**Study of biochemical medicine dose-response effect on treatment of breast cancer**

Mozhdeh Haddadi1, Dr [Soroush Sardari](https://www.researchgate.net/researcher/253619_Soroush_Sardari)2

1Department of Biochemistry, Tarbiat Modares University, Tehran, Iran

2Drug Discovery and Bioinformatics Group, Biotechnology Research Center of Institute Pasteur of Iran, Tehran, Iran

mozhdeh\_haddadi\_777@yahoo.com

**Abstract:** In this study therole of the dose level of cyclophosphamide, methotrexate, and fluorouracil in chemotherapy for breast cancer and in chemotherapy for metastatic breast cancer were investigated and the effects of TNF-α on estrogen metabolism was evaluated. There was a clear dose-response effect, indicating that CMF was useful only when given in a full or nearly full dose. Chemotherapy uses drugs to destroy cancer cells. If cancer has a high risk of returning or spreading to another part of body, it was recommended chemotherapy to decrease the chance that the cancer will recur. This was associated with a concomitant effect on the expression of detoxification enzymes COMT and NQO1 where TNF-α reduced the expression levels of these two enzymes. This may implicate a new possible explanation for inflammation associated breast cancer. Our findings indicate that it is necessary to administer combination chemotherapy at a full dose to achieve clinical benefit.

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**1. Introduction**

Breast cancer is one of the most common malignancies accounting for nearly 1 in 3 cancers diagnosed among women in the United States, and it is the second leading cause of cancer death among women (DeSantis et al., 2014). Chemotherapy is sometimes given before surgery in women with larger breast tumors. The goal is to shrink a tumor to a size that makes it easier to remove with surgery.

Chemotherapy may be recommended to try to control the cancer and decrease any symptoms the cancer is causing. The most acknowledged mechanism of estrogen carcinogenicity is its binding to its specific estrogen receptor alpha (ER-alpha) for exerting a potent mitogenic effect on cell proliferation. However, there is conceivable evidence that estrogen induced breast cancer can also occur by a non ER-alpha mediated mechanism involving the estrogen metabolic pathway. It is well established that prolonged exposure to estrogens especially estradiol, is an important risk factor for genesis and promotion of breast cancer. Chemotherapy is also used in women whose cancer has already spread to other parts of the body.

External beam radiation is commonly used after lumpectomy for early-stage breast cancer. Doctors may also recommend radiation therapy to the chest wall after mastectomy for larger breast cancers or cancers that have spread to the lymph nodes. Chemotherapy side effects depend on the drugs you receive. Common side effects include hair loss, nausea, vomiting, fatigue and an increased risk of developing infection. Rare side effects can include premature menopause, infertility (if premenopausal), damage to the heart and kidneys, nerve damage, and, very rarely, blood cell cancer. Metabolism of estrogens is characterized by two major pathways: one is hydroxylation at the 16-position and the second is hydroxylation to form the 2- and 4-catechol estrogens. In the catechol pathway, the metabolism involves further oxidation (E2) to semiquinones and quinones which react with DNA to form depurinating adducts. The apurinic sites obtained by this reaction generate mutations that may lead to the initiation of cancer.

It has become increasingly clear that inflammation plays a major role in breast cancer pathogenesis. An inflammatory tumor microenvironment consists of infiltrating immune cells and activated fibroblasts, both of which can secrete cytokines, chemokines, and growth factors, as well as DNA-damaging agents. Oxidation of catechol estrogens to their quinones normally occurs in homeostasis which minimizes their reaction with DNA. When the homeostasis is disrupted, excessive amounts of catechol estrogen quinones are formed and the resulting increase in depurinating DNA adducts can lead to initiation of cancer (Rogan, 2014). Moreover, 4-hydroxylated estrogens may exert proinflammatory roles by inducing reactive oxygen species (ROS) and DNA damage which is possibly decisive in chronic inflammation.

Some studies show evidence that chronic inflammation is linked to breast cancer recurrence and that elevated biomarkers of inflammation are associated with reduced survival among breast cancer patients (Cole, 2012; Pierce et al., 2012). In addition, experimental studies clearly indicate that inflammatory mediators promote tumor development in cancer prone animal strains. Moreover, inhibition of NF-kappaB transcription factor is proved to be protective with respect to chemical induced mammary gland carcinogenesis (Connell et al., 2014). TNF-α is a major inflammatory cytokine shown to be highly expressed in breast carcinomas. Indeed, investigations strongly suggest that the chronic expression of TNF-α in breast tumors actually supports tumor growth. Further, the in vitro activation of the TNF-α axis has induced an invasive and malignant behaviour in breast cancer cells (Balkwill, 2012).

