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Inhibitory Effects of 5-Fluorouracil on the Growth of 4-Hydroxytamoxifen-Resistant and Sensitive Breast Cancer Cells †

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Abstract: Cancer is one of the leading causes of death worldwide, accounting for about 10 million deaths a year, or nearly one in six deaths. The most common types of cancer are breast, colorectal, lung, and prostate cancers. Prolonged application of hormone drugs leads to the development of resistance. The development of agents with high activity against resistant cells is a challenge. It is important to create novel targeted compounds and search for active molecules among those previously developed. The study aimed to evaluate the sensitivity of 4-hydroxytamoxifen-resistant cells to 5-fluorouracil (5-FU) and analyse the signalling pathways that are regulated by 5-FU in breast cancer cells. Antiproliferative activity of compounds was assessed by the MTT assay, and immunoblotting was used to evaluate the expression of proteins in breast cancer cells. Activity of 5-FU was evaluated on parental MCF7 cells and a cell subline with resistance to 4-hydroxytamoxifen (HT), named MCF7/HT. The MCF7/HT cells showed high sensitivity to 5-FU. Expression of oestrogen receptor α (ERα, a key driver of breast cancer growth) in MCF7 and MCF7/HT cells was not sensitive to 5-FU treatment. In both parental and resistant cells, 5-FU induced changes in the activity of several signalling proteins. 5-FU activated AKT, extracellular signal-regulated kinase 1/2 (ERK 1/2) and upregulated cyclin D1 expression. The data suggest that 5-FU should be further investigated as a chemotherapeutic for hormone-resistant cancers; the combination of 5-FU with novel apoptosis inducer LCTA-3344 is considered effective to inhibit the growth of breast cancer cells, including those that are hormone-resistant.

Keywords: 4-hydroxytamoxifen; 5-fluorouracil; breast cancer; resistance; oestrogen receptor alpha

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1. Introduction

Hormone therapy is one of the most common treatments for tumours, including breast, ovarian, endometrial and prostate cancer. Hormone therapy is based on the principle of creating a deficiency of hormones which are necessary for the growth of hormone-dependent tumours, such as oestrogens (for tumours of the female reproductive system) and androgens (for prostate tumours). This effect is mainly achieved in two ways: (I) by reducing the concentration of endogenous hormones by inhibiting their synthesis (aromatase inhibitors) or (II) by replacing hormones with their inactive analogues (anti-oestrogens or anti-androgens) [1].

According to the World Health Organization, more than 2.2 million women worldwide were diagnosed with breast cancer in 2020; the disease caused 685,000 deaths worldwide. About eight million women diagnosed with breast cancer in the past 5 years continue to fight the disease. Breast cancer is the most common cancer in the world, and about 70% of breast tumours contain oestrogen receptors (ERα). ERα expression status in breast cancer is a prognostic parameter for response to hormonal therapy. Tamoxifen (2-[4-[(Z)-diphenibut-1-enyl]phenoxy]-N,N-dimethylethanamine), developed in the 60s, is an anti-hormonal agent belonging to SERMs, which has been widely distributed [2]. In breast cancer cells, tamoxifen stabilises ERα and prevents its translocation into the nucleus [3]. It is crucial to understand the mechanisms of tamoxifen resistance and to look for ways to overcome this type of resistance [4].

5-FU is an aromatic organic compound with a structure similar to that of the pyrimidine molecules of RNA and DNA; it is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen [5]. 5-FU is a widely used anticancer drug, and it is applied for the treatment of various cancers. The 5-FU agent can inhibit breast cancer progression by a variety of different mechanisms. To date, the question regarding which resistant cancers may be sensitive to 5-FU treatment remains open.

This work aims to assess the sensitivity of 4-hydroxytamoxifen-resistant breast cancer cells to 5-FU, analyse the signalling pathways regulated by 5-FU, and evaluate this drug in combination with novel apoptosis inducer LCTA-3344.

2. Materials and Methods

2.1. Synthesis, Reagents and Cell Lines

HT and 5-FU (Figure 1a) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA); drugs were dissolved in DMSO (PanEco, Moscow, Russia) and then the solutions were stored at −70 °C. The MCF7 (HTB-22) cell line was obtained from the ATCC (Manassas, VA, USA). The MCF7 and MCF7/HT cells were maintained in DMEM medium (PanEco, Moscow, Russia) with 10% fetal bovine serum (HyClone, Phoenix, AZ, USA) at 37 °C, 5% CO_2 and 80–85% humidity (NuAire CO_2 incubator, Plymouth, MN, USA). The synthesis of LCTA-3344 (2,3-dioxo-1-(4-((trifluoromethyl)thio)benzyl)indoline-5-sulfonamide) involved the following steps: firstly, 4-aminobenzenesulfonamide was converted to isatin-5-sulfonamide using Sandmeyer's methodology. In the final step, isatin-5-sulfonamide was alkylated using 4-((trifluoromethyl)thio)benzyl chloride, as described in the work [6].

