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Natural Compound Honokiol and Its Application against Fulvestrant-Resistant Breast Cancer Cells: An In Vitro Challenge †

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Abstract: The history of the use of natural compounds as therapeutic agents goes back many centuries. Being the first objects of interest in the early days of medicine, natural compounds are still of great relevance, considering the improvement of methods for isolation, chemical transformation, and synthesis. They are also used in oncology, with the advantage of preventing the development of toxicity to normal cells and resistance in tumor cells. One of the promising classes of natural compounds with antitumor activity is lignans. We studied a number of lignans (arctiin, honokiol, matairesinol, pinoresinol, myrislignan, enterodiol, and enterolactone) in the breast cancer cell line MCF7 and the subline MCF7/FUL with acquired resistance to the antiestrogen fulvestrant. Antiproliferative activity was assessed using the MTT test. An analysis of the level of intracellular proteins was carried out via immunoblotting. Based on the results of the screening, the most active compound was honokiol; it had the lowest IC_{50} value for both MCF7 and MCF7/FUL cells, 19.7 μM and 9.1 μM, respectively. The revealed antiproliferative activity of honokiol against resistant cells prompted us to study its effects on intracellular proteins associated with proliferation and cell death. Honokiol suppressed the expression of Bcl-2 (an inhibitor of apoptosis) and cyclin D1 (a cell cycle regulator) in both cell lines, but this effect was more pronounced in the resistant subline. The decrease in the expression of antiapoptotic and proliferative proteins induced by honokiol is consistent with its antiproliferative effect, which is more pronounced in resistant subline MCF7/FUL.

Keywords: lignans; honokiol; breast cancer cells; resistance; Bcl-2; cyclin D1

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1. Introduction

All over the world, there is a steady increase in new cases of cancer, of which breast cancer is a widespread health problem. According to the WHO and the International Agency for Research on Cancer (IARC), in 2020, 2.3 million new cases of breast cancer were registered in the world, which is 11.7% of the total number of detected malignant neoplasms [1]. Among the female population in Russia, on average, breast cancer accounts for about a quarter of cases of all types of oncological diseases, which means that this pathology remains an urgent target in the search and development of new drugs.

Breast cancer is a complex heterogeneous disease that, depending on its histological and molecular features, can be divided into three groups, among which the luminal subtype, in which estrogen receptor α (ERα) expression is observed, is the most common (more than 70% of cases). Endocrine therapy, which is the basis of the treatment of hormone-dependent breast cancer, includes the use of steroid drugs. Among them are aromatase inhibitors (AIs), selective estrogen receptor modulators (SERMs), as well as selective estrogen receptor

degraders (SERDs), a member of which, fulvestrant, is the drug of choice for metastatic breast cancer and is included in the clinical recommendations in many countries [2,3].

Despite the similar efficacy and better toxicity profile of endocrine therapy compared to chemotherapy [3], breast cancer cells can develop resistance due to the action of many molecular mechanisms, among which increases in the expression of apoptosis inhibitors [4] and cell cycle regulators play a significant role [5], thereby causing disease progression or recurrence, especially in metastatic conditions.

Overcoming drug resistance is a critical problem in modern cancer therapy that requires special attention not only in clinical practice but also in the development of new drugs. One of the possible solutions to the problem of multidrug resistance (MDR) in tumor cells is the use of natural compounds of a polyphenolic structure, lignans, which have proven antitumor activity [6]. The results of numerous preclinical studies indicate that honokiol, a lignan isolated for the first time from plants of *Magnolia* spp., can be considered a potential antitumor agent, since it can modulate numerous intracellular signaling pathways associated with the emergence and progression of malignant clones, including breast cancer [7,8], as well as the development of multidrug resistance [8].

The aim of this study was to estimate the antiproliferative effects of lignans on the breast cancer cell line MCF7 and the subline MCF7/FUL with acquired resistance to the antiestrogen fulvestrant, and to reveal the molecular mechanisms of the antiproliferative action of the leader compound.

