Isodeoxyelephantopin (IDOE) Retards Tumor Cell Migration by Downregulating MMP-2/9 Expression in Triple Negative Breast Cancer

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ABSTRACT

Introduction: Isodeoxyelephantopin (C₁₉H₂₀O₆), a sesquiterpene lactone extracted from Elephantopus Scaber Linn., have tremendous contributions in inhibiting the proliferation and metastasis of cancer cells.

Materials and Methods: The cell viability assay was shown to be procured half-maximal inhibitory concentration of isodeoxyelephantopin on MDA-MB-231 cell line. Apoptosis was screened by AO/EB staining and it was confirmed by annexin V staining. The DNA damaging property of IDOE was examined by comet assay. The cell cycle arrest determined using flow cytometry analysis and the growth inhibition due to the modulation of cell cycle regulatory protein p53 were substantiated by RT PCR. Scratch wound migratory assay was done to evaluate the migratory effect of IDOE on MDA-MB-231 cell line. RT-PCR analysis showed that expression of MMP-2/9 was inhibited by IDOE treatment.

Result: Anti-proliferative and anti-migratory effect of IDOE was determined by cell cycle analysis and annexin V staining. Cells were arrested in the G2/M phase and it is due to the modulation of cell cycle regulatory protein p53. The mRNA level expression studies of matrix metalloproteinases, MMP-2/9 provide supporting data to prove the anti-migratory property.

Keywords: Apoptosis, Cell proliferation, Isodeoxyelephantopin, Migration, Triple-negative breast cancer.
of IDOE in cancer cell proliferation, migration, invasion, and metastatic rate. Gene expression studies have evidently documented the regulatory role of isodeoxyelephantopin in the exertion of matrix metalloproteinase-2/9 (MMP-2/9) and tumor suppressor gene (p53) during cancer progression along with its constitutive and inducible suppressing activity in NF-κB signaling. Current investigation attempts to reveal the anti-cancerous property of isodeoxyelephantopin in tumor-associated processes along with its down regulating property in MMP-2/9 expression on the aggressive phenotype, triple-negative breast cancer cell line MDA-MB-231.

**Materials and Methods**

*E. Scaber* whole plants are taken, and 12 hours of chloroform extraction is performed followed by silica gel column chromatography and eluted with hexane-ethanol acetate gradient. The purified drug in crystalline form (99%) was obtained from NIIST Trivandrum (Laboratory of Dr. Mangalam S. Nair - CSIR – NIIST, Trivandrum, India) and dissolved in dimethyl sulfoxide (DMSO). Serial dilutions made using DMEM from 100 mM stock.

**Cell line and cell culture conditions**

The human triple-negative breast adenocarcinoma cell line MDA-MB-231 were gifted from Rajiv Gandhi Centre for Biotechnology. Required culture medium, DMEM (GIBCO, USA) supplemented with 10% (v/v) fetal bovine serum (GIBCO, USA) and 1% penicillin/streptomycin (GIBCO, USA). Cell cultures were incubated in a humidified atmosphere of 95% oxygen and 5% carbon dioxide at 37°C.

**Cell viability assay**

Cell viability of MDA-MB-231 triple-negative breast cancer cells was treated with IDOE assessed using 3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen, USA). Cancer cells were seeded at the density of 10,000 cells/well in 96 well microplates and incubated for 24 hours. Various concentrations (100 – 12.5 µM) of IDOE in medium were prepared and added to the cultured cells. After 48 hours of incubation, medium was removed and fresh medium along with 20 µL of MTT (5 mg/mL) were added to each well. After incubation of 3.5 hours at 37°C in the dark, formazan crystals formed were solubilized with acidified isopropanol. The color developed was quantified (measuring wavelength: 570 nm; reference wavelength: 630 nm) with 96 well plate reader (BIORAD, USA). The concentration which inhibited 50% of cellular growth (IC₅₀ value), was determined and calculated by the following formula:

$$\text{Survival rate} = \frac{\text{OD of drug treated}}{\text{OD of control}} \times 100$$

The cytotoxicity of IDOE on MDA-MB-231 was expressed as IC₅₀ values (the drug concentration, reducing the absorbance of treated cells by 50% with respect to untreated cells). The experiments were carried out in triplicate. DMSO (0.1%) was used as the negative control.

