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Hsps responsible for apoptosis induction failure in cervical cancer cells upon osthole and tamoxifen treatment

Hsps odpowiedzialne za oporność komórek raka szyjki macicy na indukcję apoptozy pod wpływem ostolu i tamoksyfenu

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Aim: The aim of the present study was to investigate the efficacy of osthole (7-metoxy-8-isopenthenocoumarin) alone and combined with tamoxifen (TAM) in the elimination of human cervical cancer cells via programmed death. The involvement of heat shock proteins, i.e. well-known molecular chaperones, will be investigated.

Material/Methods: Three human cervical cancer cell lines, infected with human papilloma virus (HPV), i.e. HeLa (HPV 18), SiHa (HPV 16), and CaSki (HPV 16 and 18), were used in the experiments. After osthole and TAM treatment, cells stained with fluorochromes were analyzed microscopically according to apoptotic, autophagic, and necrotic morphology. Hsp27, Hsp72, and Hsp90 levels were analyzed by immunoblotting. Transfection with specific siRNA was used for blocking of Hsp expression.

Results: In the HeLa, CaSki, and SiHa cell lines, osthole and TAM applied alone had no significant effect on cell death induction. This was correlated with an overexpression of heat shock proteins 27, 72, and 90. In the case of a combination of both drugs, the level of apoptosis was elevated only in SiHa cells. Preincubation with osthole followed by TAM addition as well as simultaneous incubation with both drugs was the most effective. This was correlated with the inhibition of Hsp27, Hsp72, and Hsp90 expression. Blocking of Hsp expression with specific siRNA increased the sensitivity of the studied cell lines to the induction of apoptosis, but not to autophagy or necrosis.

Conclusions: Our results indicated that the elimination of heat shock proteins from cervical cancer cells sensitized them to initiation of apoptosis after osthole and tamoxifen treatment.

Keywords: **osthole • tamoxifen • apoptosis • heat shock proteins • cervical cancer**

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INTRODUCTION

Over the years, numerous derivatives of coumarin have been used as anticoagulant agents due to their resemblance to the structure of vitamin K. They have been used as antimicrobial, antibacterial, antifungal, antioxidant, antitumor, anti- HIV, anti-hypertension, anti-inflammatory, anti-arrhythmia, and antiseptic agents. They have been identified to exhibit anticancer activity [3, 10]. Therefore, they may be candidates as potential alternative medicine to conventional chemotherapy.

Cnidium monnieri L. (Apiaceae) is a traditional Chinese medicinal plant commonly used for gynecological disease, ringworm, and nephritis. One of active monomers found in Cnidi fruits is osthole (7-metoxy-8-isopenthenocoumarin). It has been reported to have anti-viral, anti-osteoporotic, anti-bacterial, anti-allergic, and anti-inflammatory effects. In vitro experiments have also revealed that osthol inhibits the proliferation and migration of various cancer cells, e.g. leukemia (HL-60), cervical cancers (HeLa), breast cancers (MCF7, MDA-MB-231), non-small cell lung carcinoma (A549), hepatocellular carcinoma (HCC), larynx carcinoma (RK33), and medulloblastoma (TE671) through the initiation of apoptosis or cell cycle arrest [9, 18]. The molecular mechanism of osthole action is not well studied. It has been shown that osthole induced G2/M cell cycle arrest and apoptosis in lung carcinoma and ovarian cancer cells, where it was associated with the inhibition of cyclin B1, p-Cdc2, and p-Akt expression and up-regulation of the Bax/Bcl-2 ratios. Osthole can also down-regulate fatty acid synthase (FASN) expression and induce apoptosis in HER-2-overexpressing breast cancer cells through the inhibition of the phosphorylation of Akt and mTOR. It can effectively inhibit the matrix metalloproteinase-2 (MMP-2) promoter and enzyme activity, which in consequence may lead to the inhibition of migration and invasion of breast cancer cells [10, 17, 18]. In medulloblastoma cells, osthole arrested the cell cycle in the G1/S phase and decreased cell cycle progression by increasing the expression of TP53 and CDKN1A genes [9].

