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Research Article
Inhibitory Effect of Enterolactone on Indoleamine 2,3 Dioxygenase in HCT-116 Cancer Cells

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Abstract

Background and Objective: Indoleamine 2,3 dioxygenase 1 is an intracellular enzyme that catabolizes tryptophan into kynurenine. Cancer cells often express Indoleamine 2,3 dioxygenase 1 and can be induced by inflammatory cytokines such as Interferon-Gamma. This study investigated the impact of enterolactone lignan on the indoleamine 2,3 dioxygenase 1 enzyme in colon cancer cells. **Materials and Methods:** In this study, HCT-116 colon cancer cells were stimulated with Interferon-Gamma (IFN-γ) to induce IDO1 expression, followed by treatment with various concentrations of Enterolactone for 48 hrs. The IDO1 expression was measured using ELISA, while apoptosis was evaluated by flow cytometry. The study employed cell culture techniques, cytokine induction enzyme assays, with a focus on understanding the effects of Enterolactone on IDO1 expression. **Results:** Indoleamine 2,3 dioxygenase 1 was expressed by HCT-116 cancer cells and its expression increased after exposure to interferon-gamma. Additionally, It was found that high concentrations of enterolactone inhibited the expression of indoleamine 2,3-dioxygenase 1 (IDO1) in HCT-116 cancer cells. **Conclusion:** These findings suggest that enterolactone is an inhibitor of Indoleamine 2,3 dioxygenase 1 expression in colon cancer cells, without affecting cell viability or inducing apoptosis. Therefore, enterolactone should be further investigated due to its ability to modulate indoleamine 2,3 dioxygenase 1.

Key words: Indoleamine 2,3-dioxygenase 1, Enterolactone, lignan, HCT-116, anti-cancer effect

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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

Colon cancer often abbreviated as CRC, specifically targets the colon or rectum, vital components of the digestive system[1]. In the year 2020, the number of new cases of colorectal cancer (CRC) that were diagnosed worldwide was roughly 1.93 million. The number of deaths was approximately 935,173, accounting for 10% of the total global cancer incidence[2]. In 2023, there will be an estimated 153,020 new cases of CRC, including 106,970 tumors in the colon and 46,050 tumors in the rectum[3].

The progression of CRC is a multifaceted and multistage process, arising from a combination of genetic mutations and environmental factors[4]. Previous studies demonstrated that elevated Indoleamine 2,3 dioxygenase 1 (IDO1) expression played a role in the progression of CRC and was associated with an adverse clinical outcome[5,6]. Previous research also has established that in the Stage I group, 15 out of 30 cases (50%) showed a positive expression for IDO1, whereas in the Stage IV group, 9 out of 30 cases (30%) were positive for IDO1[7].

The IDO1 is a cytosolic enzyme that contains two alpha-helical regions separated by a prosthetic heme group and these regions play a role in the tryptophan (Trp) catabolic pathway by converting Trp into N-formyl kynurenine[8,9]. The IDO1 function was first discovered in mammals dependent on IDO1-mediated immunosuppressive mechanisms to prevent embryonic rejection in utero[10]. The research conducted by Munn, Mellor and their colleagues revealed that IDO1 is expressed in placental cells, which prevents the fetus from being destroyed by maternal T-cell destruction during pregnancy[11,12]. The IDO1 is a critical component in the progression of cancer, as demonstrated by Moon *et al.*[13]. It facilitates inflammation within the tumor microenvironment, promotes immune tolerance to tumor antigens within stromal and immune cells, inhibits T and natural killer cell activity, enhances the development and activation of regulatory T cells and myeloid-derived suppressor cells and promotes tumor angiogenesis[14]. The overexpression of IDO1 is one of the mechanisms that can lead to immune escape in cancer. The main way immune escape occurs through the IDO1 pathway is by decreasing the number of T cells that enter the tumor microenvironment, resulting in poorer oncologic outcomes[8].