**Acridine orange/Ethidium bromide staining**

MDA-MB-231 cells grown in 96 well plates were treated with 50 µM IDOE for 24 hours. After washing once with PBS, the cells were stained with a mixture of 100 µL acridine orange and ethidium bromide (1:1; 4 µg/mL) solution. Cells were immediately washed with PBS and viewed under Olympus inverted (IX-51) microscope using respective filters. The differential uptake of AO/EB allows the identification of viable and non-viable cells by morphological features like chromatin condensation and the formation of apoptotic bodies.

**Cell cycle arrest by flow cytometry**

After IDOE treatment at the indicated time, DNA content-based cell cycle analysis was assessed using flow cytometry (Muse™ cell analyzer, Merck, Millipore) and Muse cell cycle kit according to the manufacturer’s instruction.

**Quantitative RT-PCR**

After treatment with IDOE (50 µM for 24 hours) for the indicated time points, total RNA was isolated using TRI reagent (SIGMA, USA) following the manufacturer’s protocol. The purity and concentration of total RNA was determined. The cDNA was synthesized using cDNA preparation kit (Thermo scientific, product code – AB1453A, Verso cDNA synthesis kit). Real-time PCR was carried out using SYBR green master mix (APPLIED BIOSYSTEM, Life technologies). Specific primers used are listed in table. (Table 1). Beta-actin, a non-regulated housekeeping gene, was used as an internal control to normalize input cDNA.

**Scratch Wound Assay**

Scratch wound assay is an easy and low-cost method to measure cell migration in vitro. MDA-MB-231 cells were seeded with 5,00,000 cells per well in a 24 well plate, incubated for 24 hours in serum-free media. A 10 µL pipette

| Gene   | Forward primer (5’-3’)                                | Reverse primer (5’-3’)                   |
|--------|------------------------------------------------------|-----------------------------------------|
| p53    | CGCCCTCTGGCCCCCTGTACATCTTG                          | GCAAGCCGCTCCACACCTCGCAT                 |
| MMP2   | GGCTGCTAGTGGCTGGTTGA                                 | AGATCTCTTCTTCTCAAGGACCCTGGTT            |
| MMP9   | GGGAGATGGGACACCACGCTGTA                              | GAGCGGCTGTGACACCCAC                   |
| Beta-actin | TACCCCACTTGCCCATCTACG                              | CAGCGGACCACGCTCATTTGCAATGG          |

Table 1: Sequence of forward and reverse primers used for RT-PCR.
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Journal of Applied Pharmaceutical Sciences and Research, April-June, 2021; 4(2)

Cells were seeded in 96 well plates and then treated our in

staining. The IDOE (50 µM) treated cells MDA-MB-231 (50.9%) revealed that the average mean number of Et Br positive nucleus differed significantly as a function of the concentration of IDOE. *p < 0.05; **p< 0.01; ***p< 0.001

Results

IDOE inhibits proliferation of triple negative breast cancer cell line MDA-MB-231

IDOE inhibited the proliferation of triple-negative breast cancer cell line MDA-MB-231 in a concentration-dependent manner (100–12.5 µM) for 48 hours (Figure 2 A). This clearly indicates that treatment of IDOE (50 µM) for 48 hours decreased cell proliferation by 50%. We selected 50 µM concentrations for further studies.

