p53, (B) Bid, (C) caspase-3, (D) caspase-8, (E) Bax and (F) Bcl-xL gene expression in MDA-MB-231 cell line. Bars represent the mean ± SEM of three independent experiments (*P<0.05).

Figure 6. Western blot analysis of (A) Bcl-2 and (C) Bax protein expression in MDA-MB-231 cell line treated with paclitaxel (10 nM) and curcumin (30 µM). β-actin was used as control for loading. Western blotting results were quantified with Adobe Photoshop program and were expressed as average density to β-actin. (B and D) Bars represent the mean ± SEM of three independent experiments (*P<0.05).
(120 nM) significantly (P<0.05) decreased c-Ha-Ras, Rho-A, p53 and Bcl-xL gene expression in comparison to control and substances alone in MCF-7 cells as seen in Fig. 2A-D. Gene expression indicated that curcumin (15 µM) and paclitaxel (120 nM) alone significantly (P<0.05) increased Bax expression in comparison to control and substances alone in MCF-7 as seen in Fig. 2D.

Curcumin and paclitaxel alone and both combined significantly (P<0.05) decreased CCND1 (Fig. 3A) and NF-κB (Fig. 3B), whereas curcumin alone decreased IκBα and Stat-3 gene expression (Fig. 3C) and paclitaxel alone and combined increased them (Fig. 3D) in MCF-7 cells in comparison to control.

Effect of curcumin and paclitaxel on protein expression by western blot analysis in MCF-7 breast cancer cell line. Results indicated that either paclitaxel at 120 nM or curcumin at 30 µM alone and combined (Fig.4A and B) significantly decreased Bcl-2 and increased Bax protein expression (Fig. 4C and D) in MCF-7, in comparison to control corroborating the apoptotic effect of these substances. Normalized fold protein expression is presented in graphs of Fig. 4B and D.

Effect of curcumin and paclitaxel on gene expression in MDA-MB-231 breast cancer cell line. Gene expression analyzed by RT-qPCR in MDA-MB-231 cells is shown in Fig. 5 where results indicate that curcumin at 30 µM alone and combined with paclitaxel at 10 nM significantly (P<0.05) increased p53 (Fig. 5A), Bid (Fig. 5B), caspase-3 (Fig. 5C), caspase-8 (Fig. 5D) and Bax (Fig. 5E) gene expression, whereas Bcl-xL decreased such expression (Fig. 5F) in comparison to control.

Effect of curcumin and paclitaxel on protein expression by western blot analysis in MDA-MB-231 breast cancer cell line. Results indicated that paclitaxel at 10 nM and curcumin at 30 µM combined significantly (P<0.05) decreased Bcl-2 protein expression in comparison to control and either substance alone. However, either substance alone and combined increased Bax protein expression in MDA-MB-231 corroborating the apoptotic effect of these substances (Fig. 6A and C). Normalized fold protein expression is presented in graphs of Fig. 6B and D.

Discussion

Chemotherapy in breast cancer fails due to resistance to drugs. Chemotherapeutic strategies are now making use of combined active compounds where they are believed to be more active as compared to the single agent itself. Therefore, the efficacy of treatment would increase and the possibility of toxic effect might be lowered due to the extremely low usage of drug (42). A phytochemical such as curcumin can be used in a therapeutic modality as it elicits antitumor effects. Curcumin is a natural compound derived from turmeric (Curcuma longa) and exhibits an antitumorigenic effect on various cancers. Curcumin and paclitaxel effect was evaluated with the luminal MCF-7 and the basal-like MDA-MB-231 cells, respectively, these cell lines are positive and negative for hormonal receptors (ER+ PR+ and HER2+), respectively.

Curcumin combined with paclitaxel decreased c-Ha-Ras, Rho-A and p53 gene expression in comparison to control and substances alone in MCF-7 cell line. This is the first report that demonstrated that an antioxidant as curcumin and a chemotherapeutic drug reduces c-Ha-Ras, Rho-A and p53 gene expression. Gene expression levels supported the idea that paclitaxel induces changes in important signaling pathways.