Unfortunately, little is known about the effect of osthole on the expression of Hsps, i.e. well-known molecular chaperones protecting cells against death. Such activity is desirable in normal cells but poses a serious problem in cancer cells. Heat shock proteins block the anticancer potential of chemotherapeutics by preventing cells to enter the death pathway [5]. Therefore, the aim of the present study was to investigate the effect of osthole on the expression of Hsp27, Hsp72, and Hsp90, mostly involved in cancer cell protection against apoptosis. Human cervical cancer lines infected by different types of viruses were chosen as an experimental model. The CaSki cell line is reported to contain an integrated human papilloma virus type 16 genome (HPV 16) as well as sequences related to HPV 18. SiHa was constructed on the basis of HPV 16, while HeLa on the basis of HPV18 infection. It is known that single therapy is less effective than combined therapy. Therefore, we decided to include tamoxifen (TAM) in the experiments, which is a non-steroidal drug exhibiting anti-estrogenic activity. It arrests the cell cycle in the G0/G1 phase and induces apoptosis by modulating the microenvironmental level or cellular production of cytokines and growth factors [2, 15]. Therefore, the aim of our study was to investigate the effect of tamoxifen on cervical carcinoma and its effectiveness after additional osthole treatment.

MATERIALS AND METHODS

Cells and culture conditions

A human carcinoma cell line (HeLa B, ECACC No 85060701; Ca Ski, ATCC No CRL-1550 and SiHa, ATCC No HTB-35) cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 5% fetal bovine serum (FBS) (GIBCO BRL) (v/v) and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml, amphotericin B 0.25 µg/ml) was used. Cells seeded in Falcon vessels (immunoblotting) or Labtec chamber slides (cell death analysis), both at a density of 1×10^6 cells/ml, were incubated at 37°C in humidified atmosphere with 5% CO_2.

Osthole isolation

Osthole was obtained for the experiments after being isolated from a petroleum extract of Cnidium monnieri L. fruits, according to a method described previously [9]. Two-phase solvent systems made of n-heptane, ethyl acetate, methanol, and water (HEMWat) with a volume ratio 3:2:3:2 were chosen as the most proper for the purification of target compounds (K = 1.8). After injection of 600 mg of a crude oil extract, 2 mg of the target com-

pound were obtained. The identification of the isolated compound was carried out by a comparison of retention time and UV-DAD spectra with those obtained by standards under the same conditions. The purity of osthole was 99% (established with the HPLC-DAD method).

Drug treatment

Osthole (50 μM, isolated from Cnidi fruits) and tamoxifen (25 μM, Sigma) were dissolved in DMSO. Studied concentrations were chosen on the basis of preliminary experiments estimated by the staining method described below (section Detection of apoptosis, autophagy, and necrosis; data not shown). Cancer cells were treated with osthole or with tamoxifen separately or in combination (at the same time) for 24h. As controls, HeLa, CaSki, and SiHa cells were incubated only with 0.01% of DMSO. This concentration of DMSO had no effect on cell viability.

Detection of apoptosis, autophagy, and necrosis

Apoptosis, autophagy, and necrosis were identified microscopically, after earlier staining with fluorochromes Hoechst 33342 (Sigma), acridine orange (Sigma), and propidium iodide (Sigma) respectively, as described previously [8]. A fluorescence microscope (Nikon E – 800) was used for the morphological analysis of dead cells. At least 1.000 cells in randomly selected microscopic fields were counted under the microscope. Each experiment was repeated three times with each 1.000 cells.

Immunoblotting

Whole cell extracts were prepared according to a method described previously [8]. Proteins were separated by 10% SDS-PAGE and electroblotted onto an Immmobilon P membrane (Sigma). After blocking with 3% low fat milk for 1h, the membranes were incubated overnight with primary mouse monoclonal antibodies: anti-Hsp27 (Santa Cruz Biotechnology, sc-59562, dilution 1:500), anti-Hsp72 (Santa Cruz Biotechnology, sc-32239, dilution 1:500), and anti-Hsp90 (Santa Cruz Biotechnology, sc-51966, dilution 1:500). After three washes with PBS enriched with 0.05% Triton X-100 (Sigma), the membranes were incubated with secondary antibodies conjugated with alkaline phosphatase (AP) for 2h. Proteins were detected with AP substrates: 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT) (Sigma) in N,N-dimethylformamide (DMF, Sigma). The quantitative analysis of protein bands was performed using the Bio-Profil Bio-1D Windows Application V.99.03 program. The value was expressed as relative intensity of band thickness, width, and color depth. The data was normalized relative to β-actin (data not shown). Three independent experiments were performed.

