Overexpression of ABCC2 and NF-κB/p65 with reduction in cisplatin and 4OH-tamoxifen sensitivity in MCF-7 breast cancer cells: the influence of TNF-α

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Abstract

**Background:** TNF-α, as a pro-inflammatory cytokine in the tumor microenvironment is able to regulate the expression and function of various ATP binding cassette (ABC) transporters involved in clinical drug resistance and among them, ABCC2 transporter is represented to contribute to cancer multidrug resistance (MDR) by drug efflux.

**Methods:** In this study, we aimed to evaluate the effects of TNF-α and/or E2 (17β-estradiol) on the mRNA and protein expression levels of ABCC2 and NF-κB (p65) transcription factor in estrogen receptor positive (ER+) MCF-7 cells by QRT-PCR and Western blot analysis. Also, we used MTT assay to study the cell sensitivity against the active form of tamoxifen (4OH-TAM), a hypothetical substrate and Cisplatin (Cis), a well-known substrate for ABCC2 used in endocrine and chemo-therapy of breast cancers, respectively. Data were analyzed by one-way ANOVA and Tukey tests. Significance was considered in P-values < 0.05.

**Results:** The expression levels of ABCC2 and the active form of NF-κB (p65) were significantly increased following 20-day concomitant treatment with TNF-α and E2, compared to untreated cells as control. Also, the viability assay showed that 20-day TNF-α+E2 treatment led to more sensitivity reduction of MCF-7 cells to Cis and 4OH-TAM compared to E2-treated and untreated cells.

**Conclusion:** Based on our findings, there is a positive correlation between ABCC2 overexpression, over-activity of NF-κB/p65 and decreasing the sensitivity of MCF-7 cells to Cis and 4OH-TAM following TNF-α treatment in MCF-7 cells. Further experiments are needed to elucidate possible mechanistic relationship of these findings and their clinical significance in order to circumvent the drug-resistance in breast tumors.
Key words: Breast cancer, TNF-α, 17β-estradiol, ABCC2, NF-κB/p65, Cisplatin, 4OH-Tamoxifen
Introduction

With no doubt, there is a strong connection between inflammation and cancer.\textsuperscript{1} TNF-\(\alpha\), one of the outstanding pro-inflammatory cytokines, initially found as a result of its antitumor activity, has now been represented to be one of the important mediators of inflammation. It is present in cancer microenvironment and is secreted by infiltrating immune and stroma cells (mainly by tumor-associated macrophages) or even tumor cells themselves. TNF-\(\alpha\) expression is high in biopsies and in the plasma of patients with multiple advanced cancers.\textsuperscript{2} There is a link between chronic, low level TNF-\(\alpha\) exposure and the acquisition of some tumor properties such as tumor proliferation, angiogenesis and metastasis.\textsuperscript{3} On the other hand, it can influence the sensitivity of the tumor cells to drug treatment which is associated with both endocrine and chemo-therapy failures.\textsuperscript{4-6}

MDR is a major problem in cancer therapy. In this phenomenon, exposure of cancer cells to a chemotherapeutic drug may result in resistance to it and also cross resistance to many other structurally and mechanically different chemotherapeutics.\textsuperscript{7} MDR may occur due to different mechanisms including decreased drug accumulation and increased drug efflux, alterations in drug target, over-activity of drug detoxifying enzymes, activation of DNA repair mechanisms, alterations in cell cycle regulation, evasion of apoptosis and the overexpression of ATP-binding cassette (ABC) transporters that pump out chemotherapeutics.\textsuperscript{8-10}

ABCC2 or the multidrug resistance protein 2 (MRP2) called canalicular multi-specific organic anion transporter (cMoat) is a transmembrane ABC transporter placed at the canalicular domain of hepatocytes. It is also found in most excretory organs such as renal, small intestine, colon, gall bladder, and lung.\textsuperscript{11,12} Notably, ABCC2 is expressed in malignant tissues and plays a pivotal role in the transport of chemotherapeutic agents (e.g. methotrexate, vinca alkaloids and anthracyline) and different conjugated metabolites.\textsuperscript{13,14}
Cis (Cis-dichlorodiammine platinum (II) (CDDP)) is a well-known and the first platinum-containing chemotherapeutic drug used to treat a wide range of human malignancies for example testicular, head and neck, esophageal, lung, ovarian and breast. Various factors influence the uptake of Cis including sodium and potassium ion concentrations, pH, the attendance of reducing agents, and especially transporters. ABCC2 is one of the key actors of Cis transporting across the cell. From the past until now, many evidences have highlighted the importance of ABCC2 efflux pump in drug resistance in different cell lines such as human embryonic kidney (HEK-293), Cis-resistant human cell lines (T24IDDP1O, P/CDP5and KB/KCP4), and two human gastrointestinal tumor cell lines (HepG2 and PANC-1). The expression of ABCC2 transporter could also be considered as an important biomarker for Cis chemotherapy response in different solid tumors.

