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Research Article
Effect of Enterolactone on the Regulation of Indoleamine 2,3-Dioxygenase in MCF-7 Cancer Cells

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Abstract

Background and Objective: Indoleamine 2,3-dioxygenase 1 (IDO1) plays a key role in immune escape and tumor survival in various cancers. Numerous studies have shown that several antioxidants inhibit the expression of IDO1 in cancer cells. The purpose of this work was to examine how enterolactone (ENL), a bioactive metabolite from dietary lignans effects the IDO1 enzyme in breast cancer (MCF-7). **Materials and Methods:** The Erα-positive human breast cancer cell line, Michigan Cancer Foundation-7 (MCF-7) was utilized and induced to produce IDO1 by stimulating the cells with IFN-γ. Subsequently, the cells were cultured in the presence of enterolactone and their response was evaluated by measuring IDO enzymatic activity using the Enzyme-Linked Immunosorbent Assay (ELISA). Furthermore, the impact of ENL on the cytotoxicity and apoptosis of cancer cells was assessed. **Results:** The cell viability assay show that ENL had no impact on the viability of MCF-7 cells. The MCF-7 cancer cell line expresses IDO1, as detected by ELISA and its expression is further increased in response to IFN-γ treatment. The IDO1 activity increased with the different concentrations used of ENL and is significantly increased with 100 μM ENL. Apoptosis assay revealed no significant difference between the control and treated cells. **Conclusion:** This work provides the first proof of the impact of ENL on the immunosuppressive enzyme IDO1 in MCF7 cells activated by IFN-γ. Interestingly, it was discovered that high ENL concentrations increased IDO1 activity. Because ENL has IDO1-induced activity, more research on them is necessary.

Key words: IDO1, enterolactone, breast cancer, MCF-7, interferon-gamma

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Breast cancer is a significant global health concern, representing 11.7% of cancer cases in 2020 and ranking fifth in cancer-related deaths worldwide[1]. It is classified into subtypes based on hormone receptor expression: Estrogen receptor-positive, progesterone receptor-positive, human epidermal growth factor receptor-positive and triple-negative breast cancer[2].

The IDO1 is an intracellular cytosolic enzyme with a prosthetic heme group that plays a crucial role by allowing tumors to evade the immune system[3,4]. The IDO1 is responsible for initiating and controlling the breakdown of tryptophan, serving as the primary enzyme in the kynurenine pathway, where tryptophan is metabolized[5]. It is necessary for the synthesis of protein and niacin and is a precursor of serotonin and melatonin[6].

While IDO1 is mainly expressed by mucosal tissues, including the lung and placenta where it's expressed by endothelial cells, in the woman's genital tract by epithelial cells and also in lymphoid tissues in normal conditions[7], its overexpression leads to an increase in kynurenine/tryptophan ratio, which is used as a prognostic marker for cancer progression and invasiveness[3]. Depletion of tryptophan and the production of kynurenine promote immunosuppression in various types of tumors[8]. Tryptophan depletion ultimately reduces T-cell proliferation as T-cells are extremely sensitive to tryptophan deficiency[9]. Additionally, toxic downstream metabolites of IDO1 can cause macrophages and dendritic cells to develop an immunosuppressive phenotype[10].

The IDO is expressed in breast cancer[4], colorectal cancer[11] and prostate cancers[12] as proven by multiple researchers. Cancer cells have the ability to express IDO[7], either by itself or when tumor-infiltrating immune cells release inflammatory cytokines such IFN-γ[13]. The control of IDO1 expression is thought to be mediated by several immunological factors IFN-γ which is one of the major inducers of IDO in a variety of human cell types[14].

Lignans are naturally occurring phytoestrogens that have beneficial effects on health in treating several diseases, including cancer[15]. Flaxseed has large concentrations of secoisolariciresinol diglucoside (SDG)[16]. The ENL is a mammalian lignan, created during flax SDG digestion by the gut flora[17]. The ENL is widely recognized for its anti-breast cancer activity[18].

The aim of this study was to assess the effect of ENL using different concentrations on MCF-7 cells after inducing the cells for IDO1 production.