The IDO1 can be expressed by cancer cells[15], either by itself or in response to the release of inflammatory cytokines such as Interferon-gamma (IFN-γ) by immune cells that have infiltrated the tumor[16]. The expression of IDO1 is regulated by several immunological factors, with IFN-γ being one of the primary inducers across various human cell types[17]. The IDO1 expression has been observed in several cancers, including breast cancer[18], colorectal cancer[19] prostate cancers[20] as demonstrated by various researchers. The IDO1 inhibition is a particularly good approach to restore or enhance cancer immunosurveillance. The IDO1 inhibitors have shown effectiveness in conjunction with immunotherapy, radiation, or chemotherapy, even in cancers that often show resistance to these therapies[21]. Natural compounds stand out as essential sources of pharmacological agents. In the initial stages of IDO1 inhibitor discovery before 2010, natural compounds provided crucial structural information for the design rational of IDO1 inhibitors[22].

The research found a relationship between the anti-inflammatory properties of natural products and IDO1 activity[23]. The lignans are a class of secondary metabolites of a large group of polyphenols found in plants and human food sources[24]. There is a growing interest in lignans due to their potential bioactive properties in foodstuffs[25]. Flaxseed is the most abundant source of the plant lignan secoisolariciresinol diglucoside (SDG)[26]. The gut microflora is responsible for converting SDG into enterolactone (ENL) and enterodiol (END), which serves as the primary biologically active metabolite[27]. Preclinical studies have suggested that ENL prevents cancer progression by reducing inflammation, tumor development, angiogenesis, metastasis and the induction of cancer cell apoptosis[28]. Previous studies demonstrated the immune-modulatory effect of ENL on the IDO1 enzyme, wherein high concentrations of ENL were found to enhance the activity of IDO1 in MCF-7 breast cancer cells *in vitro*[29].

The study aimed to examine the effects of Enterolactone at different concentrations on the Indoleamine 2,3-dioxygenase1 enzyme in IFN-γ stimulated HCT-116 colon cancer cells.

MATERIALS AND METHODS

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

Cell culture: The HCT-116 colon cancer cell line was kindly provided by (King Fahd Center for Medical Research, KAU University, Jeddah, Saudi Arabia). A DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin was utilized to culture colon cancer cells. The cells were maintained at 37°C in a humidified incubator with 5% CO_2. To sub-culture the cells when they reached 80-90% confluence, they were washed with PBS and detached using 1XTrypLE™ Express Enzyme for 2-5 min at 37°C. Then, a complete serum medium was added to

inactivate the enzyme. The cell pellet was obtained by centrifuging the cells for 5 min at 1500 rpm. The supernatant was removed, the cell pellet was resuspended in a fresh media. Finally, the cell suspension was transferred to a new culture flask containing fresh medium.

Enterolactone preparation: A concentrated stock solution (1 mM) of ENL was prepared using 5% dimethyl sulfoxide (DMSO) by dissolving 5 mg of ENL in 16.76 mL of 5% DMSO. The solution was then stored at 4°C in the dark. Before use, a fresh dilution of the concentrated stock with complete DMEM media was prepared to achieve a working concentration of ENL. It is important to note that the final concentration of DMSO in the working solution never exceeded 0.5% (v/v).

Interferon-gamma preparation: The IFN-γ was prepared by dissolving 100 µg of IFN-γ in 500 µL of sterile deionized water. Upon use; it was diluted with DMEM media to 100 ng/mL.

MTT assay: The MTT Cell Proliferation and Cytotoxicity Assay Kit was used to evaluate the effect of ENL on cell viability. Cells were seeded into 96-well plates at a density of 5,000 cells per well, with 180 µL of medium added to each well. After 24 hrs, IFN-γ was added to the cell culture at a concentration of 100 ng/mL. The next day, the medium was removed, the cells were treated with varying concentrations of ENL (25, 50 100 µM) in complete media for 48 hrs, as per previous work[30]. After treating the cells with ENL for 48 hrs, the supernatant was discarded. Subsequently, 10 µL of MTT solution and 90 µL of fresh culture medium were added to each well. The plate was incubated at 37°C for 4 hrs. Following incubation, the medium was removed, the formazan crystals formed were dissolved by adding 110 µL of Formazan solution. Following a 10 min incubation, the absorbance of each well was measured at 490 nm using a (Synergy HT) microplate reader. Each group contained three replicate wells. The percentage of viable cells was calculated using the formula below[31]:

