**University of Szeged**

**Faculty of Pharmacy**

Institute of Pharmacodynamics and Biopharmacy

# *In vitro* and *in vivo* evaluation of the antineoplastic potential of novel D-ring-modified estrone derivatives

Summary of Ph.D. Thesis

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**Szeged**

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**University of Szeged**
**Faculty of Pharmacy**

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**Sándor Bartha**

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**2026**

## 1. Introduction

Cancer remains a leading cause of morbidity and mortality worldwide, with approximately 20 million new cases and 9.7 million deaths reported in 2022. The global cancer burden is projected to increase substantially in the coming decades, largely driven by population aging and lifestyle changes. Incidence and mortality rates vary markedly across regions: high-Human Development Index (HDI) countries generally report higher incidence due to longer life expectancy and widespread screening, but lower mortality owing to early detection and advanced treatment options. In contrast, low- and middle-HDI regions often experience higher mortality, reflecting late-stage diagnosis and limited access to healthcare. Cancer arises from a complex interplay of extrinsic factors, including environmental exposures such as tobacco, alcohol, diet, and infections, and intrinsic factors, notably genetic predisposition. Germline mutations in tumor suppressor genes such as *BRCA1*, *BRCA2*, and *TP53* markedly increase lifetime cancer risk. Importantly, nearly 90% of cancer-related deaths result from metastatic disease rather than the primary tumor. Metastatic progression involves enhanced migratory and invasive capacities of cancer cells, their survival in circulation, and colonization of secondary sites. Moreover, it represents the primary clinical challenge in cancer management. Consequently, understanding the molecular mechanisms driving metastasis and designing new agents targeting this aspect is critical for improving patient outcomes and reducing cancer mortality.

Breast cancer is the most frequently diagnosed malignancy and the leading cause of cancer-related death among women worldwide, with 2.3 million new cases and more than 665,000 deaths reported in 2022. Its development is a multistep process driven by genetic alterations, hormonal signaling, tumor-microenvironment interactions, and immune evasion. Breast cancer is classified pathologically and molecularly into subtypes with distinct prognostic and therapeutic implications, including hormone receptor-positive (HR+), HER2-positive, and triple-negative disease. Approximately 70–80% of breast cancers are estrogen receptor-positive (ER+), a subtype characterized by estrogen-dependent growth and generally better prognosis.

A central feature of HR+ breast cancer, particularly estrogen receptor-positive (ER+) tumors, is its dependence on estrogen signaling for growth. Endocrine therapy remains the mainstay for managing ER+ breast cancer, both in the adjuvant and metastatic settings. In premenopausal women, tamoxifen, a selective estrogen receptor modulator (SERM), is the

primary agent of choice. In postmenopausal women, aromatase inhibitors (AIs), such as anastrozole, letrozole, and exemestane, are preferred.

For patients at higher risk of recurrence, extended endocrine therapy up to 10 years is now standard, as longer durations have been shown to reduce recurrence risk and improve survival. However, resistance to endocrine therapy, either intrinsic or acquired, is a major barrier to durable responses in the metastatic setting.

In efforts to overcome recurrent ER+ breast cancer, fulvestrant, a pure antiestrogen and one of the earliest selective estrogen receptor degraders (SERDs), represented a significant milestone in this area. However, fulvestrant suffers from poor bioavailability and requires special administration, warranting the development of novel SERDs with improved oral availability and superior pharmacological profiles.

Steroids represent a diverse group of biologically active compounds, generally associated with favorable pharmacological properties, including low cytotoxicity and high bioavailability. The connection between steroids and cancer has been observed and studied extensively since the mid-1900s, where it has been established that sex steroids are the main drivers of cell proliferation in hormone-sensitive tissues.

Advancements in computational technologies have further enhanced our understanding of how steroids influence cancer cells. In clinical practice, several antineoplastic agents with a steroidal core structure are in use; these compounds either inhibit endogenous hormone biosynthesis (e.g., exemestane) or interfere with hormone-receptor interactions (e.g., fulvestrant, cyproterone acetate). Given their direct involvement in cancer development, researchers are actively exploring new steroidal compounds, particularly those structurally related to sex hormones with potential growth-inhibitory properties.

Concerning the biological effects of estrogen-related compounds, our research group (Zupkó and colleagues) has been investigating the *in vitro* antiproliferative and antimetastatic potential of this particular group of molecules for more than a decade. Structural modifications, such as introducing heterocycles, altering the steroidal side chains, or incorporating heteroatoms within the steroid framework, are known to modulate their biological properties.

In this study, we focused on the investigation of the *in vitro* antiproliferative effects of aryl-substituted oxazolines fused to the estrane D-ring at the sterically accessible 16α and 17α positions. Moreover, we also explored the biological evaluation of organophosphorus derivatives of 3-methyl and 3-benzyl ethers of the core-modified 16-methylene-13α-estrone lacking estrogenic activity due to the conformational change of C-13.

## 2. Aims and Objectives

The primary aim of this study was to determine the antiproliferative potential of several D-ring-modified estrone 16α,17α-oxazoline and 16-methylene-13α-estrone derivatives. Initial screening of the test compounds was conducted through an *in vitro* viability assay using a panel of human adherent cell lines of female breast, gynecological, and oropharyngeal origin. Further testing of the most prominent compound included several additional *in vitro* and *in vivo* methodologies to gain insight into its antiproliferative and antimetastatic properties, as well as its mechanism of action.

Accordingly, detailed objectives of the experiments are clarified as follows:

- To assess the antiproliferative potential of nine D-ring-substituted estrone 16α,17α-oxazoline derivatives against a panel of human adherent gynecological and female breast cancer cell lines by means of standard MTT assay. Furthermore, the assessment of the half maximal inhibitory concentration ($IC_{50}$) values of the most potent test compounds.
- To determine the antiproliferative potential of fourteen 16-methylene-13α-estrone derivatives against a panel of human adherent gynecological cancer cell lines by means of standard MTT assay. Furthermore, the assessment of the $IC_{50}$ values of the most potent test compounds.
- To evaluate the estrogenic activity of the most effective test compound *in vitro* using a luciferase reporter gene assay in a commercially available, stably transfected T47D cell line.
- To assess estrogenic activity *in vivo* and to gain insight into the hormonal properties of the test compound using a uterotrophic assay.
- To investigate the cell cycle arrest potential of the test compound using propidium iodide-based cell cycle analysis.
- To evaluate the antimetastatic properties of the most potent test compound using wound healing and Boyden chamber assays.