The number of cells expressing TNF-α in inflammatory breast carcinoma was found to be correlated with increasing tumor grade and node involvement, and TNF-α expression was suggested to play a role in the metastatic behavior of breast carcinomas. Furthermore, patients with more progressed tumor phenotypes were shown to have significantly higher TNF-α serum concentration. We thus investigated the effect of the TNF-α, a hallmark of inflammation, on the estrogen metabolic pathway in MCF-7 estrogen dependent breast cancer cells. Our choice of MCF-7 as our study model was built on several bases: First, studies have shown that regular use of nonsteroidal anti-inflammatory drugs (NSAIDS), such as aspirin, significantly reduce the risk of ER-positive but not ER-negative breast cancers. The tumor-promoting functions of TNF-α may be mediated by its ability to induce proangiogenic functions, to promote the expression of matrix metalloproteinases (MMP) and endothelial adhesion molecules, and to cause DNA damage via reactive oxygen, the overall effect of which is promotion of tumor related processes.

Side effects of radiation therapy include fatigue and a red, sunburn-like rash where the radiation is aimed. Breast tissue may also appear swollen or more firm. Rarely, more-serious problems may occur, such as damage to the heart or lungs or, very rarely, second cancers in the treated area. In the current study we explored a new mechanism by which inflammation may influence breast carcinogenesis through the estrogen metabolic pathway. Second, it was recently suggested that inflammation may promote more aggressive ER-positive tumors and that this may be one of the mechanisms by which a portion of ER-positive breast tumors fail to respond to endocrine therapy (Baumgarten et al., 2012). In general, we sense that further research is required to fully elucidate the mechanisms of action of TNF-α on breast carcinogenesis because TNF-α activity may vary under different physiological conditions and in a cell-type-dependent manner which contributes to a sense of ambiguity regarding its tumor effects. Most of the reports examining the effects of TNF-α on MCF-7 breast cancer cells demonstrated its ability to induce apoptosis, inhibit proliferation and promote migration, invasion as well as resistance to chemotherapeutic drugs. These effects may however vary with other estrogen dependent (T47D) or independent (MDA-MB-231) cell lines (Goldberg and Schwerfeger, 2013).

Other studies have pointed that the effect of TNF-α on estrogen metabolism and homeostasis is mediated by its effect on the coordinated expression of the enzymes that are involved in estrogen biosynthesis and metabolism. For example, it has been suggested that TNF-α increases the local estrogen biosynthesis in human endometrial glandular epithelial cells and directs estrogen metabolic enzymes to produce more hormonally active and carcinogenic metabolites (Salama et al., 2011). What encouraged us more to explore this area of research is that not many studies have examined the role of inflammation on the estrogen metabolic pathways in general in spite of this pathway being an important cause of carcinogenesis. In some of these studies, TNF-α has been found to have an important role in regulating estrogen synthesis in peripheral tissues, including normal and malignant breast tissues.

Collectively, the above mentioned information stimulated our interest to have a closer look on the estrogen metabolic pathway in MCF-7 breast cancer cells as a whole and examine the role played by TNF-α in this pathway aiming to enrich our knowledge about how inflammation may influence breast carcinogenesis. Understanding the mechanisms by which inflammatory mediators promote breast cancer may shed the light on these cytokines being explored as potential therapeutic targets and hopefully will lead to novel therapeutic regimens to treat this devastating disease.

**2. Materials and Methods**

The levels of estrogen and estrogen metabolites (EM) were determined by testing the conditioned media from cells treated with E2 alone or with different doses of TNF-α according to the method described by Xu et al. (2010). Briefly, 5 µL of the stable isotope labelled estrogen and estrogen metabolite (SI-EM) working internal standard solution was added to cell medium aliquot followed by 500 μl sodium acetate buffer (pH 4.6) containing L-ascorbic acid. Dichloromethane (6 ml) was added to the sample, which then underwent inverse extraction at 8 rpm (RKVSD™, ATR, Inc., Laurel, MD, USA) for 30 minutes. The human breast cancer cell line MCF-7 was purchased from (Rockville culture collection, Md, USA). The cells were maintained at 37ºC in a 95% humidified, CO2 atmosphere in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum, 1% L-glutamine, sodium pyruvate, and penicillin and streptomycin (Gibco BRL, Life Technologies, Long Island, NY, USA). To study the effect of TNF-α on the profile of estrogen metabolism and DNA adduct analysis, the cells were grown in serum free, phenol red-free media for 24 hours. The cells were then treated with 10 nM of E2 with or without 5ng/ml TNF-α for 48 hours. The media were collected for estrogen metabolite measurements and DNA adducts analysis.