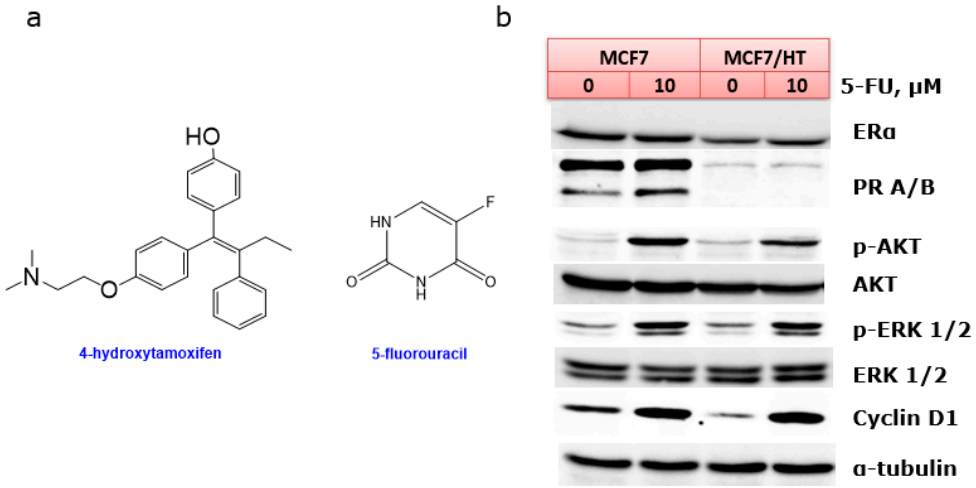


Figure 1. (**a**) 4-hydroxytamoxifen and 5-fluorouracil. (**b**) Immunoblotting analysis of signalling pathways in MCF7 and MCF7/HT cells treated with 5-FU (24-h treatment). α-Tubulin was used as a loading control for immunoblotting.

2.2. The Evaluation of the Antiproliferative Activity

The analysis of the antiproliferative activity of the drugs was performed by the MTT test [7] as described earlier in [8]. Absorbance was measured at 571 nm with a MultiScan reader (ThermoFisher, Waltham, MA, USA). The half-maximal inhibitory concentrations (IC_{50}) were determined with GraphPad Prism 9.0 (GraphPad Software, Boston, MA, USA).

2.3. Statistical Analysis

Data are presented as mean values and standard deviation (mean ± std. deviation). Student's *t*-test (GraphPad Prism 9.0, Boston, MA, USA) at $p < 0.05$ was considered to indicate a statistically significant result.

2.4. Immunoblotting

Immunoblotting with modifications was performed as described earlier [9]. ERα, PR (progesterone receptor), phosphorylated AKT, AKT, phosphorylated ERK 1/2, ERK 1/2, and cyclin D1 expression were evaluated using Cell Signaling Technology (Danvers, MA, USA) antibodies. The detection was performed using secondary antibodies to rabbit Ig conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA) and ImageQuant LAS 4000 imager (GE Healthcare, Chicago, IL, USA), as described in Mruk and Cheng's protocol [10].

3. Results and Discussion

The MCF7/HT [11] cells were obtained by long-term cultivation of MCF7 breast cancer cells with anti-oestrogen HT (Figure 1a), which is one of the major active metabolites of anti-oestrogen tamoxifen [12,13]. The HT sensitivity of cells was assessed using the MTT test. The established MCF7/HT subline lost sensitivity to HT which was confirmed by changes in the IC_{50} values (Table 1) as described in [11].

Table 1. Characteristics of parental and hormone-resistant breast cancer cells (HT—4-hydroxytamoxifen; 5-FU—5-fluorouracil; * $p < 0.05$ versus MCF7 cells, #—ratio of the IC_{50} value of MCF7/HT cell line to the IC_{50} value of MCF7 cell line).