## 3. Material and Methods

### 3.1. Chemical structures of novel D-ring modified estrone derivatives

The tested estrone 16α,17α oxazoline derivatives (Fig. 1) and organophosphorus 16-methylene-13α-estrone derivatives (Fig. 2) were synthesized by Kiss et al. and Mernyák et al., respectively, as previously reported.

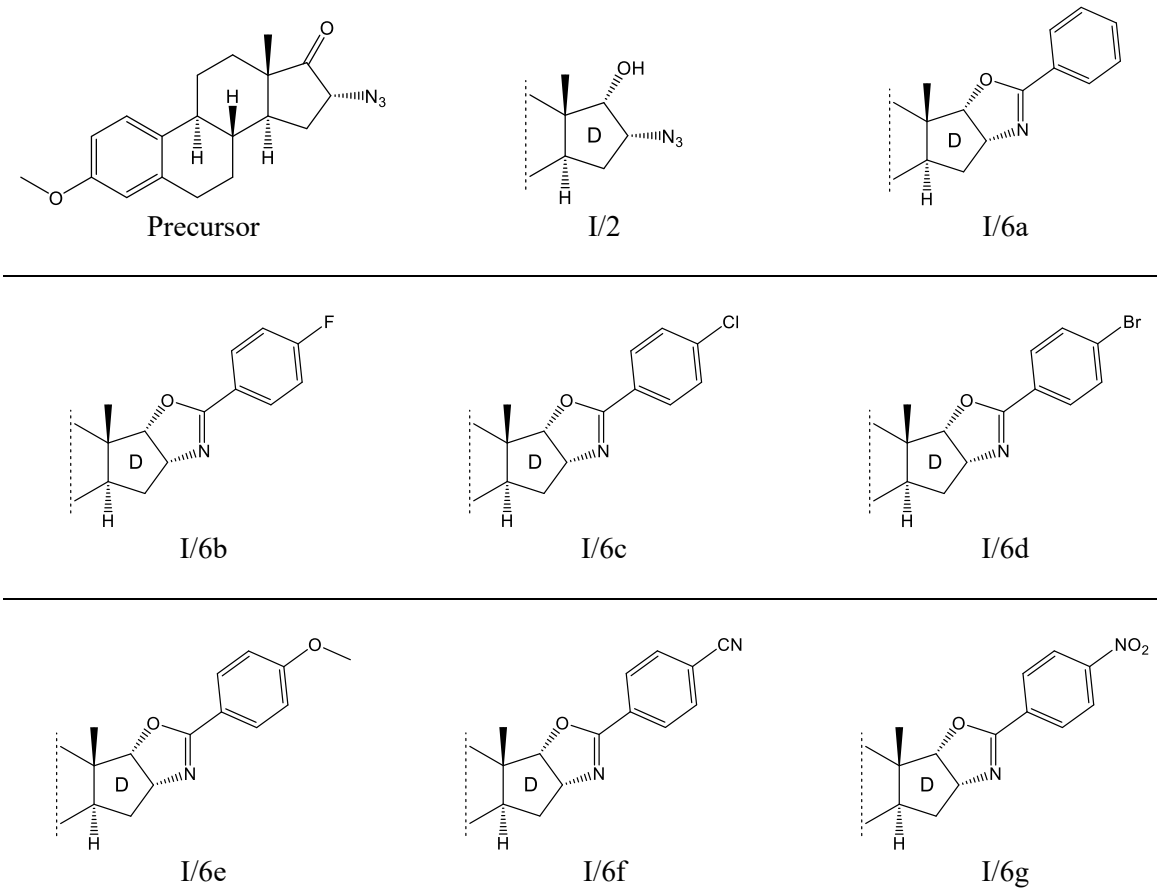



**Figure 1:** Chemical structures of the tested estrone 16α,17α-oxazoline derivatives

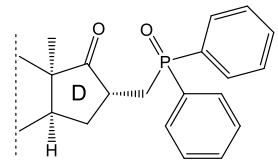
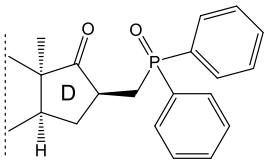
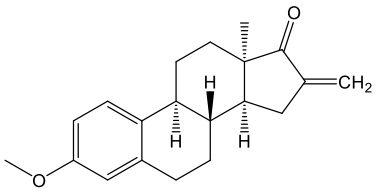
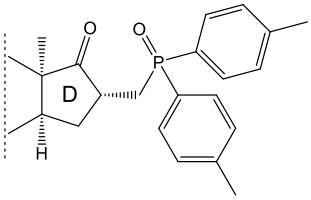
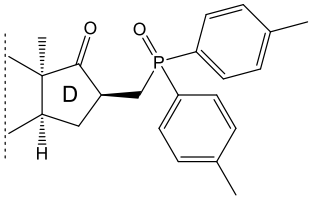
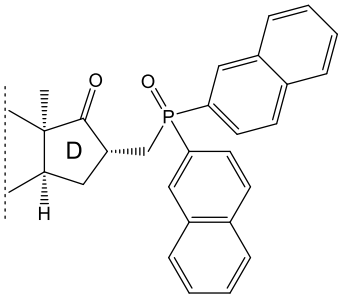
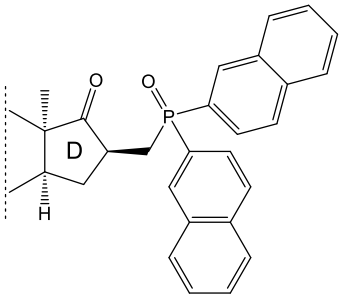
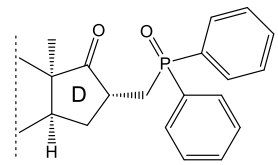
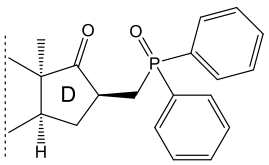
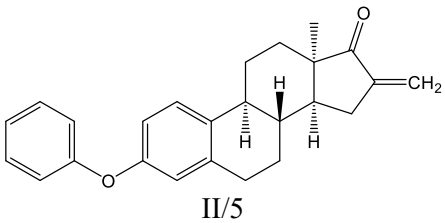
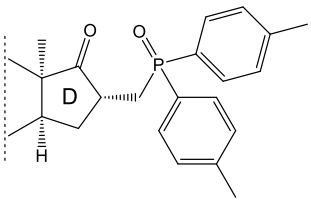
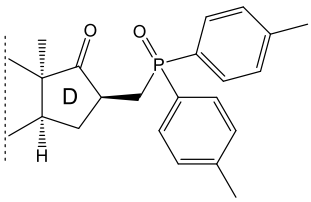
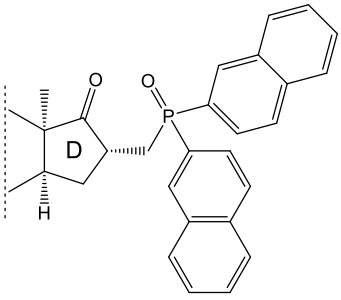
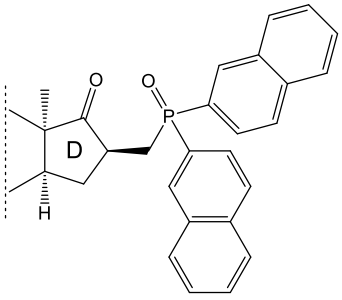
| Precursors | C-16 | |
|---|---|---|
| | α | β |
| |  II/9 (EDPO) |  II/10 |
|  II/4 |  II/13 |  II/14 |
| |  II/17 |  II/18 |
| |  II/11 |  II/12 |
|  II/5 |  II/15 |  II/16 |
| |  II/19 |  II/20 |

**Figure 2:** Chemical structures of the tested 16-methylene-13α-estrone derivatives

3.2. Cell cultures

In this study, human adherent carcinoma cell lines of gynecological (HeLa, SiHa, C33A and A2780), female breast (T47D, MCF-7 and MDA-MB-231), and oropharyngeal (UPCI-SCC-131, UPCI-SCC-154) origin were used to assess the antiproliferative properties of the test compounds. Mouse fibroblast (NIH/3T3) cells were used to determine tumor selectivity. Cells were cultured and maintained in a minimum essential medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 1% L-glutamine and 1% antibiotic-antimycotic mixture. Moreover, the T47D-KBluc cell line was used to determine the antiestrogenic effect. Cells were cultured in phenol red-free RPMI medium supplemented with 10% FBS, 1% non-essential amino acids, 1% L-glutamine, and 1% antibiotic-antimycotic mixture. All cell lines were incubated at 37°C in humidified atmospheric air containing 5% $CO_2$.

3.3. Antiproliferative (MTT) assay