After extraction, the aqueous layer was discarded and the organic solvent portion was transferred into a clean glass tube and evaporated to dryness under a stream of nitrogen gas at 60°C (Reacti-Vap III™, Pierce, Rockford, IL, USA). The dried sample residue was then redissolved in sodium bicarbonate buffer. After sonication, the sample was heated at 60°C (Reacti-Therm III™ Heating Module, Pierce, Rockford, IL, USA) for 5 min. Absolute concentrations of individual EM were normalized against protein concentrations.

***Sample preparation***

Stably transfected cells containing the reporter gene vectors with the promoters of interest were grown in RPMI media, distributed in 6 well plates and treated with different concentrations of TNF-α. After 48 hours, the cells were harvested and luciferase activities were determined using luciferase enzyme assay systems according to the supplier’s protocol (Promega, Madison, WI, USA). The luciferase activity was normalized against protein concentration using Bradford protein assay procedure.

MCF-7 cells were treated as described above. The growth media was used to measure the level of depurinating estrogen−DNA adducts according to the method described by Zahid et al. (2011). Briefly, cell culture media was extracted by using Varian C8 Certify II solid phase extraction cartridges (Varian, Harbor City, CA, USA) which were preequilibrated by sequentially passing 1 ml methanol, distilled water, and potassium phosphate buffer through them. Forty-eight hours post-transfection, the media was replaced with Genecitin-containing media. Individual colonies were picked and propagated following 2 weeks of selection and were screened for luciferase activity. Colonies which showed positive luciferase activity indicated that they were successfully stably transfected and thus were selected and maintained in liquid nitrogen for further experiments.

Analysis of all samples was conducted on an HPLC system equipped with dual ESA Model 580 autosampler, and a CoulArray electrochemical detector (ESA, Chelmsford, MA, USA). The system was controlled and the data were acquired and processed using the CoulArray software package (ESA). Peaks were identified by both retention time and peak height ratios between the dominant peaks and the peaks in the two adjacent channels. The metabolites, conjugates and depurinating adducts were quantified by comparison of peak response ratios with known amounts of standards. The level of adducts were normalized against cell numbers and the DNA contents.

Data are presented as mean±Standard error (SE) of at least two or three individual experiments as indicated under the results section. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey test as a post-ANOVA multiple comparison test. Unpaired *t*-test was used for comparison between two groups.  *P*<0.05 was considered statistically significant.

**Targeted drugs**

Targeted drug treatments attack specific abnormalities within cancer cells. Targeted drugs used to treat breast cancer include:

• Pertuzumab (Perjeta). Pertuzumab targets HER2 and is approved for use in metastatic breast cancer in combination with trastuzumab and chemotherapy. This combination of treatments is reserved for women who haven't yet received other drug treatments for their cancer. Side effects of pertuzumab may include diarrhea, hair loss and heart problems.

• Trastuzumab (Herceptin). Some breast cancers make excessive amounts of a protein called human growth factor receptor 2 (HER2), which helps breast cancer cells grow and survive. If your breast cancer cells make too much HER2, trastuzumab may help block that protein and cause the cancer cells to die. Side effects may include headaches, diarrhea and heart problems.

• Ado-trastuzumab (Kadcyla). This drug combines trastuzumab with a cell-killing drug. When the combination drug enters the body, the trastuzumab helps it find the cancer cells because it is attracted to HER2. The cell-killing drug is then released into the cancer cells. Ado-trastuzumab may be an option for women with metastatic breast cancer who've already tried trastuzumab and chemotherapy.

• Medications that block hormones from attaching to cancer cells. Selective estrogen receptor modulator (SERM) medications act by blocking estrogen from attaching to the estrogen receptor on the cancer cells, slowing the growth of tumors and killing tumor cells.

• Bevacizumab (Avastin). Bevacizumab is no longer approved for the treatment of breast cancer in the United States. Research suggests that although this medication may help slow the growth of breast cancer, it doesn't appear to increase survival times.