TAM as a selective estrogen receptor modulator (SERM) is the basis of anti-estrogen therapy for ER+ breast cancers composed of 75% of breast tumors. Acquired resistance to TAM is a major obstacle to effective endocrine therapy in breast cancer patients. To our knowledge, tamoxifen could be a substrate for ABC transporter proteins and its intracellular accumulation is affected by efflux transporters.

Increasing number of studies showed that the expression levels of ABCC2 is higher in TAM-resistant in comparison with TAM-sensitive breast cancer cells; Therefore, it has been speculated that ABCC2 might participate in efflux of the active metabolites of TAM (e.g.4OH-TAM) from breast cancer cells. Furthermore, it has been shown that ABCC2 is also overexpressed in breast tumors and it has some effects on TAM clinical efficacy.
Plenty of studied have revealed that the expression or activity of different ABC transporters at various levels of expression (transcriptional, post-transcriptional, translational, and post-translational levels) might be influenced by proinflammatory cytokines such as TNF-α. 33-35 NF-κB transcription factor is an important regulator of inflammation and its constitutive activation have reported in tumors with a high risk for recurrence. 36,37 On the other hand, NF-κB is one of the proved downstream mediator of TNF-α signaling and it has shown to be involved in the alteration of ABC transporters expression mediated by TNF-α. 38,39

As TNF-α proved to be present in the microenvironment of breast tumors, 3 for the first time we evaluated its effects on mRNA and protein expression of ABCC2 transporter in long term (20-day) treatment in MCF-7 (ER+) breast cancer cells, in the presence of E2 as one of the represented growth stimulators in ER+ breast carcinoma cells. 40 We also checked the sensitivity of TNF-α treated cells against breast cancer chemotherapeutic agent, Cis, which is a well-known ABCC2 substrate. 18 Furthermore, a probable substrate of this transporter, 4OH-TAM, used widely as the basis of anti-estrogen therapy for ER+ breast cancers was investigated. 26 Then, the possible correlation between change in expression levels of ABCC2 transporter, NF-κB/p65 nuclear transcription factor and TNF-α/E2 treatment was investigated.

**Materials and Methods**

*Cell culture and Reagents*

MCF-7 cell line was provided from Pasteur Institute, Tehran, Iran. Cells were cultured in RPMI-1640, supplemented with FBS (Fetal Bovine Serum) 10% (v/v) (GIBCO, Grand Island, NY, USA), penicillin (100 units/ml), and streptomycin (100 μg/mL) (GIBCO, Grand Island, NY, USA) at 37°C in the presence of 5% CO2.
TNF-α and 4OH-TAM were purchased from Millipore, USA. 5mM 4OH-TAM stock was prepared by Ethanol 100% (v/v) as solvent and stored in -80 °C. Cis vial for injection (10mg/10ml) was purchased from MYLAN (France).

**Cell treatments and viability assay**

The influence of TNF-α in the presence of E2 on cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Morphologic changes were examined under an INVERSO-TC100 phase-contrast microscope (Medline Scientific Limited, Oxfordshire, United Kingdom).

**20-day TNF-α and/or E2 treatment**

Cells were treated with TNF-α (10ng/ml) +E2 (10nM) or E2 (10nM) alone for 20 days. The media was replaced with fresh one containing TNF-α and/or E2 every 3 days. Control cells were treated only with culture medium.

*Cis-treatment:* After 20-day TNF-α/E2 treatment, the sensitivity of MCF-7 cells to Cis was analyzed by MTT assay. Cells were cultivated in 96-well plates at a density of 1.0×10⁴ cells/well and then treated with increasing concentrations of Cis (12.5, 25, 50 and 100 μM) for 48 h.