MATERIALS AND METHODS

Study area: The study was carried at King Fahd Medical Research Centre, King Abdulaziz University, Jeddah, Saudi Arabia from July, 2022 to February, 2023.

Cell culture: Breast cancer cell line human MCF-7 was kindly supplied (King Faisal Specialist Hospital and Research Centre, Jeddah, Saudi Arabia). The cells were cultured in Dulbecco's Modified Eagle Medium DMEM (11965092, Gibco, USA) supplemented with 10% fetal bovine serum FBS (26140087, Gibco, USA) and 1% penicillin-streptomycin (10,000 U mL^{-1}; 15140122, Gibco, USA) at 37°C humidified incubator with 5% CO_2. For sub-culturing at 80-90% confluence, cells were washed with phosphate-buffered saline PBS (25-507B, Quality Biological, USA) and detached with 1X TrypLE™ Express Enzyme (12605036, Gibco, USA) for 2 to 5 min at 37°C. A complete serum medium was added to stop 1X Tryple Express enzyme function. The cell pellet was obtained by centrifugation the cells for five minutes at 1500 rpm. After removing the supernatant, the cell pellet was resuspended in a fresh media. The cell suspension was transferred to a new culture flask with a fresh medium.

Enterolactone preparation: 1 mM concentrated stock of ENL (MW of 298.33 g mol^{-1}) (45199-5MG-F, Sigma-Aldrich, USA) was prepared with 5% Dimethyl sulfoxide (DMSO) (161954, PanReac AppliChem, USA) and stored at 4°C in the dark. Upon use, freshly dilution of the concentrated stock with complete DMEM media to reach a concentration of working solutions was prepared. The concentration of DMSO never exceeded 0.5% (v/ v).

MTT assay: Effect of ENL on cell cytotoxicity was analyzed with MTT Cell Proliferation and Cytotoxicity Assay Kit (Solarbio, Beijing, China). Cells were seeded in 96-well plates with 180 µL in each well at a density of 5,000 cells per well. After 24 hrs Recombinant Human IFN-gamma Protein (285-IF-100, R&D systems) was added to the cell culture at 100 ng mL^{-1}. The next day medium was removed and the cells were treated with 25, 50 and 100 µM of ENL in complete media for 48 according to Mali *et al.*[19].

After 48 hrs from adding ENL, the supernatant was removed, 90 µL of new culture medium and 10 µL of MTT solution were added and the plate was incubated for 4 hrs at 37°C. After that, the medium was removed and 110 µL of formazan solution was used to dissolve the formazan crystals. Each well's absorbance was measured at 490 nm in a microplate reader (synergy HT) after 10 min.

Each group has a set of three replicate wells. The following formula was used for calculating the percentage of viable cells[20]:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treatment}}{\text{Absorbance of control}} \times 100$$

IDO enzymatic activity: The MCF-7 was plated at 7×10^5 cells/T25 flasks and allowed to attach overnight. The following day, IDO1 expression was induced by adding IFN-γ to the cell culture at a final concentration of 100 ng mL^{-1} and were incubated at 37°C and 5% CO_2[21]. After 24 hrs, the cell culture medium is then replaced with 5 mL of assay medium containing ENL in different concentration. Cells were incubated for 48 hrs at 37°C in a 5% CO_2 incubator. Positive controls were MCF-7 cultures stimulated with IFN-γ (100 ng mL^{-1}) only. The MCF-7 was lysed directly in the flask by adding 250 µL cell extraction buffer per flask. Cell lysates were collected, incubated on ice for 15 min, spun down at 18.000xg (20 min, 4°C). The supernatants were transferred to clean Eppendorf and diluted 1:2 using cell extraction buffer. Concentrations of IDO1 were measured in cell lysates by using enzyme-linked immunosorbent assay Human IDO ELISA Kit (Abcam, # ab245710) and following the procedures described in the kit.