HeLa, SiHa, and CaSki transfection with siRNA

The cells at a density of $2x10^5$ were incubated for 24h at 37°C in a CO_2 incubator to reach 60-80% of confluence. After washing with the RPMI 1640 medium without serum and antibiotics, the medium was aspirated. The cells were overlaid with transfection probes containing 2 μl of specific anti-Hsp27, anti-Hsp72, or anti-Hsp90 small interfering RNA (siRNA) (Santa Cruz Biotech) and 2 μl of Transfection Reagent (Santa Cruz Biotech). After 5 hours of incubation at 37°C in a CO_2 incubator, the medium was supplemented with medium containing 20% of fetal bovine serum and 200 μg/ml of antibiotics. Incubation was performed for an additional 18h. After changing the medium to fresh normal growth one, cells transfected in this way were used in further experiments.

Statistical analysis

A one-way Anova test followed by Dunnett's multiple comparison analysis was used for statistical evaluation. $P < 0.05$ of data presented as mean ± standard deviation (SD) was taken as the criterion of significance.

RESULTS

1. The effect of osthole and tamoxifen on induction of apoptosis, autophagy, and necrosis in CaSki, SiHa, and HeLa cells

A staining method with dyes specific for apoptosis, autophagy, and necrosis was used for detection of cell death. Microscopic observations revealed that HeLa and CaSki cells were not sensitive to osthole and tamoxifen treatment and the percent of dead cells was not significant (Figure 1a, b). Only HeLa cells preincubated with osthole followed by TAM treatment was an exception, but the number of apoptotic cells was still not satisfactory (6%). SiHa cells were much more sensitive to apoptosis induction upon osthole and tamoxifen action in comparison to HeLa and CaSki cells (Figure 1c, d). The preincubation with osthole followed by TAM addition was the most effective and initiated programmed cell death in about 27% of the SiHa population. The simultaneous drug application induced apoptosis in 18% of cells, while preincubation with tamoxifen in 9%. In both these experimental variants, apoptosis was accompanied by necrosis – about 9% in the variant with osthole preincubation and 5% when both drugs were applied at the same time.

Autophagy was not significant in all the cell lines in all the experimental variants.

2. Hsp27, Hsp72, and Hsp90 expression upon osthole and tamoxifen treatment

As revealed by the immunoblotting technique, the level of Hsp27, Hsp72, and Hsp90 expression after the osthole and tamoxifen treatment was cell line specific. The results presented in Figure 2 suggest inhibition of Hsp27, Hsp72, and Hsp90 expression in the SiHa cell line after the treatment with the tested drugs, which was observed in all the experimental variants (Figure 2c). In the case of Hsp27 expression, the inhibitory effect was the most intense after cell incuba-