Studies have indicated that curcumin is a pharmacologically safe compound, it has a therapeutic potential in preventing breast cancer metastasis possibly through suppression of NF-κB (43). Since curcumin suppresses NF-κB activation and most chemotherapeutic agents activate genes that mediates cell survival, proliferation, invasion, and metastasis (43), we analyzed whether curcumin would potentiate the effect of chemotherapy in breast cancer cell lines. Thus, curcumin decreased expression of NF-κB supporting the conclusion of other reports (44) that the mechanism of growth inhibition induced by combined effect of paclitaxel and curcumin is through NF-κB inhibition. It was demonstrated that curcumin suppressed the paclitaxel-induced NF-κB pathway in breast cancer cells by inhibiting lung metastasis of human breast cancer in nude mice (43).

Curcumin combined with paclitaxel decreased Bcl-xL gene expression in comparison to control and either substance alone in MCF-7 cell line. Other authors reported on the capability of inducing apoptosis in MCF-7 by several drugs (45). On the other hand, either substance alone increased Bax expression in comparison to control and either substance alone in MCF-7 cells. Curcumin, paclitaxel and combination of both decreased CCND1, whereas either substance alone decreased IκBα and Stat-3 gene expression in MCF-7 cells in comparison to control. Results indicated that either substance alone as curcumin and paclitaxel as well as combined decreased Bcl-2 and increased Bax protein expression in comparison to control in MCF-7 cells, corroborating the apoptotic effect of these substances. It has also been suggested that inhibition of activity of proteins may improve the efficacy of chemotherapeutic agents (44). It has been shown that curcumin induced apoptosis in breast cancer cells and delayed the outgrowth of mammary tumors in neu transgenic mice (46). The combination of docosahexaenoic acid and curcumin inhibited 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumorigenesis in mice (47). In addition, curcumin can reverse multidrug resistance in human colon carcinomas and lung cancer cells in vitro and in vivo (48,49). Authors have shown that curcumin can inhibit epithelial mesenchymal transition by affecting transcription factors as E-cadherin and Vimentin genes and invasive capabilities in breast cancer cell lines (50,51).
When MDA-MB-231 cell line was analyzed it was found that curcumin alone and combined with paclitaxel increased p53, Bid, caspase-3, caspase-8 and Bax gene expression, whereas Bcl-xL decreased such expression in comparison to control. Results indicated that paclitaxel and curcumin combined decreased Bcl-2 protein expression in comparison to control and either substance alone. However, either substance alone and combined increased Bax protein expression in MDA-MB-231 corroborating the apoptotic effect of these substances. Curcumin decreased expression of apoptotic genes such as caspase-3, caspase-8, and other genes such as cyclin D1.

We demonstrated that curcumin increased the sensitivity of MCF-7 and MDA-MB-231 cells to chemotherapeutic drugs as paclitaxel. It has been proposed that combined effect of natural products may improve the effectiveness of treatment in the process of proliferation of cancer cells (42).

Several studies have aimed to delineate the complex molecular background of breast cancer, which has advanced personalized treatment approaches and enabled the development of several agents that target specific molecular aberrations associated with breast cancer. Nevertheless, tumor heterogeneity poses obvious impediments to the successful clinical development of targeted agents. The combined anticancer activities of a compound of natural origin such as curcumin has been examined in combination with a chemotherapy drug such as paclitaxel and it was observed that paclitaxel-curcumin compound exhibited synergistic growth inhibition and induced significant apoptosis in MCF-7 and MDA-MB-231 cell lines. In conclusion, curcumin may be of considerable value in synergistic therapy of cancer since it may reduce the associated toxicity and it has a therapeutic potential with a drug such as paclitaxel in breast cancer.

Acknowledgements

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References