IDOE induces nuclear condensation in MDA-MB-231 cell line

The ability of the IDOE to induce apoptosis was initially screened by using acridine orange/ethidium bromide staining. The IDOE (50 µM) treated cells MDA-MB-231 (50.9%)...
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Andrographolide

Figure 3: A) Effects of IDOE on DNA damage in MDA-MB-231 cells by using comet assay. Cell suspensions of 1*10⁶/5-30 µL were incubated with 0-50 µM IDOE for 24 hours and then examined for DNA damage using the Comet assay. B) As described in materials and methods comet tail length was calculated, quantified and expressed in mean ± SD for at least three replicates. All results were expressed as the mean percentage of control ± SD of quadruplicate determinations from three independent experiments. The differences among the mean values were analyzed using one-way ANOVA followed by Tukey’s post hoc t-test analysis. The one-way ANOVA revealed that the average mean values of comet tail formation between control and IDOE treated cells did not differ significantly. *p < 0.05; **p < 0.01; ***p< 0.001. C) Effect of IDOE on DNA fragmentation in MDAMB 231 cells. Cells (1 x 10⁶ cells/well; 96-well plates) were plated in DMEM medium + 10% fetal bovine serum (FBS) with 0 and 50 µM of IDOE for 24 hours. The cells were collected by centrifugation and then DNA was isolated for DNA gel electrophoresis.

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showed obvious nuclear condensation after 24 hours treatment, similar to doxorubicin treated cells (37.9%). Control cells (8.9%) showed a bright green nucleus with uniform intensity and had not taken up ethidium bromide, where the apoptotic cells appeared orange in color (Figure 2B, C). Based on the above cytomorphological changes and cell death, the effect of IDOE in these cells were indicative of apoptosis.

IDOE does not induce DNA Damage in MDA-MB-231 cell line

DNA damaging properties of IDOE were evaluated using comet assay (tail formation) and DNA laddering (DNA strand breaks). MDA-MB-231 cells were incubated with 50 µM IDOE for 24 hours to assess DNA tail formation and DNA laddering. We did not observe a significant increase in tail formation in IDOE treated cells compared to control, which represents extent of DNA damage (Figure 3. A,B,C). This indicates there is no significant increase in DNA damage in IDOE treated MDA-MB-231 cells.

IDOE induces cell cycle arrest and modulates p53

Further, we checked whether IDOE-induced cell growth inhibition was due to blockade of cell cycle. Cell phase distribution analysis showed a 1.4-fold and 1.5-fold increase in the number of cells at the S phase and G2/M phase respectively upon treatment with IDOE with a corresponding decrease in the number of cells at the G1 phase (Figure 4. A, B). Distribution of cells in untreated culture were G0 (1.6%), G1 (44.8%), S (16.9%) and G2/M (14.5%). In cells treated with 50uM IDOE for 24 hours, cell cycle distribution was G0 (2.6%), G1 (30.1%), S (24.8%) and G2/M (22 %). We also checked whether IDOE-induced cell growth inhibition was due to modulation of the cell cycle regulatory protein p53. In an effort to find the upstream cell cycle regulatory molecules, we checked the expression of p53 at mRNA level. Our results revealed that IDOE induces an up-regulation of p53 at mRNA level (Figure 4. C).

IDOE Inhibits the Migration of MDA-MB-231 cells via Inhibition of MMP-2/9

The inhibitory effect of IDOE on MDA-MB-231 was evaluated by scratch wound migration assay. A concentration of 50 µM IDOE for 24 hours effectively reduced the migration of cells into the wounded area, which was determined as a movement of cells (mm) into the wound (Figure 5. A, B). Real-time-PCR analysis showed that IDOE inhibited expression of MMP-2/9 in MDA-MB-231 cells was inhibited by IDOE at 50 µM (Figure 5. C). We could observe nearly fourfold decrease in the expression of MMP-2/9 in IDOE-treated MDA-MB-231 cells.

Discussion

Cancer cells are known to evade cell death by various means; persistence of cancer or being dormant in adverse conditions and thereby avoiding detection is one among them. These hidden cells later contribute to cancer relapse and metastasis. So novel candidates for cancer treatments need to be analyzed thoroughly for their efficacy in ensuring cancer elimination.