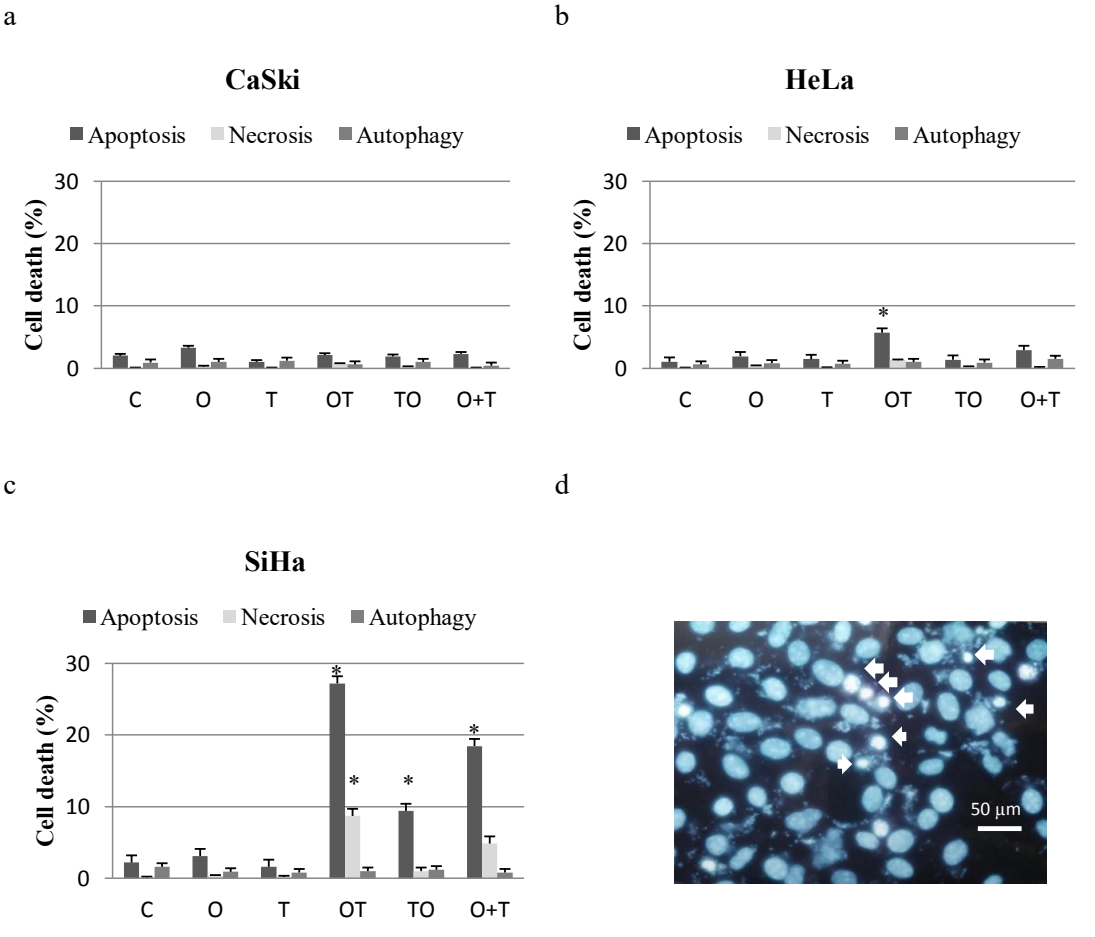


Fig. 1. Effect of osthole (O) and tamoxifen (T) on the level of apoptosis, necrosis, and autophagy in CaSki (a), HeLa (b), and SiHa (c, d) cell lines; C- control cells; OT – pre-incubation with osthole followed by tamoxifen incubation; TO – pre-incubation with tamoxifen followed by osthole treatment; O+T – simultaneous drug application; * $p < 0.05$;d - apoptosis (pointed by arrows) in SiHa cells after osthole and tamoxifen treatment

tion with TAM alone and after TAM preincubation followed by osthole treatment. The level of Hsp72 and Hsp90 was the lowest after the treatment with both drugs in all the variants, while their expression after a separate drug application was comparable to the control. In the CaSki and HeLa cell lines, TAM and osthole did not have such strong inhibitory potential on Hsp expression as that observed in the SiHa cells (Figure 2a, b). In the CaSki cells, TAM and osthole applied alone or in combination had no significant effect on the level of Hsp27. In the case of Hsp72, the effect of both drugs on the expression of the chaperone depended on the sequence of application of the chemicals. Preincubation with TAM or osthole induced the expression of Hsp72, while their simultaneous application inhibited the protein level. The separate drug treatment had no effect on the amount of this chaperone. The level of the Hsp90 expression seemed unchanged in comparison to the control in most of the experimental variants. Only TAM incubation with osthole (separately or in combination) elevated the level of this protein.