The antiproliferative effects of the test compounds were assessed on the previously described panel of cell lines using MTT assay. Tumor selectivity was evaluated using a mouse fibroblast cell line, NIH/3T3. Cells were treated with treated with 10 or 30 μM solutions of the compounds, followed by a 72-hour incubation period. Subsequently the samples were subjected to MTT assay, and absorbance was measured by a microplate reader at 545 nm. In the case of active compounds (i.e., cell growth inhibition reached 50% at either 10 or 30 μM), the assay was repeated with a series of dilutions (0.1–30 μM), and sigmoidal concentration-response curves were fitted to the obtained data, and $IC_{50}$ values were calculated using GraphPad Prism 5.01. Cisplatin was used as the reference agent.

3.4. Luciferase reporter gene assay

The *in vitro* antiestrogenic potential of the test compound EDPO and its 3-benzyloxy counterpart (3-BEDPO) were evaluated using the T47D-KBluc cell line, an ERα+ breast cancer model that is stably transfected with an ERE-luciferase reporter gene cassette. Cells were exposed to 17β-estradiol (E2) and fulvestrant or the test compounds for 24 hours. Luminescence was measured using a FLUOstar OPTIMA luminometer. The resulting Relative Luminescence Units (RLU) were normalized to percentage values, and sigmoidal concentration-response curves were generated using GraphPad Prism 9.5.1.

3.5. *In vivo* uterotrophic assay

Following the results obtained from the *in vitro* luminometric assay, we sought to assess the antiestrogenic activity of the test compound *in vivo*. Immature Sprague-Dawley rats were assigned to five experimental groups: vehicle (vegetable oil); E2 (0.1 μg/g E2); E2 + FULV (0.1 μg/g E2 + 0.3 μg/g fulvestrant); E2 + EDPO (60) (0.1 μg/g E2 + 60 μg/g EDPO); E2 + EDPO (600) (0.1 μg/g E2 + 600 μg/g EDPO), with 6–7 animals per group. After three consecutive daily treatment, on the fourth day, uterine tissues were surgically removed under deep anesthesia. Wet uterine weights were recorded, and the results were compared to those of the positive control group. The experiment study was approved by the National Food Chain Safety Office (permit number: IV./397/2023).

3.6. Cell cycle analysis

To investigate the potential mechanism of action of EDPO, cell cycle distribution was analyzed in the ERα+ breast cancer cell line T47D by propidium iodide-based flow cytometry. Cells were treated with either fulvestrant or EDPO for 12, 24, 48, and 72 hours. DNA content was then quantified using a CytoFLEX Flow Cytometer equipped with CytExpert software, acquiring 20,000 events per sample. Data were analyzed using ModFit LT 3.3.11 and GraphPad Prism 5.01. Untreated cells served as controls. The sub-G1 cell population was identified as apoptotic cells.