The anticancer properties of IDOE have already been demonstrated previously, and its effects are attributed to several cellular pathways. In lung cancer cell lines, inhibition of autophagy combined with IDOE treatment showed increased levels of apoptosis through regulation of Nrf2 feedback loop.[18,19] Another work showed that IDOE could suppress osteoclastogenesis induced by RANKL dependent pathways.[20] In the present study the effect of IDOE on triple-negative breast cancer MDA-MB-231 is analyzed and found that IDOE is capable of causing cell death in a dose-dependent manner with an IC₅₀ value of 50µM at 48 hours. The observation from the MTT assay is comparable to the analysis done on MDA-MB-231 with “Andrographolide” - which is also herbal in origin from the plant Andrographis paniculata.[21] We further conducted a series of experiments to...
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After treatment, cells were labeled with PI and analyzed by flow cytometry. Data indicate the percentage of cells in each phase of the cell cycle. All experiments were performed in triplicate with similar results and analyzed by Student’s t-test. * p < 0.05; ***p < 0.001

Figure 4: (A) Cell cycle analysis of MDA-MB-231 cells after treatment with IDOE. Cells were synchronized at the G0 stage by serum starvation. After treatment, cells were labeled with PI and analyzed by flow cytometry. Data indicate the percentage of cells in each phase of the cell cycle. All experiments were performed in triplicate with similar results and analyzed by Student’s t-test. * p < 0.05; ***p < 0.001

(B) Untreated and IDOE treated MDA-MB-231 cells were stained with PI, and flow cytometry was used to analyze the cells at different phases of cell cycle.

(C) Analysis of p53 in IDOE treated MDA-MB-231 cells: Real-time RT-PCR analysis of p53 inhibition by IDOE. The real-time PCR was performed by 1 μg of cDNA and 2 pmol primers per reaction in Quant studio 3 PCR system using SYBR Green qPCR Master mix for SYBR Assay. Fold increase of the target mRNAs were normalized with that of β-actin and is plotted as a graph. Each value is presented as the mean ± SD of determinations from two independent experiments. The mean fold change in the IDOE treated group was significantly higher than the control group as analyzed using One-way ANOVA followed by Tukey’s post hoc t-test analysis. ***p < 0.001.

specifically identify the mechanism of induction of cell death by IDOE in the TNBC. Acridine Orange/Ethidium Bromide (AO/EB) assay showed IDOE induces nuclear condensation and annexin V binding assay confirmed the structural disruption of the plasma membrane, suggesting activation of apoptosis pathways by IDOE. Chromatin condensation is an important morphological change that is observed usually in dying, aged, or senescent cells. In the context of cancer cells, senescence has many implications, and thus confirmatory assays are necessary for cross-checking the cell death induction potential of the candidate drugs. Even though annexin V staining is recently observed in necrosis and other non-apoptotic cell death, previous study in IDOE has proved the drug enhances apoptosis through NF-κB pathway inhibition in leukemia, adenocarcinoma, and other cell lines. Though p53 is considered as the main protein involved in apoptosis, several studies described p53 independent apoptosis mechanisms in breast carcinomas when treated with different anticancer agents. We, therefore, examined the expression level of p53 and found a significant upregulation of p53 in IDOE treated cells, and hence ruled out the possibility of p53 independent mechanisms of apoptosis at least triple-negative breast cancer cell lines.
Cancer cells are known to use multiple mechanisms to bypass tumor-suppressing activities and one of the mechanisms is using a mutant version of p53 itself. It has been shown that the MDA-MB-231 cell line harbours a higher number of mutant p53 which facilitates the survival of cancer cells. The observed upregulation of p53 in IDOE treated cells needs to be studied further to verify whether IDOE is upregulating the wild-type p53 or the mutant counterpart.

Dysregulation of repair pathways is a hallmark of cancers. Therefore, interacting with DNA repair and causing DNA damage is employed as a therapeutic option and even used in combination with radiation as a better treatment option. But recent reports of chemo-resistance suggest DNA damage can no longer be considered as a remarkable feature of drugs. In addition to this, it has been reported recently that cancers, including TNBCs make DNA damage by self-inducing double-strand breaks through leakage of cytochrome c for their survival benefits. Our gel-based DNA fragmentation and comet assays showed IDOE is not inducing DNA breaks, making it a better therapeutic option.