*TAM-treatment:* After 20-day TNF-α/E2 treatment, TAM sensitivity was assessed by MTT method. Cells were cultivated in 96-well plates at a density of 3.0×10³ cells/well and then treated with increasing concentrations of 4OH-TAM (0.001, 0.01, 0.1, 0, 1, 10 and 15μM) for 5 days. (Control cells: treated only with the culture medium and ethanol 1% (v/v)).

At the end of incubation period with 4OH-TAM and Cis, 20 μL MTT solution was appended to each well for 3 h. Thereafter, to solubilize the formazan crystals, 200 μL of dimethyl sulfoxide (DMSO) was added to each well and the absorbance was determined at 570 nm on an ELISA plate reader (Biotek®). Values were corrected for background absorbance at 630 nm. Three independent
experiments, at least three wells for each time and concentration point, were evaluated; and cell viability was calculated as a percentage of negative controls.

**Quantitative reverse transcription polymerase chain reaction (QRT-PCR)**

The mRNA levels of ABCC2 transporter and NF-κB/p65 transcription factor were examined by quantitative RT-PCR. Total RNA was extracted from treated and control cells. RNA isolation and then cDNA synthesis with the final evaluation step of expression, RT-PCR, were done according to the manufacturer’s instructions (Yekta Tajhiz Azma kit (Iran)) using specific primers (Table 1). PCR was performed for 40 cycles following thermal cycling conditions: 95 °C for 3 min (initial denaturation), 95 °C for 5 s (denaturation) and 60 °C for 1 min (annealing/extension).

At the end of the PCR cycles, the PCR products were examined using a melting curve analysis by slowly increasing the temperature from 60 °C to 95°C to indicate the absence of non-specific products detected by SYBR Green dye. Standard curves were delineated for the target (ABCC2, NF-κB/p65) and reference (β-actin) genes. Calibrator-normalized relative quantification with efficiency correction was performed using the Applied Biosystems Software (StepOne software). The results were represented as the target/reference ratio of the cytokine+E2 or E2-treated samples divided by the target/reference ratio of the calibrators (untreated sample).41,42

**Western blot analysis**

Western blot analysis was used to assess the levels of cytoplasmic (ABCC2) and nuclear protein expression (NF-κB/p65) in treated and control cells using nuclear protein extraction kit (abcam® (USA), CAT. NO: ab113474). According to kit protocol, 100 µL of 1X Pre-Extraction Buffer containing DithioThreitol (DTT) and Protease Inhibitor Cocktail (PIC) at a 1:1000 ratio was added to cell pellet. After vortexing for 10 s and incubation on ice for 10 min, cytoplasmic proteins were
carefully removed from nuclei by centrifugation for 1 minute at 12,000 rpm and then transferred to another micro-centrifuge vial. Nuclear protein extract was prepared according to protocol of nuclear protein extraction kit. DTT Solution and PIC was appended to Extraction Buffer at a 1:1000 ratio. After adding 2 volumes of Extraction Buffer containing DTT and PIC to nuclear pellet (about 10 μL per 10^6 cells), the extract was incubated on ice for 15 minutes with vortex every 3 minutes. The extract can be further sonicated for 3 x 10 seconds to increase nuclear protein extraction. Before transferring the supernatant into a new micro-centrifuge vial, the suspension was centrifuged for 10 minutes at 14,000 rpm at 4°C and the protein concentration of the nuclear extract was measured. The Bradford Reagent was used to measure the protein concentration of the nuclear and cytoplasmic extracts. The fractionated proteins were then sample transferred electrophoretically to (poly vinylidene difluoride) (PVDF) membranes (Bio-Rad, Hemel Hempstead, UK) and the proteins were immunoblotted with the specific antibodies. Primary antibodies against β-actin, ABCC2 and NF-κB/p65 (all purchased from Cell Signaling Technology, Beverly, MA, USA) interacted with secondary horseradish peroxidase–linked anti-mouse or anti-rabbit conjugate antibodies (Table 2). Protein bands were appeared by Enhanced chemiluminescence (ECL) reagent (Pierce Rockford, IL, USA) and Alliance 4.7 Geldoc (UK).^{34,43}

Statistical analysis

Results were expressed as mean ± SD (standard deviation) of at least three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) and Tukey-Kramer test with GraphPad Prism5. Significance was considered in P-values < 0.05.