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

IDO enzymatic activity: The cancer cells were plated at a density of 7×10^5cells/T25 Flasks and allowed to adhere overnight. The next day, IFN-γ was added to the cell culture at a final concentration of 100 ng/mL and the cells were incubated at 37°C with 5% CO_2 to promote IDO1 expression[32]. Following 24 hrs, 5 mL of Assay Medium containing varying doses of ENL is added to the cell culture medium. The cells were cultured in an incubator with 5% CO_2 for 48 hrs at 37°C. Only cancer cell cultures treated with IFN-γ (100 ng/mL) served as positive controls. To lyse the cancer cells, 250 µL of cell extraction buffer was added to each flask. Following a 15 min ice-incubation period, cell lysates were collected and spun down at 18.000×g (20 min, 4°C). After being transferred to a clean Eppendorf, the supernatants were diluted 1:2 with cell extraction buffer. The Enzyme-linked Immunosorbent Assay Human IDO ELISA Kit was used to determine the concentrations of IDO1 in cell lysates and the kit's instructions were followed. In brief, the prepared standards and 50 µL of diluted samples were added to the appropriate wells. Each well was then supplemented with 50 µL of the Antibody Cocktail. The plate was sealed and incubated for 1 hr at room temperature on a plate shaker set to 400 rpm. Following incubation, 350 µL of 1X wash buffer was dispensed into each well, the wells were washed three times by aspirating and refilling with the buffer. The wash buffer was allowed to remain in the wells for at least 10 sec. After the last wash, the plate was inverted and a gentle tap against clean paper towels removed excess liquid. After adding 100 µL of TMB Development Solution to each well, the plate was placed on a plate shaker set to rotate at 400 rpm and incubated in the dark for 10 min. Subsequently, 100 µL of stop solution was added to each well the plate was shaken for 1 min to mix. The absorbance at 450 nm was measured using a microplate reader (synergy HT).

Apoptosis assay: The experiment was conducted using the FITC Annexin V Apoptosis Detection Kit I. A 6-well plate was seeded with 1×10^5 cancer cells per well and the cells were left to incubate overnight. Following a 24 hrs induction period with IFN-γ, the cells were incubated for 48 hrs in a medium containing three different doses of ENL (25, 50 100 µM). The IFN-γ was the only treatment given to positive control cells. Following trypsinization and centrifugation, the cells were twice washed with cold PBS on the day of the experiment. After that, 10^6 cells/mL were resuspended in 300 µL of 1X binding buffer and moved to a 5 mL FACS tube. The mixture was then incubated for 15 min at room temperature (25°C) in the dark with 3 µL of FITC Annexin V and 2 µL of PI added. Within an hour, the samples were examined using the BD FACSAriaTMIII Flow Cytometer (BD Biosciences, US).

Statistical analysis: The data are displayed as the Mean±SEM using GraphPad Prism 10 software. Comparison of different groups was performed using unpaired t-tests and one- and two-way analysis of variance (ANOVA), with $p<0.05$ considered statistically significant.

RESULTS

Effects of ENL on the viability of cancer cells: To examine the impact of ENL on the viability of HCT-116 cancer cells in the presence of IFN-γ, the MTT assay was employed. Following a 24 hrs incubation with 100 ng/mL of IFN-γ, the cells were treated with varying concentrations of ENL (25, 50 100 µM) for 48 hrs. Cells not exposed to IFN-γ were used as a negative control, while those treated with IFN-γ alone served as a positive control. The data represent the Mean±standard error from three replicate wells. The results indicate that ENL did not affect the viability of HCT-116 cancer cells after 48 hrs of treatment (Fig. 1).

IDO1 expression in colon cancer cell: To evaluate IDO1 expression in HCT-116 cancer cells, cells were treated with 100 ng/mL IFN-γ for 24 hrs. As shown in (Fig. 2), ELISA results indicated that IDO1 expression was very low in cancer cells that were not treated with IFN-γ, in comparison to cancer cells that were treated with IFN-γ. Statistical analysis revealed that the differences in IDO1 expression between the control and IFN-γ-treated groups were statistically significant *p<0.05; compared with the control.