Apoptosis assay: The FITC Annexin V Apoptosis Detection Kit I (556547, BD Pharmingen, USA) was used for the experiment. MCF-7 cells were seeded in a 6-well plate at a density of 1×10^5 cells per well and incubated overnight. After induction of the cells with IFN-γ for 24 hrs, the media was replaced with media containing three concentrations of ENL 25, 50 and 100 µM and incubated for 48 hrs. Positive control cells were treated with IFN-γ only. On the day of the assay, after trypsinization and centrifugation, Cold PBS was used to wash the cells twice. As 300 µL of 1X binding buffer were used to resuspend cells/mL, which were then transferred to a 5 mL FACS tube. After that, 3 µL of FITC Annexin V and 2 µL of PI were added to the mixture and incubated for 15 min at room temperature (25°C) in the dark. Samples were analyzed by BD FACSAria™ III Flow Cytometer (BD Biosciences, US) within 1 hr.

Statistical analysis: The data are presented as the Mean±SEM using the GraphPad Prism 10 software. Both One-way Analysis of Variance (ANOVA) and unpaired t-tests were used for the comparison of the different groups as $p<0.05$ was considered statistically significant.

RESULTS

Effects of enterolactone on the viability of MCF-7 cells: The effect of ENL on breast cancer cell viability in the presence of IFN-γ, was examined using MCF-7 cancer cell line which models the ERα-positive. Briefly, after incubating the cells with 100 ng mL^{-1} of IFN-γ for 24 hrs, MCF-7 cancer cells were treated with different concentrations of ENL (25, 50 and 100 µM) for 48 hrs. Viability was assessed using the MTT assay. The results showed that ENL had no effect on the viability of MCF-7 cells after 48 hrs (Fig. 1).

The cells that have not been treated with IFN-γ served as a negative control while those treated with IFN-γ only served as a positive control. The data represent the average standard error mean of three replicate wells.

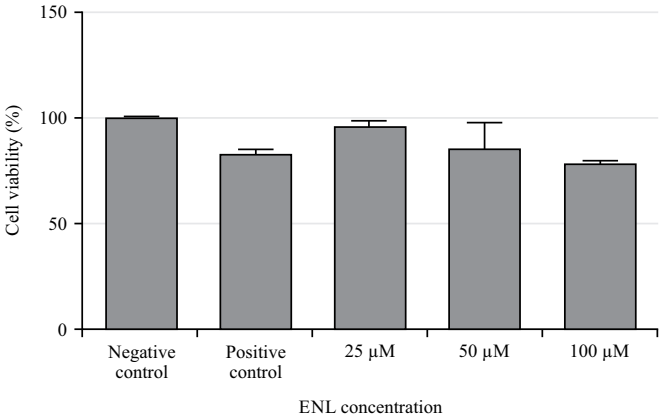

Fig. 1: IFN-γ treated MCF-7 cell viability was measured by MTT assay after treatment with various ENL concentrations 25, 50 and 100 µM for 48 hrs. The cells not exposed to IFN-γ were used as a negative control, while the cells exposed to 100 ng mL^{-1} IFN-γ were used as a positive control. The experiments were performed in triplicate

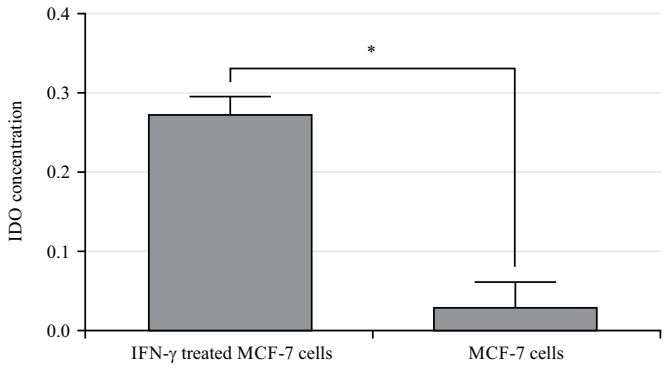

Fig. 2: IDO1 activity in MCF-7 cells cultured with or without 100 ng mL^{-1} of interferon-γ for 72 hrs