Results

Effect of TNF-α/E2 on Cis-sensitivity of MCF-7 cells
Following 20-day TNF-α and/or E2 treatment, concentration dependent reduction in cell viability was observed after 48 h incubation period with increasing concentrations of Cis (12.5, 25, 50 and 100 μM). At 12.5 to 50 μM, mean cell viability was higher in MCF-7 cells treated with TNF-α+E2 (IC50: 67.186±6.5), compared to both E2 treatment alone (IC50: 40.5333±4.45) and untreated cells (IC50: 36.1233±3) (Figure 1).

**Effect of TNF-α/E2 on TAM-sensitivity of MCF-7 cells**

Following 20-day TNF-α and/or E2 treatment, and then 5-day incubation period with increasing concentrations of 4OH-TAM (0.001, 0.01, 0.1, 0, 1, 10 and 15 μM), concentration dependent reduction in cell viability was observed. At concentrations lower than 10 μM, mean cell viability was higher in MCF-7 cells treated with TNF-α+E2, compared to both E2 treatment alone and untreated cells (Figure 2 and 3).

**Effect of TNF-α on mRNA expression of ABCC2 transporter and NF-κB/p65 nuclear transcription factor**

20-day TNF-α and/or E2 treatment was associated with significant increase in relative mRNA level of ABCC2 (***p< 0.0001). Remarkable elevation in fold change of ABCC2 mRNA expression was occurred after 20-day TNF-α+E2 concomitant treatment (10.016 ± 0.1 folds); while, after 20-day E2 treatment alone mRNA overexpression was about 2.451 ± 0.2 folds, compared to untreated cells as control (Figure 4A).

To evaluate the correlation between the NF-κB/p65 nuclear transcription factor and ABCC2 gene expressions, NF-κB/p65 mRNA level was examined and compared with the patterns of ABCC2 mRNA expression. Significant elevation in NF-κB/p65 mRNA level (2.483 ± 0.3 folds) (**p< 0.004) was obtained following 20-day TNF-α+E2 treatment, compared to control. On the other hand,
treatment with E2 (without TNF-α) did not significantly alter NF-κB/p65 mRNA expression (Figure 4B) (p > 0.05).

**Effect of TNF-α on protein expression of ABCC2 transporter and activity of NF-κB (p65) transcription factor**

Western blot analysis showed that after 20-day TNF-α+E2 treatment, protein expression of ABCC2 was significantly changed, relative to untreated cells as control (2.869 ± 0.2 folds) (***p<0.001). Increase in ABCC2 expression was also seen following 20-day E2 treatment alone (1.717 ± 0.006 folds) (*p<0.05) not as much as concomitant treatment with TNF-α and E2 (Figure 5A, C). As demonstrated in Figure 5B and C, the level of active form of NF-κB (p65) in nuclear protein extract was significantly enhanced in TNF-α+E2 treated cells following 20-day treatment (2.167 ± 0.1 folds) (***p< 0.001). E2 treatment was also significantly associated with some elevation in protein expression (1.404 ± 0.04) (*p<0.05), compared to control.

As a result, TNF-α+E2 concomitant treatment induced overexpression of both ABCC2 and NF-κB/p65 proteins stronger than E2-treated and untreated MCF-7 breast cancer cells.

**Discussion**

Now, it is well understood that inflammation has a key role in all aspects of tumor biology. It has been shown that TNF-α as a pro-inflammatory cytokine is able to modulate the expression of ABC transporters involved in cancer MDR. In this way, the cytokine might be involved in endocrine and chemotherapy response and therefore, elucidating its effects and mechanisms of action in the tumor microenvironment can be helpful in the designing new strategies to circumvent ABC-related drug resistance. In this study, we tried to investigate the influence of long-term TNF-α exposure in the presence of E2 as an important hormone and one of the represented growth
stimulators in ER+ breast carcinoma cells on ABCC2 mRNA and protein expression to mimic the inflammatory condition of the tumor microenvironment and elucidating its effect on Cis and TAM sensitivity in MCF-7 breast cancer cells. In our previous preliminary studies on the expression and activity of ABC transporters modulating by TNF-α in MCF-7 cells, we found that the maximum modulation of ABC transports occurred following 20-day concomitant treatment with both the cytokine and E2 (data not shown). No further change happened in longer incubation periods. Therefore, we treated cell with the cytokine in the presence of E2 for 20 days.