Effect of ENL on IDO1 concentration in HCT-116 cancer cells: To evaluate the effect of ENL on IDO1 levels in IFN-γ-induced HCT-116 cancer cells, various doses of ENL (25, 50 and100 µM)

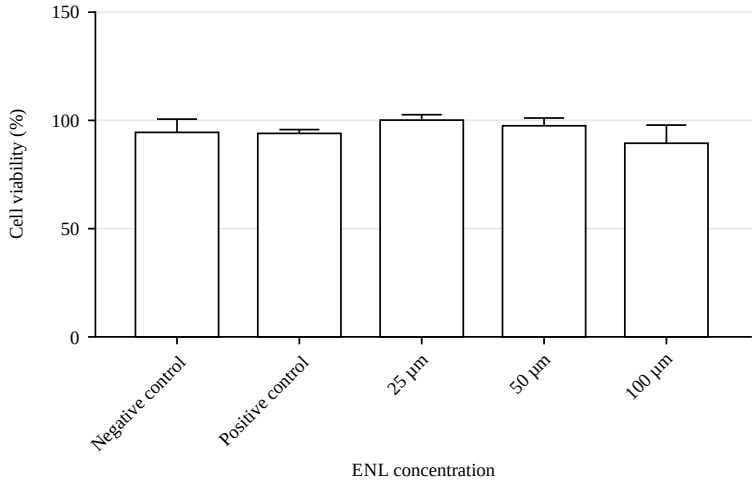

Fig. 1: Cell viability of IFN-γ-treated HCT-116 colon cancer cells assessed using the MTT assay after a 48 hrs exposure to various ENL concentrations (25, 50 and 100 µM)

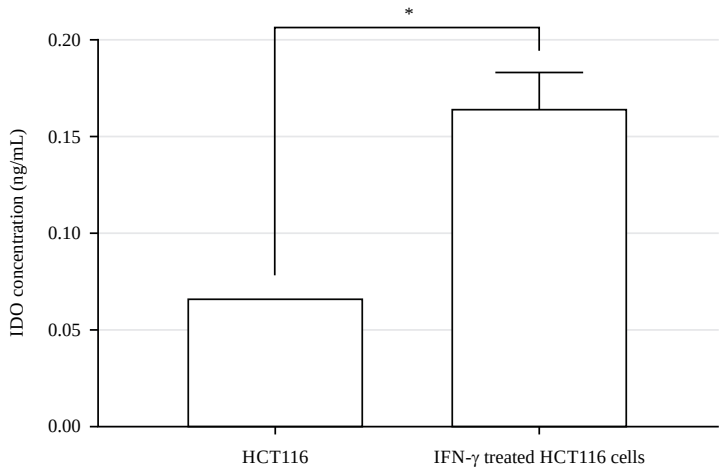

Fig. 2: IDO1 activity in colon cancer cells cultured with or without 100 ng/mL of IFN-γ for 24 hrs
Data are shown as Mean±SEM, with statistical significance indicated by *p<0.05 compared to the control

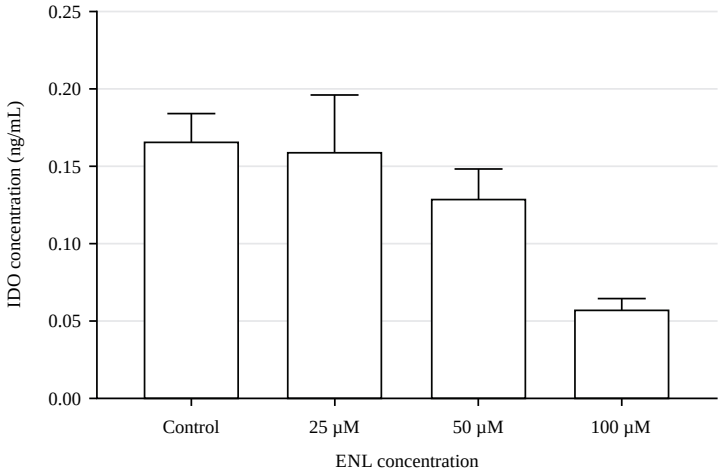

Fig. 3: Effect of ENL on IDO1 concentration in HCT-116 cancer cells

Cancer cells were treated with IFN-γ (100 ng/mL) for 24 hrs, after which ENL was added at concentrations of 25, 50 and100 µM for another 48 hrs. Following the treatment, cell extracts were collected to assess IDO1 levels

were applied for 48 hrs and the concentration of IDO1 was measured using ELISA. As shown in Fig. 3, treatment with 100 µM ENL resulted in a non-significant reduction in IDO1 concentration in HCT-116 cancer cells compared to the control.

Effect of ENL on apoptosis: To validate the results of the MTT assay assessing the cytotoxicity of ENL concentrations, an apoptosis assay was performed using Annexin V-FITC/PI. Flow cytometry analysis, as shown in (Fig. 4a-e), revealed no significant difference in apoptosis between control and treated cells, indicating that 25, 50 and 100 µM are safe concentrations of ENL.