	IC_{50} of HT, μM	**IC_{50} of 5-FU, μM**
MCF7	5.2 ± 0.6	6.3 ± 0.7
MCF7/HT	9.8 ± 1.0 *	2.1 ± 0.3 *
Resistance index #	1.9	0.3

The resistance index was estimated as the ratio of the IC_{50} value for resistant cells to that of parental cells and was found to be approximately two. Then, the antiproliferative effects of 5-FU on MCF7 and MCF7/HT cells were evaluated. The HT-resistant cells did not only lose sensitivity to the drug but also appeared to be more sensitive to it; in this case, the resistance index was much less than 1.

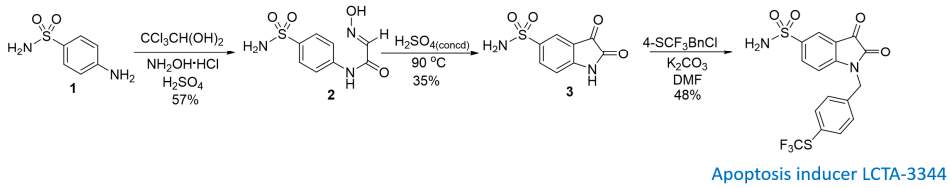
Further studies were conducted to characterize the effects of 5-FU on signalling pathways in MCF7 and MCF7/HT cells. The analysis of signalling proteins in cells was carried out using immunoblotting. It was found that ERα expression was partially decreased in HT-resistant cells as seen in Figure 1b. PR expression is employed as a biomarker of ERα function and breast cancer prognosis [14]. PR is actively regulated by oestrogens through ERα pathways [15]. PR expression is almost absent in the HT-resistant cells obtained. 5-FU did not inhibit ERα and PR expression in MCF7 or HT-resistant cells.

The PI3K/AKT pathway is one of the most commonly dysregulated pathways in breast cancer, with somatic mutations, aberrant epigenetic regulation and increased expression in various molecular types of breast cancer [16]. The chemotherapeutics can affect the activity of this pathway. 5-FU caused significant activation of AKT kinase as detected by the increased expression of p-AKT. The overall AKT level was not affected by 5-FU.

The ERK 1/2 cascade is an important signalling pathway that initiates several cellular processes and is regulated by various stimuli, including chemotherapeutics [17,18]. The expression of p-ERK 1/2 and cell-cycle regulator cyclin D1 was significantly increased in both parental and HT-resistant cells treated with 10 μM 5-FU (Figure 1b). Thus, in parental and HT-resistant cells, 5-FU activates a number of proliferative pathways that are not among its main pharmacological targets.

Considering the above-described changes in signalling pathways, we investigated effects of 5-fluorouracil combined with a novel inducer of apoptosis. The compound LCTA-3344 (Figure 2a) was obtained in our previous work [6]. After several unsuccessful attempts, a selective method for the benzylation of the heterocyclic moiety of isatin-5-sulfonamide was developed. It was discovered that using DMF as a solvent and K_2CO_3 as a base provided the highest yield of the desired 1-substituted isatin-5-sulfonamide. By following this procedure, isatin-5-sulfonamide was successfully alkylated using 4-((trifluoromethyl)thio)benzyl chloride, resulting in the LCTA-3344 with a moderate yield (48%). Apoptosis inducer LCTA-3344 demonstrated significant antiproliferative effects with an IC_{50} value of 2.6 μM for MCF7, while the HT-resistant cells were more sensitive to it, with an IC_{50} value of 1.4 μM. The MCF7 and MCF7/HT cells were then treated with 5-fluorouracil, LCTA-3344 or their combination. Figure 2b shows the selected combination of 5-FU with LCTA-3344 which caused significant suppression of the growth of breast cancer cells.

a



Apoptosis inducer LCTA-3344

b

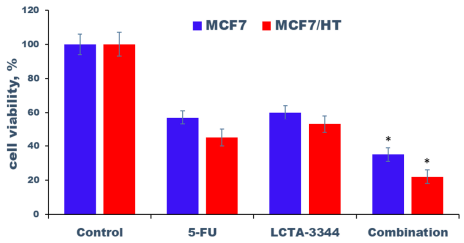


Figure 2. (**a**) Synthesis of apoptosis inducer LCTA-3344. (**b**) Drug combination study of 5-FU with compound LCTA-3344. The MCF7 and MCF7/HT cells were treated with 5-FU (5 μM), LCTA-3344 (1 μM) or their combination. The cell viability was assessed by the MTT assay. *—$p < 0.05$ versus single-agent treatment and control.