The overexpression of ABCC2 transporter is highlighted in human carcinomas such as renal cells, gastric, colon, lung, ovarian and breast carcinomas correlated with a higher histological grade and poor differentiation, compared with well-differentiated tumors and also ABCC2-mediated anti-cancer drug resistance. In support of this, there is a potential role for ABCC2 in Cis resistance by cellular efflux of Cis as a useful anti-cancer agent. Of interest is the fact that in both Cis-resistant human epidermoid cancer KB and human prostatic cancer PC-3 cell lines, the ATP-dependent active efflux system of Cis is increased. In another study, overexpression of ABCC2 increased the efflux of some chemotherapeutic agents in human embryonic kidney (HEK-293) and the resistance to GSH Cis conjugate was the maximum (10-fold) compared to other compounds. Taniguchi et al, demonstrated that in three Cis-resistant human cell lines (T24IDDP1O from human bladder cancer T24 cells, P/CDP5 from human prostatic cancer PC-3 cells and KB/KCP4 from human head and neck cancer KB cells) the mRNA of ABCC2 transporter was expressed 4- to 6-fold higher than parent cells correlated with decreased cellular Cis-accumulation. The effect of ABCC2 tumor expression on the efficacy of Cis- treatment in patients with hepatocellular carcinoma was determined by immunohistochemical and Western blot analyses. In line with Korita et al. study, ABCC2 overexpression was significantly associated with
a lower percentage of tumor necrosis by Cis chemotherapy (19% vs. 99%, P<0.05)\textsuperscript{24}. Recent studies have revealed that conferring resistance to oxaliplatin as a common clinical treatment of gastrointestinal cancers is modulated by ABCC2 efflux pump. \textsuperscript{22,51} In our study, mRNA and protein expression levels of ABCC2 were significantly greater in 20-day TNF-α+E2 treated MCF-7 breast cancer cells, compared to untreated or even relative to E2-treated cells. Cis is the well-known substrate of ABCC2 transporter \textsuperscript{18} and in line to this fact, we also observed more reduction in Cis sensitivity of MCF-7 cells treated with TNF-α+E2, compared to both E2-treated and untreated cells. Adding validity to our findings, in a recent clinical study, the expression of ABCC2 was higher in recurrent tumor compared to paired primary tumor of each patient who is received chemotherapy, highlighting ABCC2 potential role in multiple drug resistance. \textsuperscript{52}

TAM is the main anti-estrogen medicine in ER+ breast cancers. \textsuperscript{53} Unpredictable response and development of TAM resistance remain a big challenge in ER+ breast cancer management. \textsuperscript{29} It has been shown that the tumor microenvironment can modulate TAM resistance by different molecular mechanisms. A study reported that fibronectin in tumor microenvironment conferred resistance to anti-estrogen TAM via interaction with β1 integrin and through activation of some kinase pathways (phosphatidylinositol 3-kinase/serine/threonine-specific protein kinase (PI3K/AKT) and mitogen-activated protein kinase/extracellular signal–regulated kinases (MAPK/ERK)). \textsuperscript{54} In human MCF-7 breast cancer cells, TNF-α activated unliganded ERα through kinase-mediated phosphorylation events (IKKβ phosphorylation of S305), then phospho-S305 caused structural changes in ERα and as a consequence, TAM resistance occurred. \textsuperscript{4}

It has been observed that after upregulation of ABCC2 efflux transporter, the sensitivity of MCF7 breast cancer cells to TAM (1μM) reduced. \textsuperscript{28} In another study, the expression level of ABCC2 was higher in TAM-resistant breast cancer cells (TAMR-MCF-7) compared to the control cells. \textsuperscript{30}
Based on a clinical study on breast cancer patients in Japan, polymorphism in *ABCC2* gene could be a good predictor for breast cancer prognosis treated with tamoxifen. Also, nuclear expression of ABCC2 in tumor cells was linked to more aggressive and TAM-resistant breast cancers. TAM is speculated to be a possible substrate of ABCC2 pump; in this regard, in the present study we also investigated the effect of TNF-α and E2 on breast cancer cells sensitivity to 4OH-TAM and we showed simultaneous increase in expression level of ABCC2 and reduction of TAM sensitivity following concomitant treatment.