DISCUSSION

Overexpression of IDO1 has been linked to poorer survival rates in various cancer patients, such as those with cervical cancer[33], colorectal cancer[34] and lung cancer[35]. It plays a pivotal role in cancer immunotherapy by enabling tumors to evade the immune system[18]. The ENL has demonstrated considerable potential in both preventing and treating a variety of cancer types[36,37]. However, no studies have specifically investigated its impact on IDO1 activity in colon cancer cells. In previous studies, the immune-modulatory effect of ENL was demonstrated on the IDO1 enzyme, wherein it was discovered that high ENL concentrations increased IDO1 activity in MCF-7 breast cancer cells *in vitro*[29]. In the present study, it was demonstrated that ENL can inhibit IDO1 expression induced by interferon-gamma in HCT-116 colon cancer cells *in vitro*.

The current study showed that IDO1 expression was detected in HCT-116 cancer cells and was significantly increased following stimulation with 100 ng/mL of IFN-γ. This result aligns with the findings of Zhang *et al.*[38] who reported that pancreatic cancer cell lines PANC-1, CFPAC-1 and BxPC-3 expressed IDO protein, with its levels further increasing after treatment with 500 U/mL of IFN-γ for 48 hrs. Similarly, Banzola *et al.*[37] demonstrated that in an *in vitro* model using PCa cell lines (CA-HPV-10 and PC3), stimulation with 300 U/mL of IFN-γ or TNF-α resulted in consistent overexpression of IDO and IL-6 genes. Elevated IDO expression and activity have been strongly associated with colorectal cancer (CRC) progression and are linked to poor clinical outcomes[6].

The effects of ENL on the viability of colon cancer cells treated with IFN-γ were assessed in addition to IDO1 activity. The viability of HCT116 cancer cells treated with IFN-γ was found to be unaffected by varying ENL doses. Prior research on other cancer types demonstrated that ENL can reduce cell viability, which contrasted with our conclusion[39,40]. According to another study[26], treatment of MCF-7 breast cancer cells with 1 µM and 10 µM doses of ENL for 48 hrs resulted in a significant reduction in cell viability. The difference could be explained by variations in ENL concentrations, incubation times and IFN-γ addition.

Interestingly, 100 µM ENL inhibits IDO1 expression in HCT-116 cancer cells. Previous research has demonstrated that various antioxidants can inhibit the expression of IDO1 in cancer cells stimulated with IFN-γ[41]. Curcumin, a phenolic natural product, possesses antioxidant, anti-inflammatory and anticarcinogenic properties. Similar to ENL, it inhibits IDO1 expression and suppresses immunological T-cells upon IFN-γ