3.7. Cell migration (wound healing) assay

The antimigratory activity of EDPO was evaluated using a wound healing assay with specialized two-chamber inserts. T47D cells were treated with sub-antiproliferative concentrations of EDPO, subsequently, cell migration into the wound area was monitored using a phase-contrast inverted microscope (Nikon Eclipse TS100) at 0, 24, and 48 hours after treatment. Cell migration was quantified by measuring the remaining cell-free area and comparing these values to untreated controls at the corresponding time points using ImageJ software.

3.8. Transwell invasion (Boyden chamber) assay

To assess the effect of EDPO on the invasive potential of T47D cells, a Boyden chamber invasion assay was performed. Specialized inserts equipped with a thin, porous PET membrane coated with a Matrigel basement matrix were used for this purpose. T47D cells were treated with sub-antiproliferative concentrations of EDPO for 48 hours Subsequently, whole-

membrane images were taken, and cell invasion was quantified by area measurement comparing these values to untreated controls.

3.9. Statistical analysis

Statistical evaluation of the results was performed by one-way analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison Test using the GraphPad Prism 5.01 or 9.5.1. Data were expressed as mean ± standard error of the mean (SEM) or mean ± standard deviation (SD).

## 4. Results

4.1. Antiproliferative (MTT) assay

As previously described, two sets of D-ring modified estrone derivatives were included in this study. Initial screening of the test compounds was performed using standard MTT assay.

*4.1.1. Estrone 16α,17α-oxazoline derivatives*

The antiproliferative effects of newly synthesized D-ring-modified estrogen analogues were evaluated using a panel of human gynecological and female breast cancer cell lines. These test compounds, derived from 16α-azido-3-methoxyestra-1,3,5(10)-trien-17α-ol, feature a D-ring-fused oxazoline ring with a substituted 2′-phenyl group. Based on growth inhibition percentages and $IC_{50}$ values, we determined that the test compounds displayed low to moderate antiproliferative activity across all examined cell lines. $IC_{50}$ values were only determined for compounds I/6e and I/6g, as only these exceeded 75% inhibition at 30 µM: I/6e showed an $IC_{50}$ value of 13.0 µM in HeLa cells, and I/6g showed an $IC_{50}$ value of 16.5 µM in MCF-7 cells (Table 1).

| **Comp.** | **Conc. (µM)** | **Growth inhibition; % ± SEM [calculated $IC_{50}$ value; µM]** | |
|---|---|---|---|
| | | **Hela** | **MCF-7** |
| **I/6e** | 10<br>30 | 35.02 ± 2.41<br>76.75 ± 1.12<br>**[12.97]** | 51.30 ± 2.03<br>71.07 ± 1.39 |
| **I/6g** | 10<br>30 | 32.88 ± 1.43<br>52.03 ± 2.63 | 42.88 ± 2.08<br>80.76 ± 1.46<br>**[16.52]** |
| **Cisplatin** | 10<br>30 | 42.61 ± 2.33<br>99.93 ± 0.26<br>[12.43] | 66.91 ± 1.81<br>96.80 ± 0.35<br>[5.78] |

**Table 1:** Antiproliferative activity of the tested estrone 16α,17α-oxazoline derivatives

*4.1.2. 16-methylene-13α-estrone derivatives*

In the present study, the *in vitro* antiproliferative activity of twelve newly synthesized 16-substituted, 13α-estrone-based α- and β-ketophosphine oxides and their precursors were evaluated using a panel of human gynecological and female breast cancer cell lines as well as oropharyngeal squamous cell carcinoma cell lines. The non-cancerous mouse embryo fibroblast cells were used to assess tumor selectivity (Table 2).

| Comp. | Conc. (μM) | Growth inhibition; % ± SEM [calculated $IC_{50}$ value; μM] | | | | |
|---|---|---|---|---|---|---|
| | | UPCI-SCC-131 | MCF-7 | MDA-MB-231 | T47D | NIH/3T3 |
| **II/4** | 10<br>30 | 99.80 ± 0.36<br>99.88 ± 0.39<br>[3.17] | 99.52 ± 0.51<br>99.83 ± 0.44<br>[3.70] | 99.97 ± 0.71<br>94.64 ± 2.26<br>[3.97] | 99.68 ± 0.74<br>99.92 ± 0.71<br>[3.46] | 101.1 ± 0.67<br>100.9 ± 0.71<br>[2.79] |
| **II/5** | 10<br>30 | 99.54 ± 0.33<br>99.94 ± 0.44<br>[2.38] | 99.70 ± 0.43<br>100.6 ± 0.31<br>[3.35] | 97.15 ± 1.46<br>98.57 ± 0.89<br>[4.07] | 100.2 ± 0.28<br>101.3 ± 0.47<br>[3.47] | 100.8 ± 0.16<br>100.3 ± 0.25<br>[2.74] |
| **II/9 (EDPO)** | 10<br>30 | 63.42 ± 1.41<br>99.35 ± 0.27<br>**[5.30]** | 21.29 ± 2.79<br>93.08 ± 1.60<br>[13.67] | –<br>99.03 ± 0.73<br>[23.49] | 57.45 ± 3.15<br>93.77 ± 0.55<br>**[7.20]** | –<br>96.55 ± 0.86<br>[20.44] |
| **Cisplatin** | 10<br>30 | 95.63 ± 1.49<br>95.09 ± 1.57<br>[1.22] | 66.91 ± 1.81<br>96.80 ± 0.35<br>[5.78] | –<br>71.47 ± 1.20<br>[19.13] | 40.41 ± 1.25<br>56.84 ± 1.16<br>[19.24] | 76.74 ± 1.26<br>96.90 ± 0.25<br>[4.73] |

**Table 3:** Antiproliferative activity of the tested 16-methylene-13α-estrone derivatives
(–: Growth inhibition is less than 20%)

The two precursor molecules (II/4 and II/5) exerted high antiproliferative activity across all tested cell line, however, these agents also suppressed the proliferation of non-cancerous cells at concentrations comparable to those required for cancer cells, indicating a lack of tumor selectivity.