Activation of well-known NF-κB transcription factor, is associated with both endocrine and chemotherapy failures in breast tumors. It has been shown that one possible pathway in endocrine-therapy resistance is the activation of NF-κB via the PI3K/AKt signaling pathway in MCF-7 breast cancer cells. Activation of anti-apoptotic pathway via nuclear localization of NF-κB, was associated with tumor resistance to anthracyline-based chemotherapy in human breast cancers. TNF-α is one of the most influential activators of NF-κB signaling pathway. Over-activity of NF-κB in ER+ breast tumors caused both mRNA and protein overexpression of ABCG2. We previously demonstrated that over-activity of NF-κB/p65 following TNF-α treatment was concomitant with overexpression of ABCG2 in breast cancer cell lines (MCF-7, BT-474, and CAL51). Notably, functional NF-κB binding site is present in the promoter of ABCC2 gene. Not only in breast tumors, but also in other cancer cell lines, it has also illustrated a role for NF-κB in regulating the expression of ABC transporters, conferring chemoresistance. In line with these evidences, we also found that following 20-day treatment with TNF-α+E2, the mRNA and protein levels of active form of NF-κB (p65) were significantly increased, compared to untreated cells as control. These observations might propose the possibility of linkage
between over-activity of NF-κB/p65, ABCC2 overexpression and also Cis and TAM drug resistance in MCF-7 breast cancer cells.

**Conclusion**

Above-mentioned studies have highlighted that TNF-α is involved in mediating inflammation in breast tumor microenvironment leading to tumor proliferation, invasion and resistance properties. In this study, we tried to mimic the inflammatory condition of the tumor microenvironment for the MCF-7 breast cancer cells by TNF-α exposure in order to study its effect on Cis and TAM sensitivity in MCF-7 cells. Based on our findings, the cytokine could induce reduction in Cis and TAM sensitivity that was correlated with increase in expression levels of ABCC2 and NF-κB/p65 activity in MCF-7 breast cancer cells. Further experiments are needed to elucidate possible mechanistic relationship of these findings and their clinical significance in order to circumvent the drug resistance in breast tumors.

**Acknowledgment**

This work was funded by the Grant from the Scientific Board of Mashhad University of Medical Sciences.

**Conflict of interests**

The authors declare that they had no conflict of interests.
References


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Table 1: Lists of primers in QRT-PCR

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Table 2: Lists of Antibodies in western blot analysis

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**Figure Legends**

**Figure 1.** Effect of E2 and TNF-α+E2 on sensitivity of MCF-7 cells to Cis following 20-day treatment with E2 (10nM), TNF-α (10ng/ml) and then Cis (12.5, 25, 50 and 100 μM) for 48 h analyzed by MTT method. Data are represented as the mean ± SD. Statistics were calculated by one-way ANOVA. At each point: P < 0.05 *, n=3

**Figure 2.** Effect of E2 and TNF-α+E2 treatment on sensitivity of MCF-7 cells to 4OH-TAM. Following 20-day treatment with E2 (10nM) or E2+TNF-α (10ng/ml) treatment, cells treated with 4OH-TAM (0.001, 0.01, 0.1, 0, 1, 10 and 15μM) for 5 days and cell viability (% of control) was analyzed by MTT method. Data are represented as the mean ± SD. Statistics were calculated by one-way ANOVA. At each point: P < 0.05 *, n=3

**Figure 3.** Effect of TNF-α+E2 treatment on sensitivity of MCF-7 cells to 4OH-TAM. Following 20-day treatment with E2+TNF-α (10ng/ml), cells incubated with 4OH-TAM (0.001, 0.01, 0.1, 0, 1, 10 and 15μM) for 5 days and microscopic studies conducted.

**Figure 4.** Effect of E2 and TNF-α+E2 on mRNA levels of ABCC2 (A) NF-κB/p65 nuclear transcription factor (B) following 20-day treatment in MCF-7 cell line. mRNA expression was determined by QRT-PCR and normalized to β-actin. Data were analyzed by one-way ANOVA and Tukey-Kramer test. Data are represented as mean±SD. At each point: P < 0.05 *, n=3

**Figure 5.** Effect of E2 and TNF-α+E2 on ABCC2 (A) and NF-κB/p65 (B) protein expression, following 20-day treatment in MCF-7 cell line. Protein expression was determined by western blot analysis and normalized to β-actin. Protein bands were appeared by Alliance 4.7 Geldoc (UK) (C). Statistics were calculated by one-way ANOVA and Tukey-Kramer test. Data are represented as mean±SD. At each point: P < 0.05 *, n=3
Figure 1
Figure 2
Figure 3
Figure 4

mRNA expression of ABCC2

mRNA expression of NF-κB/p65

A

Relative mRNA level (fold)

20-day treatment

B

Relative mRNA level (fold)

20-day treatment