• Lapatinib (Tykerb). Lapatinib targets HER2 and is approved for use in advanced or metastatic breast cancer. Lapatinib can be used in combination with chemotherapy or hormone therapy. Potential side effects include diarrhea, painful hands and feet, nausea, and heart problems.

SERMs include tamoxifen, raloxifene (Evista) and toremifene (Fareston).

Possible side effects include hot flashes, night sweats and vaginal dryness. More-significant risks include blood clots, stroke, uterine cancer and cataracts.

• Medications that stop the body from making estrogen after menopause. Called aromatase inhibitors, these drugs block the action of an enzyme that converts androgens in the body into estrogen. These drugs are effective only in postmenopausal women.

Aromatase inhibitors include anastrozole (Arimidex), letrozole (Femara) and exemestane (Aromasin).

Side effects include hot flashes, night sweats, vaginal dryness, joint and muscle pain, as well as an increased risk of bone thinning (osteoporosis).

• A drug that targets estrogen receptors for destruction. The drug fulvestrant (Faslodex) blocks estrogen receptors on cancer cells and signals to the cell to destroy the receptors. Fulvestrant is used in postmenopausal women. Side effects that may occur include nausea, hot flashes and joint pain.

• Surgery or medications to stop hormone production in the ovaries. In premenopausal women, surgery to remove the ovaries or medications to stop the ovaries from making estrogen can be an effective hormonal treatment.

**3. Results**

To elucidate the underlying mechanisms of the differential pattern of estrogen metabolites and the rate of estrogen-induced adducts formation between TNF-α treated and non-treated cells, we assessed the effect of TNF-α on the expression of key genes and enzymes involved in estrogen metabolism. To evaluate the effects of TNF-α on estrogen metabolism, the EM levels were measured in conditioned media from MCF-7 cells treated with E2 (10 nM) alone or in combination with TNF-α (5 ng/ml) using LC/MS. It was found that TNF-α significantly increased the total EM compared with control. To further assess the effects of TNF-α on estrogen metabolism, we derived the ratios for E1/E2, which is a commonly used predictor for cancer risk (Zhang et al., 2015). Thus, we treated MCF-7 cells with TNF-α and measured its effect on enzyme promoter activity using Reporter gene assays, enzyme mRNA levels using real time RT-PCR and enzyme protein levels using Western blot analysis. TNF-α shifted the E1/E2 equilibrium towards the formation of more E2. Thus, in MCF-7 cells treated with TNF-α, the E1/E2 ratio was evaluated which is significantly lower compared with control where the ratio was evaluated. The 4-OHE1 levels were not detected in control, but increased to upon adding TNF-α. On the contrary E3 levels and 16-epiestrol levels were detected in control but not in TNF-α treated cell. There was also a non-significant increase in the rate of formation of 17-epiestriol compared to control. TNF-α also increased the rate of formation of 2-OHE2 accompanied by a decline in the rate of 2-MeOE2 by 75%. Concerning the other methylated catechols, there was non-significant increase in the levels of 2-MeoE1 and 4-MeOE2 and non-significant decrease in 4-MeOE1. There was however a significant increase in 3-MeOE1 by 2.5 folds.

**4. Discussion**

Numerous studies have linked TNF-α to breast cancer progression. As a result, the mechanisms by which NF-α promotes breast cancer have been recently explored using both in vitro and in vivo models (Goldberg and Schwertfeger, 2010). Chronic inflammation represents a major risk factor for many cancer types, including liver, breast, prostate, and pancreas, ovary, and skin, gastric, colorectal and pulmonary carcinomas. In this study we are trying to focus on the influence of TNF-α on the estrogen metabolic pathway in MCF-7 cells. This may help us better understand the mechanisms by which inflammation affects breast cancer.

One of the interesting findings in our study is that the total EM was significantly higher in MCF-7 treated cells compared to control. One reason for this may be due to that TNF-α increased the local estrogen biosynthesis in MCF-7 cells through stimulating aromatase expression as suggested by previous reports (Macdiamid et al., 2011; Morris et al., 2011). TNF-α was identified as a keyplayer in the cytokine network and a major mediator of cancer related inflammation. It exerts its action via activation of it which is the pivotal regulator of cellular inflammatory responses (Sethi et al., 2011).