In the case of HeLa cells, the effect of osthole and TAM on Hsp expression was protein type specific. Both drugs had no significant effect on Hsp27 expression (Figure 2b) but elevated the level of Hsp72. On the other hand, inhibition of Hsp90 expression was noticed after separate and simultaneous application of the drugs.

3. The effect of blocking the Hsp expression on cervical cell viability after TAM and osthole treatment

To obtain direct proof of the involvement of Hsp27, Hsp72, and Hsp90 expression in the resistance of cervical cancer cells to programmed cell death, we blocked the expression of heat shock proteins with specific siRNA. Immunoblotting analysis revealed that siRNA significantly limited the expression of Hsp27, Hsp72, and Hsp90. The subsequent osthole and tamoxifen treatment did not change the level of expression of the chaperones (Figure 3). siRNA alone as well as the transfection reagent had no significant effect on the expression of the studied heat shock proteins.

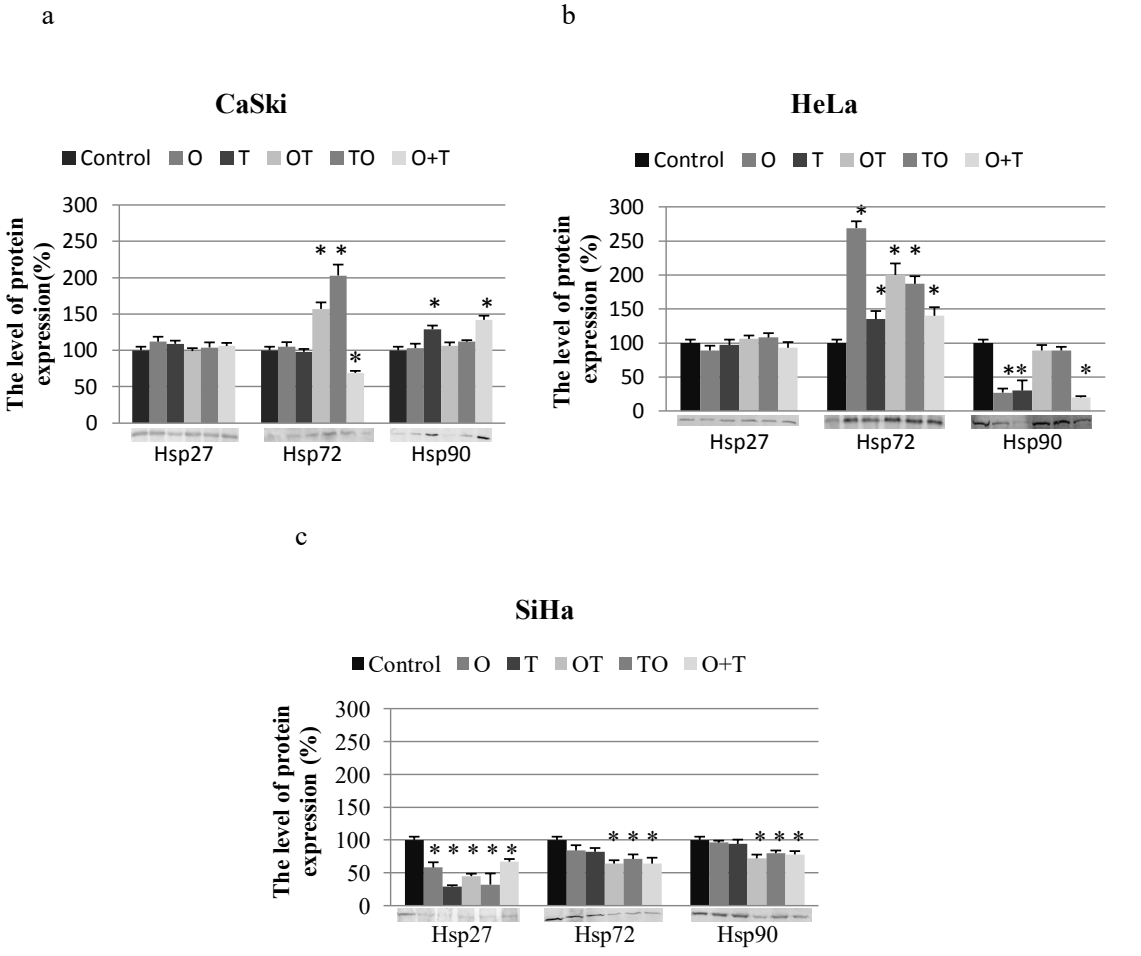


Fig. 2. Effect of osthole (O) and tamoxifen (T) on the level of expression of heat shock proteins Hsp27, Hsp72, and Hsp90 in CaSki (a), HeLa (b), and SiHa (c) cell lines; C- control cells; OT – pre-incubation with osthole followed by tamoxifen incubation; TO – pre-incubation with tamoxifen followed by osthole treatment; O+T – simultaneous drug application; ** $p < 0.05$*

It is known that the activity of Hsps is responsible for cell survival. Therefore, we wanted to estimate the effect of TAM and osthole on apoptosis, necrosis, and autophagy appearance in cervical cells with blocked Hsp27, Hsp72, and Hsp90 expression. In all the studied cell lines, inhibition of the expression of Hsps was correlated with increased