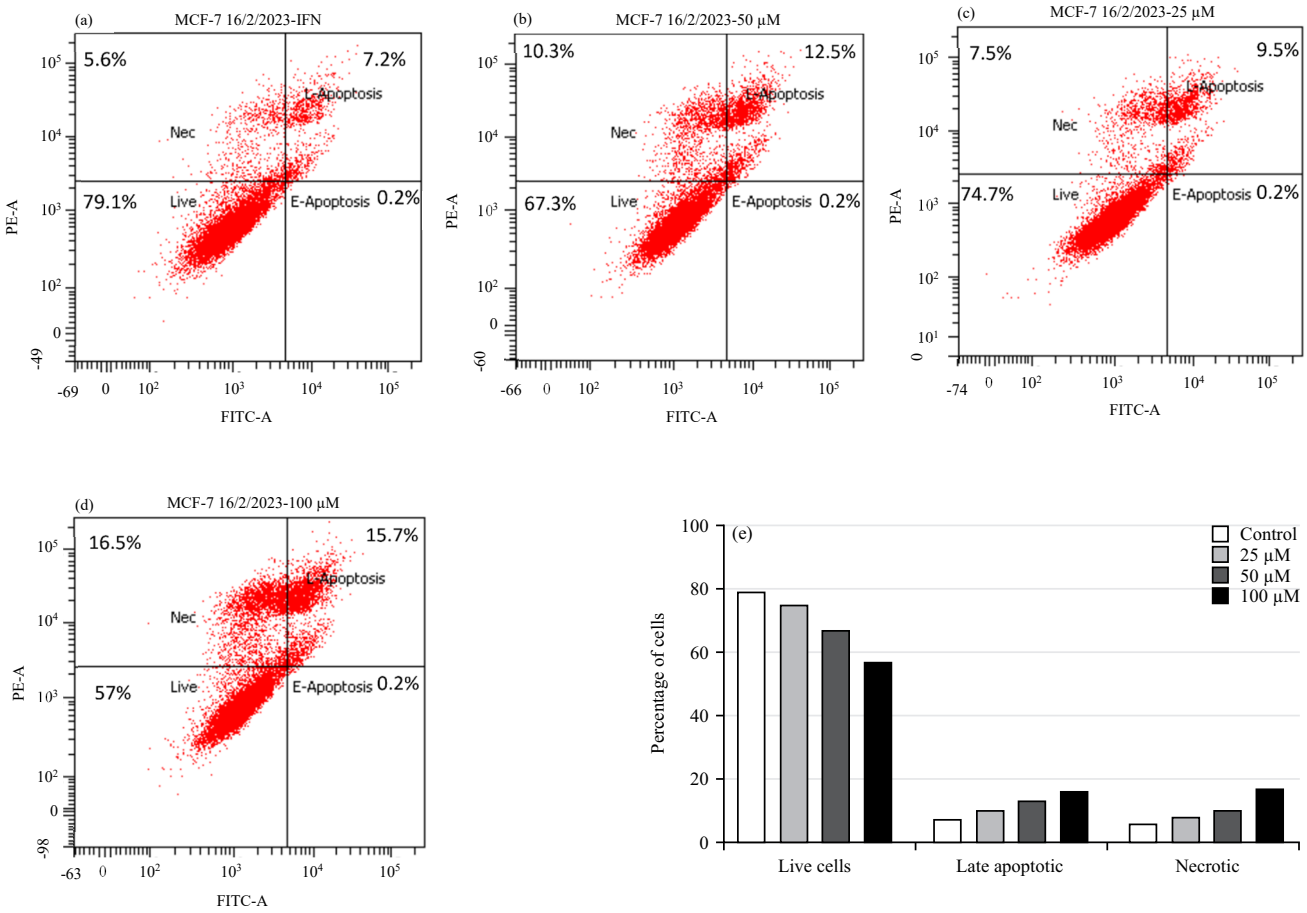

Fig. 3(a-e): Annexin V-FITC/PI apoptosis assay of IFN-γ stimulated MCF-7 cells treated with ENL, (a) Control, (b) 25 µM, (c) 50 µM, (d) 100 µM for 48 hrs and (e) A bar graph representation the summarized data of apoptosis assay result

IDO expression in breast cancer cell lines: To analyze IDO expression in MCF-7 cancer cell lines, cells were treated with 100 ng mL^{-1} IFN-γ for 72 hrs. As shown in Fig. 2, a very low IDO1 expression was detected by ELISA in MCF-7 cells without IFN-γ compared with MCF-7 that induced by IFN-γ. The MCF-7 cancer cell line expresses IDO1 and its expression is further increased in response to IFN-γ treatment. The *p<0.05, compared with the control.