However, based on our results we are adding a new mechanism where by TNF-α may be directly interfering with the estrogen metabolic pathway. Besides increasing total EM, we also found that TNF-α significantly decreased the E1/E2 ratio due to a decrease in the level of estrogen as E1 with a concomitant increase in E2 concentration. The interconversion of weakly active estrone (E1) into highly potent estradiol (E2) and their relative abundance dictate the estrogenic environment and may be contributing to the development of breast cancer. To understand why TNF-α altered the rate and pattern of estrogen metabolites, we further explored the effect of TNF-α on the expression of key genes involved in estrogen metabolism. Our data revealed that TNF-α increases the expression levels. This was associated with a concomitant effect on the expression of detoxification enzymes COMT and NQO1 where TNF-α reduced the expression levels of these two enzymes. In agreement with our results several studies reported similar effects on these enzymes (Salama et al., 2011; Tchivileva et al., 2015). This alteration in E1/E2 ratio could be explained in light of the experimental evidence that TNF-α modulates the expression of hydroxysteroid (17β) -dehydrogenase 1 (HSD17β1) in breast cancer, an enzyme that efficiently catalyzes the conversion of E1 into E2 (Zhang et al, 2015). Also, 4-OHE1, which in our study was detected only in TNF-α treated cells, was previously shown to be tumorigenic in animal models (Liehr and Ricci, 1996). It’s noteworthy that 4-hydroxy catechols have greater carcinogenic potential than 2-hydroxy catechols due to different mechanisms of adduction leading to higher DNA adducts (Cavalieri and Rogan, 2014).

Surprisingly, TNF-α was found to upregulate mRNA of CYP1A1 and to downregulate it’s promoter activity as indicated by luciferase assay. This effect may be similar to that explained by Morel et al where increasing the CYP1A1 activity in hepatoma cells elicited an oxidative stress and led to the repression of a reporter gene driven by the CYP1A1 gene promoter in a negative feedback loop. Our results also indicated that TNF-α induced the rate of formation of the catechol estrogen 2-hydroxy estradiol (2-OHE2). Some studies indicate that there’s increased risk of development of breast cancer with E3 therapy. Other studies however, support the hypothesis that E3 exert protective roles by antagonizing the carcinogenic effects of E2. Recent studies indicate that E3 acts as a G-protein-coupled receptor 30(GPR30) antagonist in estrogen receptor-negative breast cancer cells. GPR30 has been recently involved in rapid signalling triggered by estrogens.This catechol estrogen was found to redox cycle and to generate hydrogen peroxide (H2O2) and hydroxyl radicals in MCF-7. Depending on the localized concentrations of catechol estrogens and enzymes that mediate redox cycling, this may be an important mechanism contributing to the development of breast cancer (Fussel et al., 2014). Given that our results also indicated a significant downregulation of the detoxifying enzyme COMT accompanied by a reduction in the ant carcinogenic, antiangiogenic metabolite 2-MeOE2, this may create a deleterious influence on estrogen carcinogenesis. Interestingly, we also found that E3 was detected in cells treated with E2 alone while it was not detected in cells treated with TNF-α. The effect of this hormone on breast cancer has been controversial.

Indeed, our study demonstrated that treatment with TNF-α is associated with a significant increase in estrogen-induced depurinating adducts especially the Adenine adducts. It is likely that this resulted from the increased level of some catechol estrogens accompanied by downregulation of the detoxification enzymes COMT and NQO1 as demonstrated in this report. Studies on estrogen metabolism, formation of DNA adducts, carcinogenicity, cell transformation, and mutagenicity have led to the hypothesis that reaction of certain estrogen metabolites, predominantly catechol estrogen-3,4-quinones, with DNA forms depurinating adducts [4-OHE1(E2)-1-N3Adenine and 4-OHE1(E2)-1-N7Guanine]. Formation of these adducts and the concomitant apurinic sites in DNA have been shown to induce mutations that are associated with initiation of breast cancer (Naushad et al., 2014).

This may implicate a new potential mechanism for inflammation associated breast cancer and provide a plausible explanation for the effect of TNF-α on the advancement of breast cancer. Also it can be suggested that compounds which are known to have positive regulatory effects on estrogen metabolic pathways, e.g. indole-3-carbinol, can be useful in inflammation associated breast cancer. Taken altogether, it’s clear from this study that TNF-α alters the balanced homeostatic set of activating and deactivating pathways of estrogen metabolism in MCF-7 breast cancer cells. It influences key genes and enzymes involved in estrogen metabolism leading to increased DNA adduct products.

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