2. Materials and Methods

2.1. Cell Lines and Compounds

In the experiments, we used the MCF7 breast cancer cell line that was acquired from ATCC and the subline MCF7/FUL with acquired resistance to the antiestrogen fulvestrant obtained via long-term cultivation with 1 μM fulvestrant. Cells were cultivated in DMEM medium (Paneco, Moscow, Russia) with 10% of fetal bovine serum (HyClone, Logan, MA, USA) at 37 °C, 5% CO_2, and 80–85% humidity (NuAire CO_2 incubator, Minnesota, USA). Lignans (arctiin, honokiol, matairesinol, pinoresinol, myrislignan, enterodiol, and enterolactone) and fulvestrant were purchased from Cayman Chemical (Ann Arbor, MI, USA).

2.2. Antiproliferative Activity

The antiproliferative activity of lignans was assessed using the MTT test with modifications as previously described [9]. The cells were seeded on 24-well plates (Corning, Corning NY, USA), 40×10^3 cells/well. The next day after seeding, lignans (or dimethylsulfoxide (DMSO) for control cells) were added to each well in medium at concentrations of 3.1, 6.25, 12.5, 25, and 50 μM. The cell lines were cultivated with lignans or DMSO (control cells) for next 72 h. The half-maximal inhibitory concentrations (IC_{50} values) were calculated using GraphPad Prism 9.0 (GraphPad, San Diego, CA, USA).

2.3. Assessment of Protein Expression via Immunoblotting

For the assessment of the effects of honokiol on protein expression, immunoblotting was used, as previously described [9]. MCF7 and MCF7/FUL cells were seeded on 60-mm Petri dishes (Corning, Corning NY, USA) in 5 mL of DMEM medium and treated with honokiol in the concentration range of 6–25 μM for 72 h. Non-treated MCF7 and MCF7/FUL (incubated with DMSO) cells were used as a control. After incubation with honokiol, cells were lysed in 150 μL of buffer: 50 mM Tris-HCl (pH 7.5), 0.5% igepal CA-630, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 mM sodium orthovanadate, aprotinin, leupeptin, pepstatin (1 μg/mL each), and 1 mM sodium fluoride. Then, we held the samples on ice for 20 min and centrifugated them (10,000× *g*, 10 min, 4 °C). For the determination of total protein content and to standardize loading, we used the Bradford method.

2.4. Statistical Analysis

All data were obtained from three independent experiments. Data are presented as mean ± standard deviation (SD). Statistical processing of the results was carried out using GraphPad Prism 9.0 (GraphPad, San Diego, CA, USA). Student's *t*-test was used in this study. Differences were considered significant at a p value < 0.05.

3. Results and Discussion

The interest of researchers and practitioners in compounds and drugs of natural origin has been constantly growing in recent years. Natural compounds have several advantages, one of the main ones being low toxicity to normal cells. One notable group of natural compounds that have a wide range of therapeutic effects is lignans. We have chosen a number of lignans (arctiin, honokiol, matairesinol, pinoresinol, myrislignan, enterodiol, and enterolactone) based on their published anticancer activity toward different types of neoplasms [6,10]. Earlier, we studied the effects of lignans on hydroxytamoxifen-resistant cells MCF7/HT and showed that honokiol exhibited a significant antiproliferative effect [11].

The present study aimed to define the antiproliferative action of lignans on the breast cancer cell line MCF7 and to compare this effect with antiproliferative activity toward the subline MCF7/FUL with acquired resistance to the antiestrogen fulvestrant. Figure 1 shows the antiproliferative effects of lignans against MCF7 cell line (a) and MCF7/FUL cell line (b).