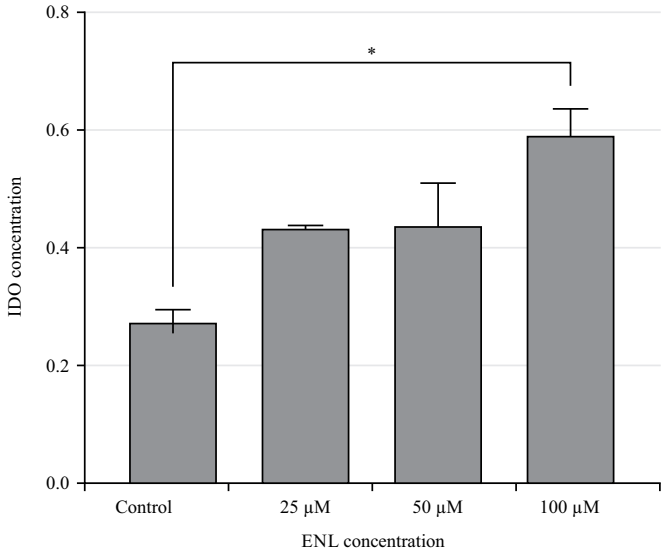

Fig. 4: MCF-7 cells were exposed to IFN-γ treatment (100 ng mL^{-1}) for 24 hrs, followed by the addition of ENL at concentrations of 25, 50 and 100 µM for an additional 48 hrs. Cell extracts were then collected to measure IDO levels

Apoptosis assay: To confirm the results from MTT assay to monitor cytotoxic effect of used concentrations of ENL apoptosis assay carried out using an Annexin V-FITC/PI. Flow cytometry was used and as shown in Fig. 3a-e. There was no significant difference between control and treated cells indicating the safe concentrations of used ENL concentrations.

Effect of intracellular IDO1 activity by enterolactone: To assess intracellular activity, the ability of ENL to regulate IDO1 in IFN-γ-induced MCF-7 cancer cells was evaluated.

To investigate the role of ENL on IDO-1 activity, different doses of ENL (25, 50 and 100 µM) for 48 hrs were used and the IDO1 activity was determined using ELISA.

As observed in Fig. 4, IDO1 activity increased with the different concentrations used of ENL and is significantly increased with 100 µM ENL compared to the control AS *p<0.05.

DISCUSSION

The IDO1 has a major role in immunological tolerance, making it a valuable emerging biomarker for cancer immunotherapy. Therefore, the creation and research of IDO1 inhibitors is essential for the treatment of tumors. The ENL has a great deal of potential for treating and preventing different kinds of cancer, therefore we are interested in learning more about how it might affect IDO activity. The findings demonstrated that MCF-7 cells produce IDO1, which is considerably upregulated in IFN-γ stimulated MCF-7 cells after exposure to high ENL concentrations. Given that ENL and estrogen have similar structures, it is possible that ENL mimics estrogenic substances by attaching to MCF-7 cells and thereby enhancing IDO1 activity, which would explain the rising activity of IDO1.

To the best of our knowledge, this study provides the first evidence of the effects of ENL on the IDO1 enzyme in MCF-7 breast cancer cells. The IDO1 overexpression has been associated with poor survival in several cancer patients, including those with cervical[22], colorectal[23] and lung[24] cancer. It plays a crucial role in cancer immunotherapy as it helps tumors evade the immune system[4]. Previous studies have shown that several antioxidants inhibit the expression of IDO1 in IFN-γ-stimulated cancer cells[25-27]. The ENL has shown significant potential in the prevention and treatment of various types of cancers[28,29], but no studies have addressed its potential effects on IDO activity.