In the case of the C-16 substituted derivatives of the precursor molecules, compound II/9 (EDPO) showed the most significant antiproliferative activity against the UPCI-SCC-131 and T47D cell lines, with $IC_{50}$ values of 5.3 and 7.2 μM, respectively. EDPO also demonstrated substantial tumor selectivity, showing an $IC_{50}$ value of 20.44 μM on NIH/3T3 cells that was approximately four times higher than on UPCI-SCC-131 cells and three times higher than on T47D cells.

EDPO was identified as the most potent and tumor-selective compound in the MTT assay, showing strong antiproliferative activity in ERα-positive cancer cell lines. It most effectively inhibited T47D and UPCI-SCC-131 cells, showed moderate activity in MCF-7 cells, and had comparatively weak effects in triple-negative MDA-MB-231 cells. EDPO was significantly more potent in cancer cells than in non-cancerous fibroblasts, demonstrating

marked tumor selectivity. The inverse correlation between ERα expression and $IC_{50}$ values suggests that EDPO preferentially targets ERα-positive cells, supporting the hypothesis that its antiproliferative effects may be mediated through ERα-dependent signaling.

4.2. Luciferase reporter gene assay

To address our hypothesis, the antiestrogenic properties of the investigational compound EDPO were evaluated in comparison with fulvestrant using the T47D-KBluc cell line. EDPO exhibited clear antiestrogenic activity, achieving concentration-dependent inhibition of ERα-mediated transcription with an $IC_{50}$ of $10^{-5}$ M, whereas 3-BEDPO showed substantially lower inhibitory effect. In comparison, fulvestrant demonstrated higher potency with an $IC_{50}$ of $10^{-9}$ M. These results identify EDPO, but not 3-BEDPO, as an effective ERα antagonist with an inhibitory profile comparable in trend to fulvestrant (Fig. 3).

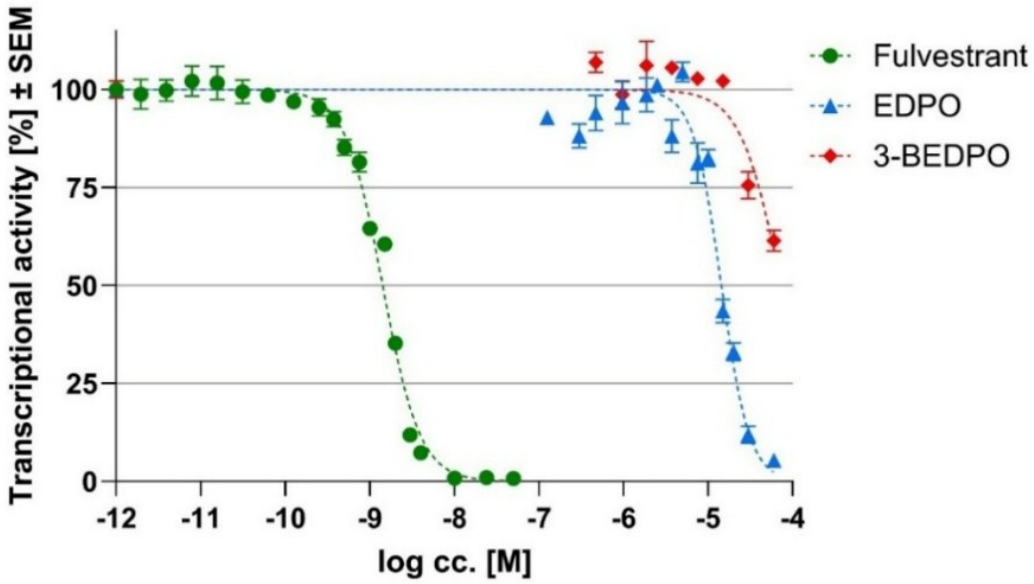



**Figure 3: Concentration-response curves for the inhibition of ERE-Luciferase by fulvestrant, EDPO, and 3-BEDPO.** T47D-KBluc cells were treated and co-incubated with E2 and either of the test compounds for 24 hours.

4.3. *In vivo* uterotrophic assay

EDPO significantly suppressed E2-induced uterine growth *in vivo* in a dose-dependent manner, comparable to the reference antiestrogen fulvestrant. While E2 treatment alone induced an approximately sixfold increase in uterine weight, co-treatment with EDPO markedly attenuated this effect, with the higher EDPO dose showing no significant difference from fulvestrant. These findings demonstrate that EDPO effectively inhibits ER-mediated uterine tissue growth *in vivo* (Fig. 4).