Despite significant progress in drug development, cancer mortality rates remain very high. One of the reasons for this state of affairs is tumour drug resistance. Resistance can be de novo or formed by exposure to drugs. Tumour resistance occurs with both standard chemotherapy and hormonal drugs. An important question for specialists to decide is which drug to prescribe for patients with resistant tumours. Tamoxifen is used for hormonal therapy in patients with breast cancer. Prolonged use of this drug leads to significant changes in tumour cell signalling pathways. Activated signalling pathways decrease the efficacy of tamoxifen and support accelerated cell proliferation. Targeted suppression of signalling pathways is effective in inhibiting the growth of resistant tumours.

In the present work, we characterized a cell line with resistance to HT, namely MCF7/HT. 5-FU had a significant inhibitory effect on the growth of MCF7/HT cells. Moreover, low PR expression was detected in the resistant cells obtained, indicating decreased ERα activity. 5-FU significantly altered the signalling pathways in the parental and

HT-resistant cells, in particular, it stimulated the activity of AKT kinase. AKT proteins regulate a wide range of cellular functions, including cell proliferation, survival, metabolism, and angiogenesis in both normal and transformed cells [19]. AKT proteins are recruited to the cell membrane by phosphatidylinositol 3,4,5-trisphosphate (PIP3) after phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) by PI3-kinase [20]. Subsequent phosphorylation by threonine and serine residues is required for complete activation of the AKT kinases. Phosphorylation of additional residues also occurs, for example, in response to insulin growth factor-1 and epidermal growth factor [20] and other growth signals. Protein phosphatases act as negative regulators of AKT proteins, dephosphorylating AKT or PIP3 [21]. 5-FU-induced AKT activity, which was mentioned above, may be involved in subsequent steps of resistance. To reduce the applied drug doses and enhance the efficacy of treatment, the combination of 5-FU with the apoptosis inducer LCTA-3344 was applied to the parental and HT-resistant cells. The synergism found suggests that such approaches are very promising.

4. Conclusions

The hormone-resistant cell subline was characterised. ERα expression was slightly reduced in the obtained cells, whereas PR expression was almost completely absent. Interestingly, HT-resistant cells acquired sensitivity to 5-FU. In both parental and resistant cells, 5-FU induced changes in the activity of a number of signalling proteins. 5-FU activated AKT and ERK 1/2 kinases and increased expression of cyclin D1. The data suggest that 5-FU should be further investigated in the therapy of hormone-resistant cancers. The combination of 5-FU with novel apoptosis inducer LCTA-3344 is considered effective against breast cancer cells, including those which are hormone-resistant.

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References

1. Mojaddami, A.; Sakhteman, A.; Fereidoonnezhad, M.; Faghih, Z.; Najdian, A.; Khabnadideh, S.; Sadeghpour, H.; Rezaei, Z. Binding mode of triazole derivatives as aromatase inhibitors based on docking, protein ligand interaction fingerprinting, and molecular dynamics simulation studies. *Res. Pharm. Sci.* **2017**, *12*, 21–30. [PubMed]
2. Kamruz, E.; Rostamizadeh, K.; Babazadeh, M. Synthesis and characterization of linear-dendritic of PEG and citric acid as a nanocarrier for tamoxifen. *Res. Pharm. Sci.* **2012**, *7*, S576.
3. Jordan, V.C. 50th anniversary of the first clinical trial with ICI 46,474 (tamoxifen): Then what happened? *Endocr.-Relat. Cancer* **2021**, *28*, R11–R30. [CrossRef] [PubMed]
4. Nabieva, N.; Fasching, P.A. Endocrine Treatment for Breast Cancer Patients Revisited-History, Standard of Care, and Possibilities of Improvement. *Cancers* **2021**, *13*, 5643. [CrossRef] [PubMed]