Current data showed that IDO1 is expressed by the MCF-7 breast cancer cell line and it was further increased in response to 100 ng mL^{-1} of IFN-γ. This finding aligned with the study of Banzola *et al.*[30] where an *in vitro* model using PCa cell lines (CA-HPV-10 and PC3) showed consistent overexpression of IDO and IL-6 genes when stimulated with IFN-γ or TNF-α at a concentration of 300 U mL^{-1}. These results were also in agreement with those obtained by Zhang *et al.*[31], where *in vitro* experiments, pancreatic cancer cells PANC-1, CFPAC-1 and BxPC-3 expressed the IDO protein. Furthermore, IDO expression levels increased after being stimulated with 500 U mL^{-1} IFN-γ for 48 hrs.

In addition to assessing IDO1 activity, the effects of ENL were evaluated on the viability of IFN-γ-treated MCF-7 breast cancer cells. It was found that different concentrations of ENL did not affect the viability of IFN-γ-treated MCF-7 cells. This finding was in contrast to earlier studies in other cancer types, which showed that ENL can decrease cell viability[32,33]. Another study also estimated that human MCF-7 cells treated with 1 and 10 μM of ENL doses for 48 hrs significantly decreased cell viability[28].

In addition to conducting a cell viability assay, an apoptosis assay was also performed. No significant cell death was recorded and ENL did not induce apoptosis. This result is partly consistent with the study Chikara *et al.*[34], where the Annexin V-FITC/PI apoptosis assay was employed to verify the results of the trypan blue exclusion assay. Non-small cell lung cancer cells (A549) treated with ENL at a concentration of 10, 50 and 100 μM for 24 and 48 hrs did not exhibit a notable increase in cell death compared to the control group. In contrast, another group found that acute myeloid leukemia cells (KG-1) treated with ENL 40 and 100 μM for 48 hrs exhibited a significant increase in apoptosis, particularly with the highest concentration (100 μM)[29].

Finally, the effect of different concentrations of ENL on IFN-γ-stimulated MCF-7 cells was examined through the secretion of IDO1. The data proved that the IDO1 activity increased in IFN-γ stimulated MCF-7 cells when using ENL and was significantly higher using 100 μM. The ENL, a phytoestrogen, exhibits anti-cancer[33] and estrogenic activity[35,36]. It can bind to estrogen receptor alpha and have modest estrogenic or anti-estrogenic effects because of the structural similarities between ENL and 17-estradiol[37]. Increased activity recorded for IDO1 with 100 μM ENL could be explained noting that ENL shares structural similarities with estrogen, as mentioned by Yoder *et al.*[37]. In the study conducted by Li *et al.*[38], significant alterations, including increased IDO expression and decreased SOCS3 expression, were observed when chorionic villi and decidua tissues were cultured with 17β-estradiol at a concentration of 10 ng mL^{-1} and estriol at a concentration of 1 μg mL^{-1}. This implies that ENL may have acted as a weak estrogenic agent by binding to MCF-7 cells and subsequently increasing the activity of IDO1.

CONCLUSION

This study represents the initial evidence of the effect of ENL on the immunosuppressive enzyme IDO1 in IFN-γ stimulated MCF-7 cells. Notably, high concentrations of ENL were found to enhance IDO1 activity. Therefore, ENL should be further investigated due to their IDO1 induced activity.

SIGNIFICANCE STATEMENT

The IDO1 is a key developing biomarker for cancer immunotherapy because it contributes significantly to immunological tolerance. Consequently, IDO1 inhibitor research and development are crucial for tumor treatment. Due to significant potential of ENL in the prevention and treatment of various types of cancers, we are interested to address its potential effects on IDO activity. The results showed that IDO1 is expressed by the MCF-7 cells and significantly increased in IFN-γ stimulated MCF-7 cells when treated with high concentration of ENL. The increasing activity of IDO1 could be explained that ENL has structure similarities with estrogen, which implies ENL may mimic estrogenic agents by binding to MCF-7 cells and subsequently increasing the activity of IDO1.

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