induction of apoptosis (Figure 4). The highest percent of cells that died via programmed death were observed in SiHa cells after blocking Hsp27 expression and subsequent TAM and osthole incubation. The Hsp inhibition had no effect on the induction of necrosis or autophagy.

DISCUSSION

Cervical cancer is a malignant neoplasm arising from cells originating in the cervix uteri. Globally, this is the second most common cancer in women. The development of this neoplasm is closely associated with several definable steps in which normal cell infection with human papilloma virus (HPV) plays a key role. Approximately 150 types of HPV have been discovered so far, of which HPV 16 and HPV 18 are the most carcinogenic, since they account for more than 50% of all cervical cancers [6]. The oncogenic potential of high risk HPVs is mediated by two viral oncoproteins, E6 and E7. They target tumor suppressor proteins, such as p53 or pRB, to induce cell proliferation, inhibition of apoptosis, and escape from the innate immune systems. Inactivated oncoproteins were the basis for discovering a vaccine strategy against HPV-induced cancer. Genetically inactivated E7 or E6 proteins are not stable and to stabilize them and minimize the risk of cell transformation as well as to enhance the immune system they should be associated with appropriate proteins, like heat shock proteins Hsp90, GRP78, or Hsp70 [1, 19, 20]. Heat shock proteins belong to molecular chaperones, which protect cells against stress and death. They control proper folding and stabilize the structure of newly synthesized proteins or help to refold proteins that were denatured or aggregated. In cancer cells, heat shock proteins are overexpressed and their high level is a signal of poor prognosis for patients