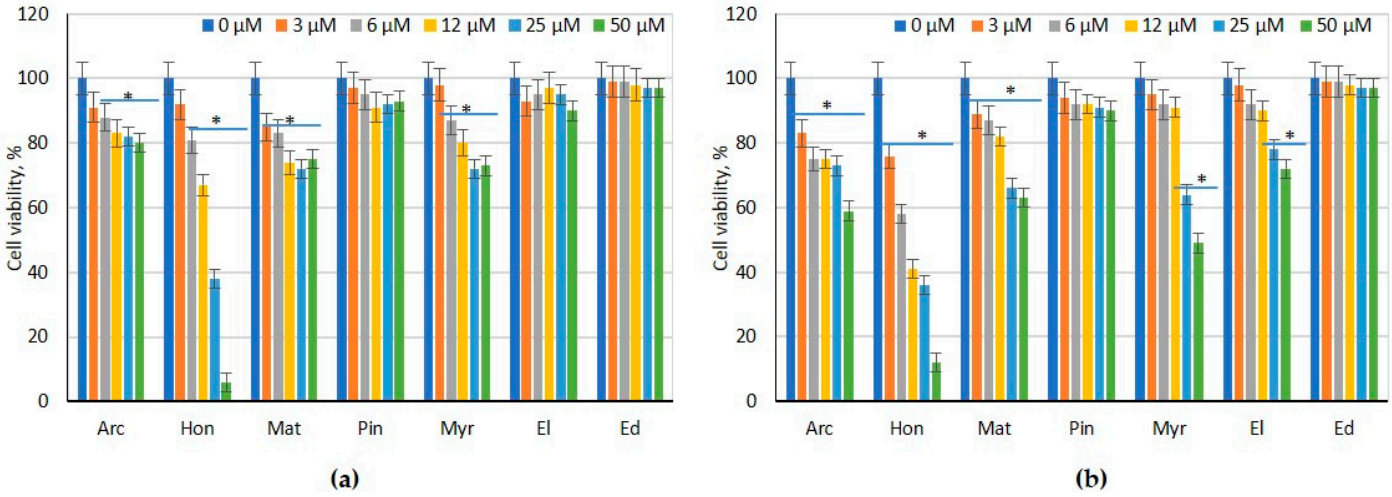

Figure 1. Antiproliferative activity of lignans arctiin (Arc), honokiol (Hon), matairesinol (Mat), pinoresinol (Pin), myrislignan (Myr), enterolactone (El), and enterodiol (Ed) at the range of concentration 3–50 μM against MCF7 (**a**) and MCF7/FUL (**b**) cells. Mean values ± standard deviation (S.D.) of cell viability (%), *—p < 0.05 versus appropriate control.

As it can be seen, only honokiol exhibited significant antiproliferative action on both cell lines and other lignans inhibited cell proliferation not more than 40% at a maximum concentration of 50 μM. IC_{50} values of honokiol were 19.7 μM and 9.1 μM for MCF7 and MCF7/FUL, respectively, while for all other tested lignans, IC_{50} values exceeded 50 μM. For this reason, honokiol was selected as a leader compound for further investigation.

One of the options for the endocrine therapy of hormone-dependent breast cancer is fulvestrant, and the effectiveness of such a therapy is largely determined by changes in the expression levels of certain biomarkers, the most common of which is the mammalian target of rapamycin (mTOR). In fulvestrant-resistant breast cancer cells, a steady increase in the expression of active components of the PI3K/AKT/mTOR signaling pathway responsible for the regulation of protein synthesis and proliferation rate was observed. Taking into account the importance of the PI3K/AKT/mTOR signaling pathway in the regulation of

the main functions of a tumor cell, the development of approaches to suppress its activity is one of the priority areas of current oncology. Honokiol inhibits the cell cycle through the PI3K/AKT/mTOR pathway by activating PTEN and P21 and downregulating p-AKT, cyclin D1/CDK4, c-Myc, Rac1 and AURKB [12].

Based on the screening results of lignans and pronounced antiproliferative effect of honokiol, we have studied its effect on intracellular proteins associated with the cell cycle and proliferation.

In Figure 2, it is shown that honokiol at concentration of 25 μM decreases the expressions of p-AKT, cyclin D1, and Bcl-2 in the MCF7 cell line and MCF7/FUL subline, but in the latter these effects are more pronounced.