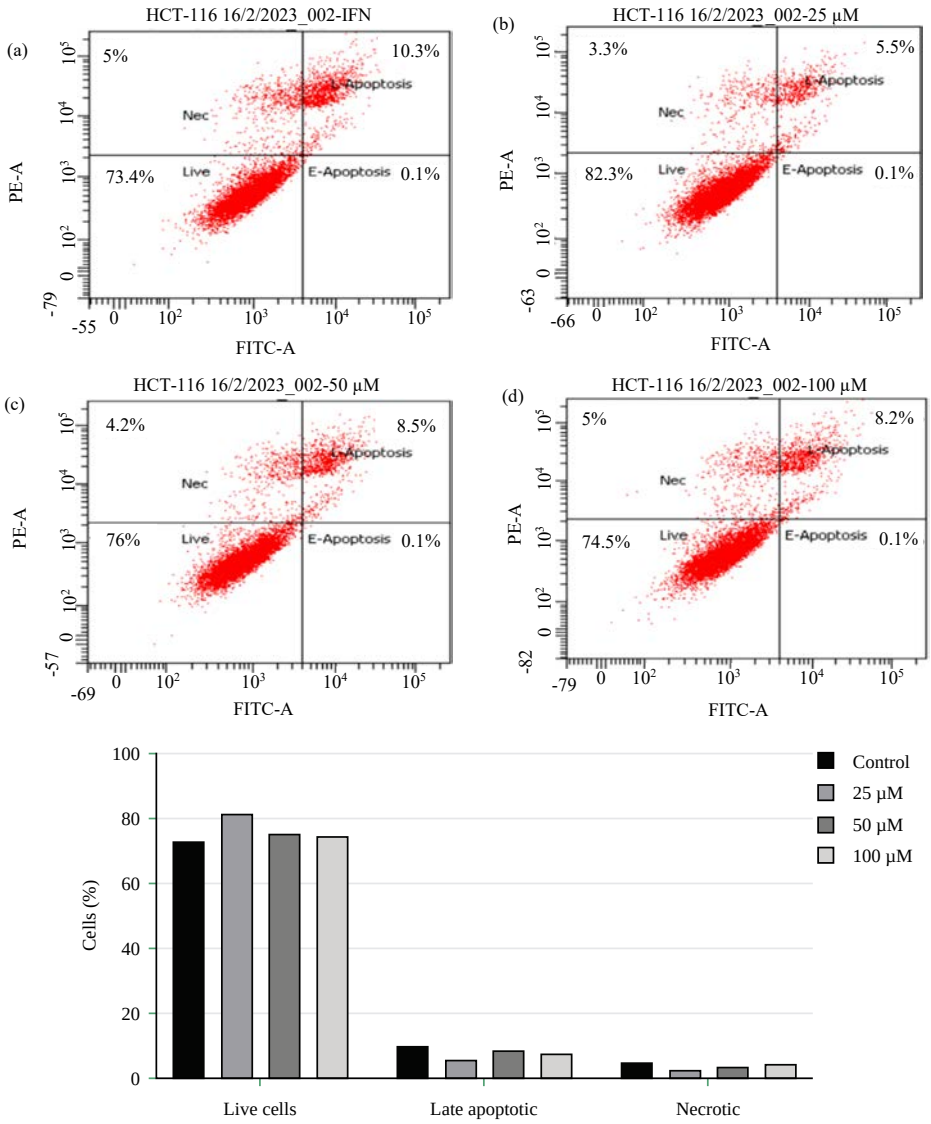

Fig. 4: The effects of different concentrations of ENL on apoptosis in HCT-116 cells, (a) Control, (b) 25 µM, (c) 50 µM, (d) 100 µM for 48 hrs and (e) Bar graph of the apoptosis assay

Annexin V-FITC/PI apoptosis assay results for IFN-γ-stimulated HCT-116 cells treated with ENL and the cells were treated with different concentrations of ENL

stimulation[42]. Likewise, flavonoids found in *S. flavescens*, which have been documented as anti-inflammatory agents and antioxidants[43], flavonoids were found to bind non-competitively with IDO1 and inhibit its activity, as confirmed by plasmon resonance assays[41]. In contrast to previous studies where 100 µM of ENL enhances IDO1 expression in IFN-γ-treated MCF-7[29]. The elevated expression of IDO1 observed at 100 µM ENL may be attributed to the structural similarities between ENL and estrogen, as reported by Yoder *et al.*[44]. This structural resemblance suggests that ENL may act as an estrogenic agent, potentially interacting with MCF-7 cells and subsequently upregulating the expression of IDO1. A likely explanation for these differing molecular responses is that ENL is a phytoestrogen and may exhibit varied effects on different cancer cell types. Phytoestrogens possess a structure and function similar to estrogens, allowing them to compete with estrogens for binding to estrogen receptors[45].

Furthermore, an apoptosis assay revealed no significant cell death or induction of apoptosis by ENL, aligning partially with the findings by Chikara *et al.*[46] where ENL-treated A549 lung cancer cells showed no notable apoptosis. Conversely, a study reported significant apoptosis in KG-1 leukemia cells treated with 40 and 100 µM ENL for 48 hrs, especially at 100 µM[36].

CONCLUSION

In conclusion, this study offers preliminary insights into the impact of ENL on the immunosuppressive enzyme IDO1 in IFN-γ-stimulated HCT-116 colon cancer cells. The study indicates that the expression of IDO1 in HCT116 increased after stimulation with IFN-γ and was subsequently inhibited by ENL. Current study results suggest that ENL is an inhibitor of IDO1 expression in colon cancer cells, without affecting cell viability or inducing apoptosis. These findings offer new perspectives on the inhibitory effects of ENL on IDO1 expression in colon cancer cells, highlighting its potential as a novel IDO inhibitor for cancer immunotherapy. Considerably more studies will be needed to determine the impact of ENL at different concentrations on IDO enzyme activity.

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