5. Rutman, R.J.; Cantarow, A.; Paschkis, K.E. Studies in 2-acetylaminofluorene carcinogenesis. III. The utilization of uracil-2-C14 by preneoplastic rat liver and rat hepatoma. *Cancer Res.* **1954**, *14*, 119–123. [PubMed]
6. Krymov, S.K.; Scherbakov, A.M.; Salnikova, D.I.; Sorokin, D.V.; Dezhenkova, L.G.; Ivanov, I.V.; Vullo, D.; De Luca, V.; Capasso, C.; Supuran, C.T.; et al. Synthesis, biological evaluation, and in silico studies of potential activators of apoptosis and carbonic anhydrase inhibitors on isatin-5-sulfonamide scaffold. *Eur. J. Med. Chem.* **2022**, *228*, 113997. [CrossRef] [PubMed]
7. Iselt, M.; Holtei, W.; Hilgard, P. The tetrazolium dye assay for rapid in vitro assessment of cytotoxicity. *Arzneimittelforschung* **1989**, *39*, 747–749. [PubMed]
8. Volkova, Y.A.; Antonov, Y.S.; Komkov, A.V.; Scherbakov, A.M.; Shashkov, A.S.; Menchikov, L.G.; Chernoburova, E.I.; Zavarzin, I.V. Access to steroidal pyridazines via modified thiohydrazides. *RSC Adv.* **2016**, *6*, 42863–42868. [CrossRef]
9. Scherbakov, A.M.; Lobanova, Y.S.; Shatskaya, V.A.; Onopchenko, O.V.; Gershtein, E.S.; Krasil'nikov, M.A. Activation of mitogenic pathways and sensitization to estrogen-induced apoptosis: Two independent characteristics of tamoxifen-resistant breast cancer cells? *Breast Cancer Res. Treat.* **2006**, *100*, 1–11. [CrossRef]
10. Mruk, D.D.; Cheng, C.Y. Enhanced chemiluminescence (ECL) for routine immunoblotting: An inexpensive alternative to commercially available kits. *Spermatogenesis* **2011**, *1*, 121–122. [CrossRef]
11. Scherbakov, A.M.; Basharina, A.A.; Sorokin, D.V.; Mikhaevich, E.I.; Mizaeva, I.E.; Mikhaylova, A.L.; Bogush, T.A.; Krasil'nikov, M.A. Targeting hormone-resistant breast cancer cells with docetaxel: A look inside the resistance. *Cancer Drug Resist.* **2023**, *6*, 103–115. [CrossRef] [PubMed]
12. Sarkaria, J.N.; Miller, E.M.; Parker, C.J.; Jordan, V.C.; Mulcahy, R.T. 4-Hydroxytamoxifen, an active metabolite of tamoxifen, does not alter the radiation sensitivity of MCF-7 breast carcinoma cells irradiated in vitro. *Breast Cancer Res. Treat.* **1994**, *30*, 159–165. [CrossRef] [PubMed]
13. Custódio, J.B.; Almeida, L.M.; Madeira, V.M. The active metabolite hydroxytamoxifen of the anticancer drug tamoxifen induces structural changes in membranes. *Biochim. Et Biophys. Acta* **1993**, *1153*, 308–314. [CrossRef] [PubMed]
14. Mohammed, H.; Russell, I.A.; Stark, R.; Rueda, O.M.; Hickey, T.E.; Tarulli, G.A.; Serandour, A.A.; Birrell, S.N.; Bruna, A.; Saadi, A.; et al. Progesterone receptor modulates ERα action in breast cancer. *Nature* **2015**, *523*, 313–317. [CrossRef] [PubMed]
15. Lee, Y.J.; Gorski, J. Estrogen-induced transcription of the progesterone receptor gene does not parallel estrogen receptor occupancy. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 15180–15184. [CrossRef] [PubMed]
16. Costa, R.L.B.; Han, H.S.; Gradishar, W.J. Targeting the PI3K/AKT/mTOR pathway in triple-negative breast cancer: A review. *Breast Cancer Res. Treat.* **2018**, *169*, 397–406. [CrossRef]
17. Lu, Y.; Liu, B.; Liu, Y.; Yu, X.; Cheng, G. Dual effects of active ERK in cancer: A potential target for enhancing radiosensitivity. *Oncol. Lett.* **2020**, *20*, 993–1000. [CrossRef]
18. Bandyopadhayaya, S.; Akimov, M.G.; Verma, R.; Sharma, A.; Sharma, D.; Kundu, G.C.; Gretskaya, N.M.; Bezuglov, V.V.; Mandal, C.C. N-arachidonoyl dopamine inhibits epithelial-mesenchymal transition of breast cancer cells through ERK signaling and decreasing the cellular cholesterol. *J. Biochem. Mol. Toxicol.* **2021**, *35*, e22693. [CrossRef]
19. Testa, J.R.; Tsichlis, P.N. AKT signaling in normal and malignant cells. *Oncogene* **2005**, *24*, 7391–7393. [CrossRef]
20. Kong, D.; Yamori, T. Advances in development of phosphatidylinositol 3-kinase inhibitors. *Curr. Med. Chem.* **2009**, *16*, 2839–2854. [CrossRef]
21. Liao, Y.; Hung, M.C. Physiological regulation of Akt activity and stability. *Am. J. Transl. Res.* **2010**, *2*, 19–42. [PubMed]