Many phytochemicals that contain sesquiterpene lactones have undergone extensive studies for their anti-proliferative abilities. Both DOE and IDOE, derived from E. scaber, are structurally similar with sesquiterpene lactone moieties. A previous study conducted in uterine leiomyoma using DOE reported cell cycle arrest in G2/M stage and we expected a similar cell cycle blockade upon IDOE treatment and our flow cytometry analysis showed cell cycle progression in MDA-MB-231 cells were severely affected with cell accumulation in G2/M phase. It suggests, IDOE is not interfering with G1 or G1/S transition stages, instead causing G2/M arrest. A slight increase in G0 cell population is observed, and this needs to be studied with importance since it has been previously identified that cancer cells in G0 phase evade chemotherapy and show enhanced invasion abilities. p53 is mostly acted on the G1/S transition stage mediating checkpoint activation but inhibits mitotic progression by inhibiting Cdc2 during G2/M transition in response to the DNA damage or replication stress. The arrest observed at G2 phase along with p53 upregulation but without DNA fragmentation may suggest the mode of cell death by IDOE may not be through canonical pathways.

Cancer severity can be accelerated with the metastatic ability of the cancer cells, and MDA-MB-231 is known for its higher level of metastatic potential. Concerted manipulation/exploitation of several pathways is required for metastatic progression. Several data indicate most cancers tend to develop into metastasis at later stages. Breast cancer cell lines adopt complex mechanisms for efficient migration, including neutrophil extracellular trap formation with extracellular DNA making it more difficult for treatment. So, drugs needed to be analyzed for their potential in inhibiting cell migration. Wound healing assay has shown IDOE is inhibiting horizontal cell migration. In addition to that, the expressions of MMP-2/9 were also significantly reduced upon treatment with IDOE. However, the mechanism of action behind the inhibition of migration and MMP-2/9 is not well understood (Figure 6).

Recent research in this direction using IDOE in MDA-MB-231 suggested NF-κB mediated inhibition of metastasis. Another study reported Formononetin-an isoflavone has shown to inhibit migration in MDA-MB-231 by reducing the expression of MMP-9 through Akt/Pi3K signaling. Cancer cells exploit pathway dependency of signaling cascades for ensuring cell survival through multiple regulations. NF-κB, being the central signaling pathway in inflammation and cancer, reported having cross talks with the Akt/Pi3K pathway. It indicates the need of future research to identify the exact mechanism by which IDOE exerts its anticancer properties.

The present study shows IDOE as a promising candidate for cancer therapeutics. Currently, many plants derived anticancer drugs are undergoing clinical trials and validation. Side effects of cancer drugs on normal cells and the reduction in the quality of patient’s life is a major problem we face in chemotherapy today, causing many patients to restrain from taking treatment on time. Reducing the dosage of drugs without interfering with the quality of treatment can be the solution, to an extent. Many phytochemicals showed enhanced therapeutic properties when they were combined or modified than their native form. DETD-35, a derivative of DOE, is found to have exhibited better anti proliferative effect in MDA-MB-231 in a relatively reduced IC50 value than DOE alone. Studies should be done more on IDOE in line with ongoing cancer research advancements to better understand its properties and make it suitable for combination therapies.

Figure 6: Schematic representation of IDOE on human triple negative breast adenocarcinoma cell line MDA-MB-231
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**CONCLUSION**

Many natural compounds retards cell proliferation in cancer tissues. However, data regarding the influence of IDOE on triple-negative breast cancer are very limited, and there is a strong need to replace chemotherapeutic treatment strategies. This paper aims to provide new insight about the possibility of using the natural compound to enhance treatment efficacy. Here, IDOE shows inhibition in G2/M phase of cell cycle, thereby elevates the expression of the tumor suppressor gene - p53; their inhibitory role also decreases the expression of MMP-2/9, showing an inhibition in the cell migration.

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