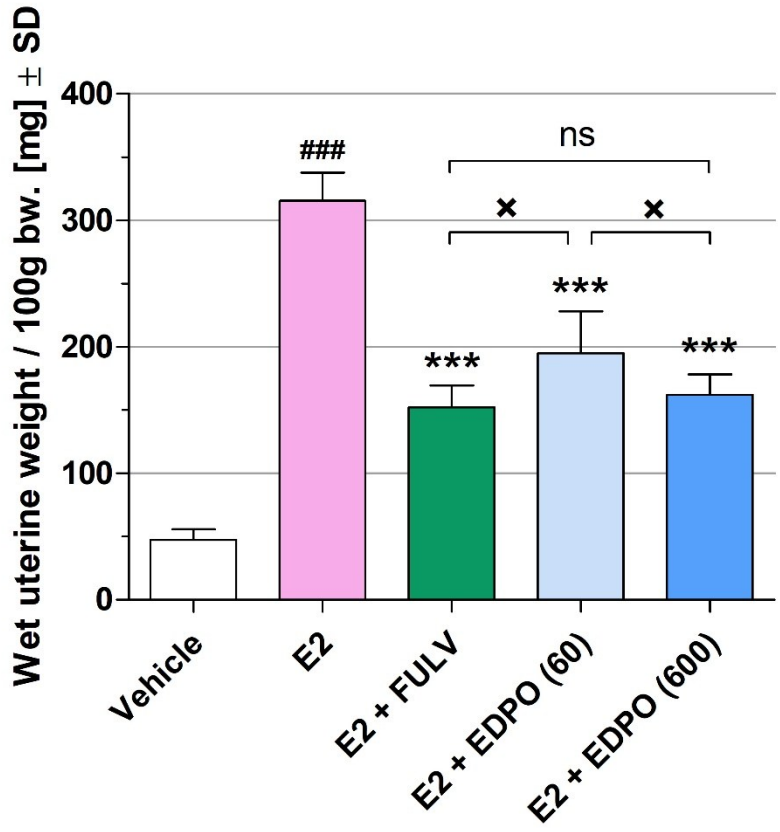



**Figure 4: Effects of fulvestrant and EDPO on uterine weight of female, immature Sprague-Dawley rats.** Wet uterine weights are represented as mean ± SD in mg normalized for 100 g of animal body weight. One-way ANOVA was used for statistical analysis, and significant differences are indicated with $^{###}$ at $p < 0.001$ vs. vehicle group; *** at $p < 0.001$ vs. E2 group; × at $p < 0.05$ for E2 + FULV vs. E2 + EDPO (60) group and for E2 + EDPO (60) vs. E2 + EDPO (600); ns: not significant, (n = 6).

## 4.4. Cell cycle analysis

EDPO induced a pronounced, time- and concentration-dependent G1-phase cell cycle arrest in T47D cells, characterized by an expansion of the G1 population and a concomitant reduction in S-phase cells. This effect was most prominent after 24 hours of treatment, at which point G1 accumulation was evident across all tested concentrations. Fulvestrant was used as positive control (Fig. 5).

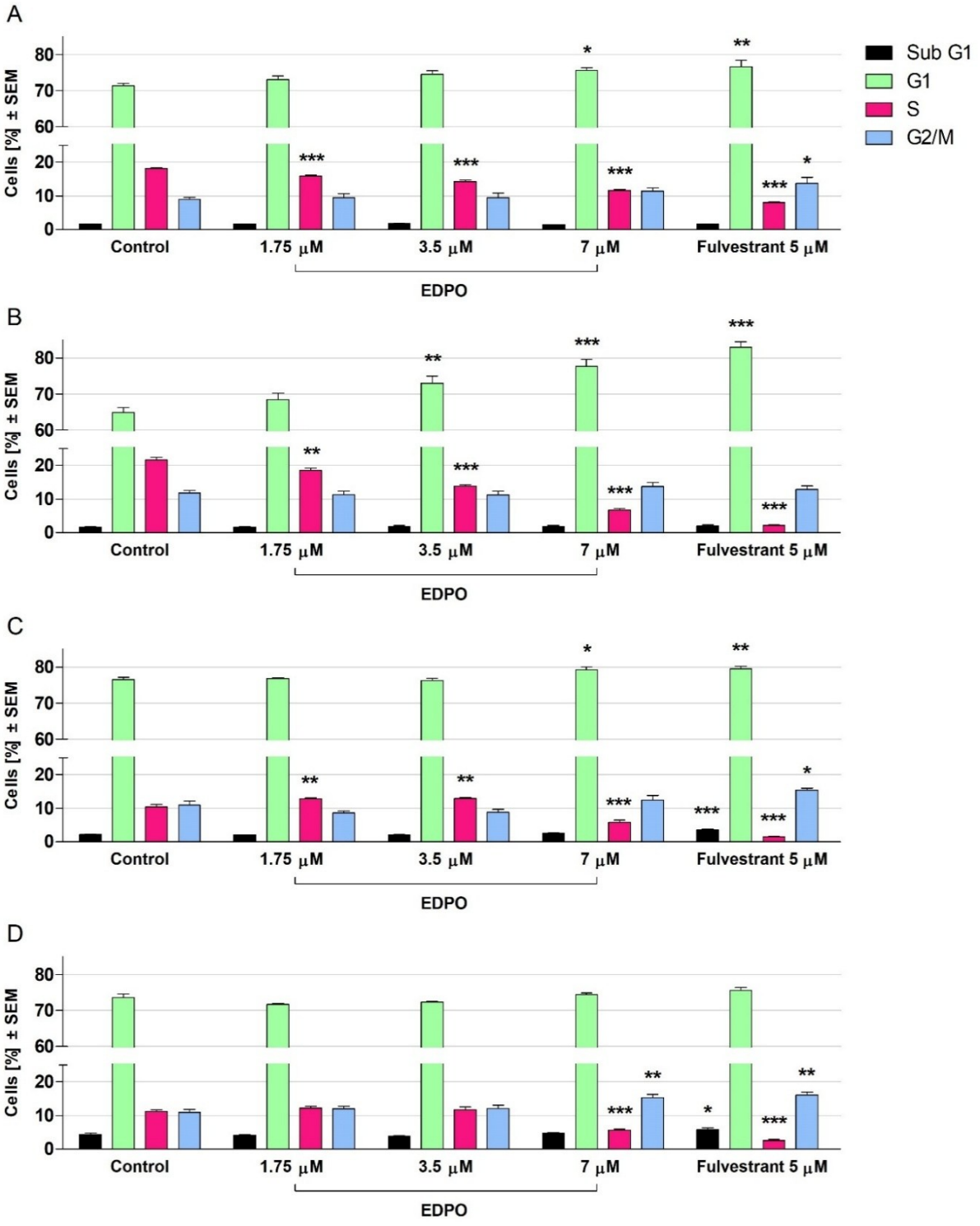



**Figure 5: EDPO-induced cell cycle disturbance in the G1 phase compared to fulvestrant observed after 12 (A), 24 (B), 48 (C), and 72 hours (D) of incubation on T47D cells.** One-way ANOVA was used for statistical analysis, with *, **, and *** indicating significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to the non-treated control samples.

4.5. Cell migration (wound healing) assay

EDPO significantly inhibited cell migration in both UPCI-SCC-131 and T47D cell lines in a time- and concentration-dependent manner, despite their differing intrinsic migratory capacities. In UPCI-SCC-131 cells, significant inhibition was observed at both concentrations after 24 hours, whereas at 48 hours this effect persisted only at the higher concentration (Fig. 6).