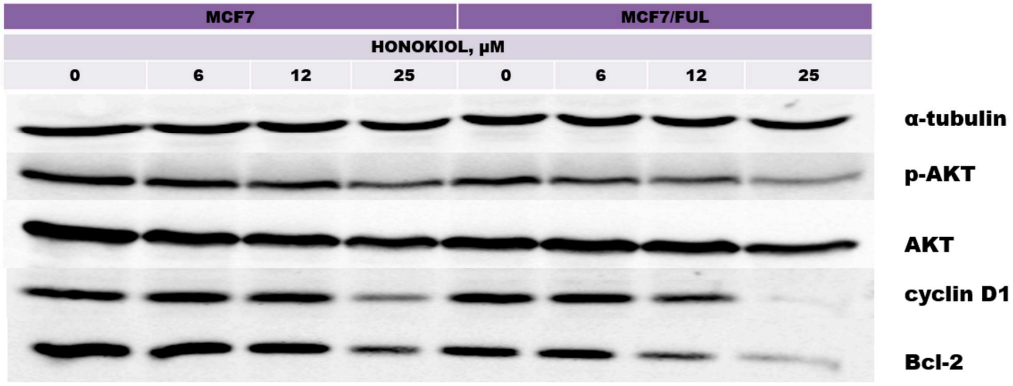

Figure 2. Effect of honokiol on intracellular proteins associated with the cell cycle and apoptosis. Cell lines MCF7 and MCF7/FUL were incubated with honokiol in a concentration range of 6–25 μM for 72 h, and then cell lysates were analyzed via immunoblotting.

This fact is consistent with literature data on the effect of honokiol on the cell cycle and the PI3K/AKT/mTOR signaling pathway. Since this signaling pathway is steadily activated in MCF7 cells resistant to fulvestrant, a more pronounced effect of honokiol toward the resistant subline is explained.

Honokiol exhibits multiple intracellular effects involving different mechanisms and proteins. The exceptional activity of honokiol as an inhibitor of the PI3K/mTOR signaling pathway was shown, which contributed to the overcoming of resistance in glioma and doxorubicin-resistant breast cancer cells [13,14]. Another major target of honokiol is the HIF-1α/NF-κB signaling pathway, the inhibition of which leads to the arrest of HIF-1α associated glycolysis [15], which allows cancer cells to meet increased metabolic demands and reduces the effect of hypoxia on ATP synthesis. In addition, by inhibiting the HIF-1α/NF-κB signaling pathway, the release of VEGF responsible for angiogenesis is blocked.

Honokiol also affects the epidermal growth factor receptor (EGFR), signal transduction, and transcriptional signaling activator 3 (STAT3), as well as broadly regulates mitochondrial function and cancer cell metabolism [16,17]. STAT3, among the various STAT proteins, is constitutively activated in many human cancer cells [18] and can be inhibited by honokiol [19], which also stops the neoangiogenesis. The inhibition of EGFR-STAT3 signaling and downregulation of STAT3 target genes is a principal mechanism for mitochondria-dependent apoptosis in multidrug-resistant cells and the increased responsiveness of tumor cells to paclitaxel therapy [20].

Tumor suppressors are general molecular components of the negative regulation of tumor cell growth and proliferation. Honokiol, by forming a strong protein complex with the product of the LKB1 gene, promotes the activation of the tumor suppressor LKB1, according to the results of in silico studies [21]. Honokiol enhances the expression of STK11, a higher expression of which positively correlates with breast cancer prognosis, due to the mediated induction of apoptosis and inhibition of tumor cell growth [22].

The solution to the problem of multidrug resistance in the treatment of hormone-dependent breast cancer is achieved by inhibiting the membrane transporter BCRP with

honokiol [23] and by influencing the functions of P-glycoprotein [24]. Thus, based on the available literature data, one may conclude that honokiol offers a valuable option for monotherapy and combined therapy of resistant breast cancer, allowing us to lower the doses of chemotherapy drugs, prevent toxicity to normal cells, and increase the effectiveness of anticancer therapy.

In our study, the pronounced antiproliferative effect of natural compound honokiol on hormone-sensitive and hormone-resistant breast cancer cells has been demonstrated. Honokiol was the most effective among all tested lignans. The possible mechanisms of its antiproliferative action are the induction of apoptosis (it decreases the Bcl-2 expression) and influence on the cell cycle (it decreases the cyclin D1 expression). Moreover, the honokiol was found to affect the activity of AKT kinase, which is involved in maintaining tumor cell survival. Honokiol is a promising agent for further research into the possibility of its use in antitumor therapies. A more in-depth study of the differences in its action on sensitive and resistant cell lines is necessary to establish the mechanisms of these differences. Studies on the activity of honokiol toward a wider range of cell lines are also necessary, and this will expand the list of indications for its use in the future. Studies of the combined use of honokiol with other chemotherapy drugs seem very promising.

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