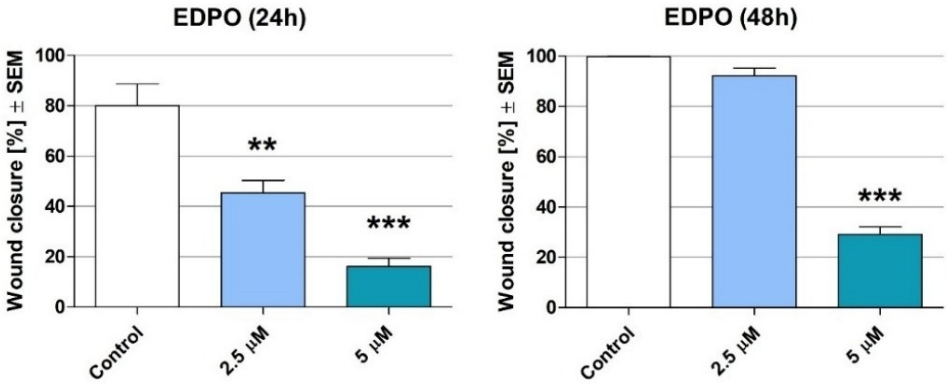



**Figure 6: Antimigratory effect of EDPO measured by wound healing assay.** Effects of EDPO on UPCI-SCC-131 cell migration after 24 and 48 hours of incubation. One-way ANOVA was used for statistical analysis, with ** and *** indicating significance at $p < 0.01$ and $p < 0.001$, respectively, compared to the non-treated control samples.

In T47D cells, although the antimigratory effect was less pronounced, migration was significantly reduced at both concentrations across all examined time points (Fig. 7). Collectively, these findings demonstrate a robust antimigratory effect of EDPO across distinct tumor cell models.

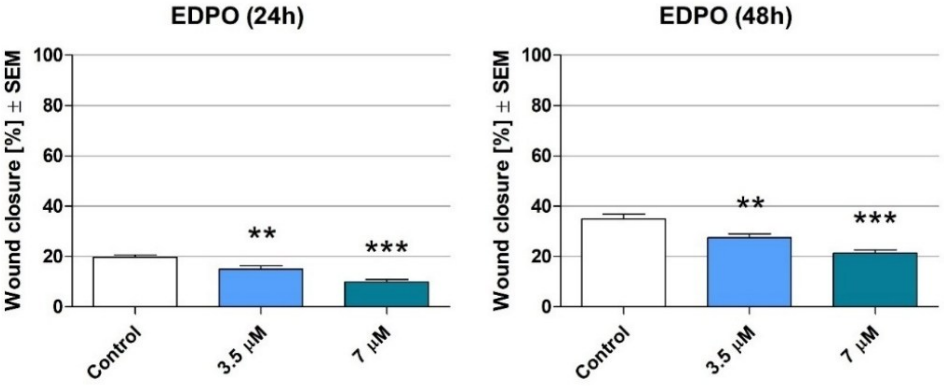



**Figure 7: Antimigratory effect of EDPO measured by wound healing assay.** Effects of EDPO on T47D cell migration after 24 and 48 hours of incubation. One-way ANOVA was used for statistical analysis, with ** and *** indicating significance at $p < 0.01$ and $p < 0.001$, respectively, compared to the non-treated control samples.

4.6. Transwell invasion (Boyden chamber) assay

EDPO significantly reduced the invasive capacity of T47D cells, decreasing the extent of cell invasion to approximately 50% of control levels at both tested concentrations. This inhibitory effect was significant but did not exhibit a statistically significant concentration-dependent relationship (Fig. 8).

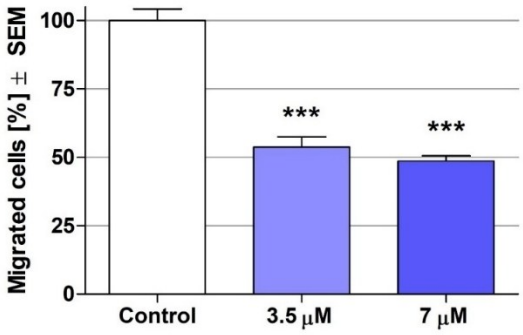



**Figure 8: Effect of EDPO on the invasive capacity of T47D cells measured by transwell migration (Boyden chamber) assay.** Effect of EDPO on T47D cell invasion after 48 hours of incubation. One-way ANOVA was used for statistical analysis, with *** indicating significance at $p < 0.001$, compared to the non-treated control samples.

## 5. Discussion

Estrogen receptor-positive breast cancer accounts for most cases of breast malignancies and remains fundamentally driven by estrogen signaling through ERα, which regulates genes involved in cell proliferation, survival, and metastatic progression. Although endocrine therapies such as SERMs, AIs, and SERDs have substantially reduced mortality and recurrence risk of the disease, intrinsic and acquired resistance to therapy is pervasive and limits long-term efficacy.

Concurrently, the clinical landscape demonstrates that even next-generation targeted endocrine agents, including oral SERDs and combination regimens (e.g., CDK4/6 or PI3K inhibitors), while advancing progression-free survival, do not fully eliminate resistance or recurrence risk, and often add significant toxicity. This ongoing challenge underscores the need for therapeutics that retain or enhance anti-ER activity while mitigating pathways of resistance and systemic adverse effects. Rational design of novel steroid-based compounds may offer a compelling strategy to address these gaps.

In the present study, two sets of D-ring modified estrone-derivatives were employed to assess their antiproliferative efficacy against various female breast, gynecological and oropharyngeal cell lines. Among the tested compounds, II/9 (EDPO) exhibited the most favorable combination of activity and selectivity, with $IC_{50}$ values comparable to cisplatin on

UPCI-SCC-131 and T47D cells. EDPO was further investigated using other *in vitro* and *in vivo* methodologies in order to determine its possible mechanism of action.

To investigate the impact of different compounds on ERα-mediated transcriptional activity, the T47D-KBluc reporter cell line was employed. In this system, the compound EDPO demonstrated a clear inhibitory effect on E2-induced transcriptional activation, comparable to that of the well-characterized antiestrogen fulvestrant. This indicates that EDPO efficiently suppresses ERα-dependent gene expression

In this study, we showed that EDPO reduces E2-induced uterine growth in a dose-dependent manner. The observed decrease in uterine weight indicates that EDPO effectively mitigates ERα activity under physiological conditions, demonstrating a potency similar to that of fulvestrant.

Moreover, we also reported, that based on *in silico* molecular docking studies, the binding profile of EDPO to ERα is comparable to that of fulvestrant. Unlike E2, which stabilizes ERα in an agonistic conformation via Helix 12, EDPO and fulvestrant form key hydrogen bonds that displace Helix 12 and block coactivator binding. EDPO mimics fulvestrant by forming stabilizing hydrogen bonds with critical residues (E353 and R394) and an additional bond with T347, likely enhancing its antiestrogenic effect.

Taken together, these findings provide strong evidence that EDPO mediates its antiestrogenic activity by interfering with the ERα and its downstream signaling pathway.

Estrogen-driven cell proliferation is mediated through the rapid induction of critical G1/S cell cycle regulators, including Cyclin D1, MDM2, and c-Myc, which collectively promote retinoblastoma protein (pRb) phosphorylation, E2F1 activation, and S-phase entry. In contrast, the p53–p21 axis functions as a central inhibitory checkpoint by suppressing cyclin-CDK activity and maintaining pRb in a growth-suppressive state. Dysregulation of this balance, often through c-Myc-mediated p21 repression and MDM2-driven inhibition of p53, favors uncontrolled proliferation in tumor cells. Consistent with previous reports showing that fulvestrant induces G1 arrest via downregulation of MDM2, both fulvestrant and EDPO elicited pronounced G1 phase arrest in T47D cells in the present study. Taken together, these findings support the antiestrogenic profile of EDPO and suggest that its antiproliferative effects are mediated through interference with estrogen-regulated cell cycle control at the G1/S checkpoint.

In ERα-positive breast cancer, estrogens not only stimulate cell proliferation but also enhances migratory and invasive behavior through ERα-dependent signaling pathways. Activation of ERα triggers a Gαi/Gβγ-mediated cascade involving the assembly of a

multiprotein complex comprising ERα, c-Src, PI3K, and FAK, leading to FAK phosphorylation and downstream activation of N-WASP. Concurrent PI3K signaling promotes cdc42 activation, which cooperates with phosphoinositide signaling to stimulate the Arp2/3 complex and drive actin cytoskeletal remodeling. These coordinated cytoskeletal changes underpin the increased motility and invasiveness characteristic of ERα-positive breast cancer cells. In our work, EDPO effectively mitigated cell migration and invasion in ERα-positive cell lines. Given that enhanced migration and invasion of cancer cells promote metastatic progression, the primary cause of cancer-related mortality, agents that effectively inhibit these processes represent promising candidates for anticancer therapy.

EDPO has been shown to effectively inhibit tumor growth in a 4T1 murine triple-negative breast cancer model, as described in our latest report, despite the absence of ERα expression in this tumor type. Although ERα-negative cancers are traditionally considered estrogen-insensitive, accumulating evidence indicates that estrogens can promote tumor progression indirectly by modulating the immune microenvironment. E2 drives the differentiation of myeloid progenitors into immunosuppressive myeloid-derived suppressor cells and directly impairs cytotoxic T lymphocyte activity, thereby fostering an immune-suppressive tumor microenvironment. In line with this, blockade of estrogen signaling with fulvestrant in the 4T1 model reduces tumor growth and enhances antitumor immunity. These observations provide a plausible mechanistic explanation for the antitumor efficacy of EDPO observed in this estrogen receptor-negative setting.

In conclusion, this work demonstrates that targeted chemical modification of the estrone scaffold can yield derivatives with improved anticancer efficacy and tumor selectivity. Among the tested compounds, EDPO emerges as a compelling lead with dual antiproliferative and antimetastatic properties mediated, at least in part, by ERα antagonism. This finding offers a solid basis for further optimization of estrone-based antineoplastic agents and provides new opportunities to generate effective therapeutic candidates against both hormone-dependent and hormone-independent malignancies.

## LIST OF PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

I. Kiss A, Jójárt R, Mernyák E, **Bartha S**, Minorics R, Zupkó I, Schneider G. Novel preparation of substituted oxazolines condensed to D-ring of estrane skeleton and characterization of their antiproliferative properties. *Steroids.* **176,** 108911 (2021).
DOI: https://doi.org/10.1016/j.steroids.2021.108911
**IF: 2.76 / Q2**

II. Mernyák E, **Bartha S**, Kóczán L, Jójárt R, Resch V, Paragi G, Vágvölgyi M, Hunyadi A, Bruszel B, Zupkó I, Minorics R. Microwave-assisted Phospha-Michael addition reactions in the 13α-oestrone series and *in vitro* antiproliferative properties. *J Enzyme Inhib Med Chem.* **36,** 1931-1937 (2021).
DOI: https://doi.org/10.1080/14756366.2021.1963241.
**IF: 5.756 / Q2**

III. **Bartha S**, Germán P, Bózsity N, Puskás LG, Börzsei R, Hetényi C, Zupkó I, Mernyák E, Minorics R. Biological and computer-aided evaluation of 3-methoxy-13α-estrone-16α-diphenylphosphine oxide as a new antiestrogenic agent. *Sci Rep.* **15,** 44295 (2025).
DOI: https://doi.org/10.1038/s41598-025-27905-x
**IF: 3.9 / Q1**

## ADDITIONAL PUBLICATIONS

i. Ristovski (Trifunović) J, Minorics R, **Bartha S**, Janković N, Zupkó I. The evaluation of the anticancer activity of the Biginelli hybrids and pharmacokinetic profiling based on their retention parameters. *J Mol Struct*. **1254,** 132373 (2022).
DOI: https://doi.org/10.1016/j.molstruc.2022.132373.
**IF: 3.8 / Q2**

**Cumulative impact factor: 16.216**