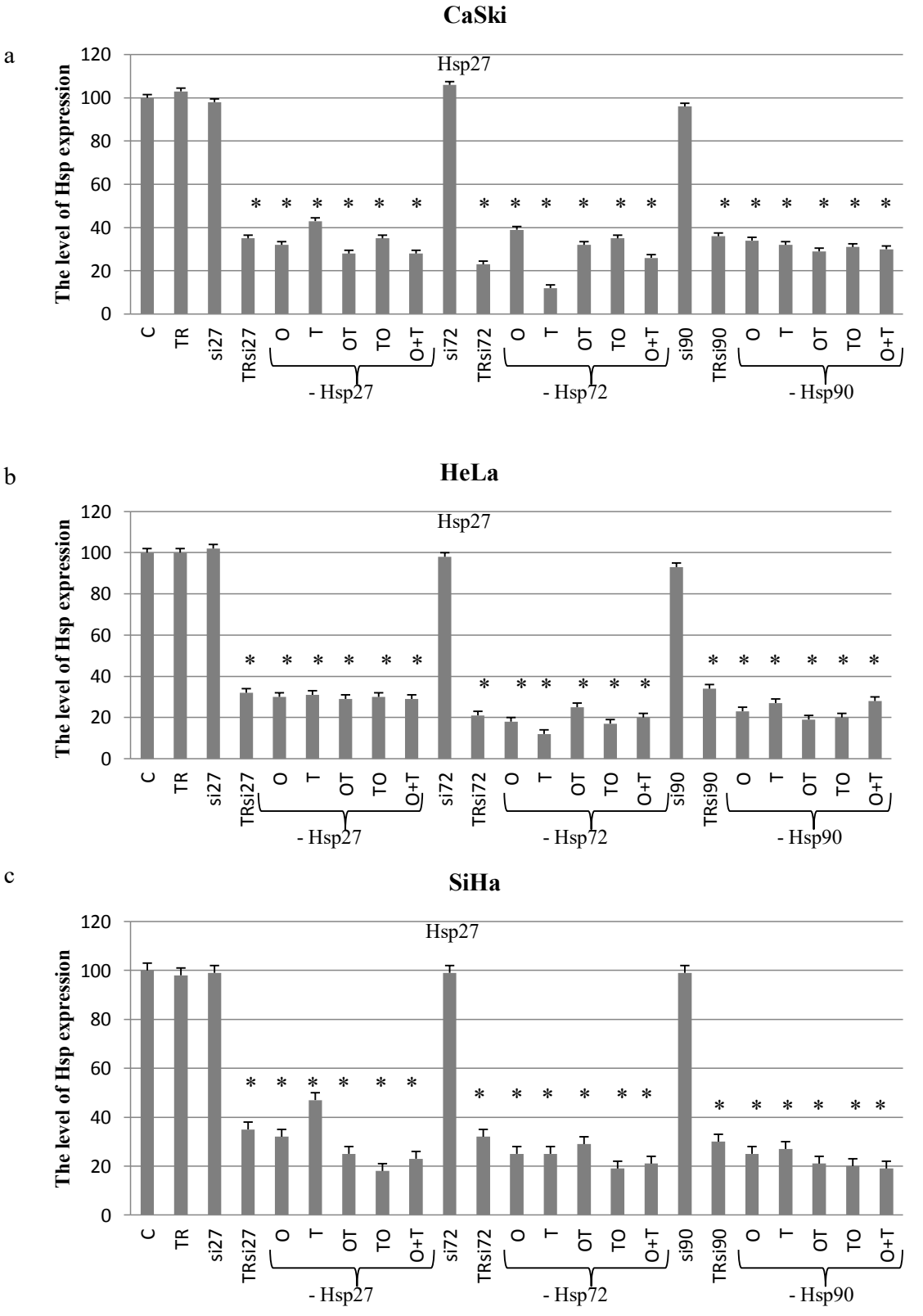
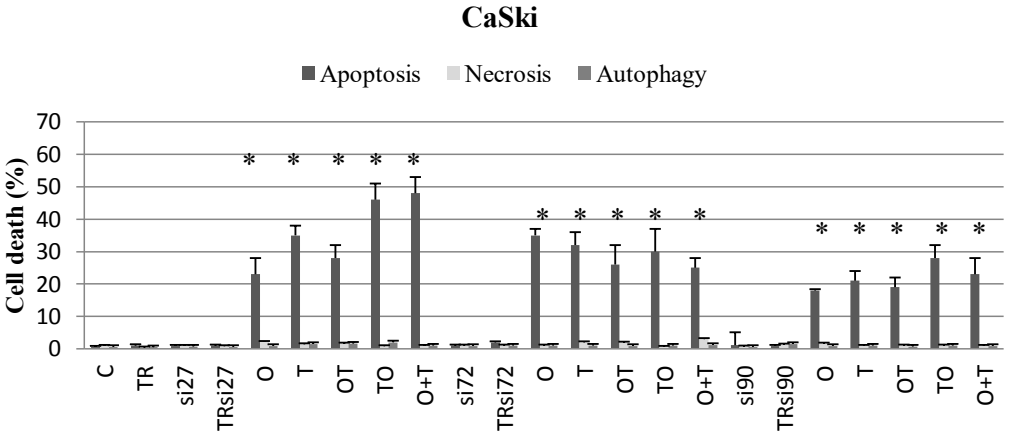


Fig. 3. Level of Hsp27, Hsp72 and Hsp90 expression in CaSki (a), HeLa (b), and SiHa (c) cells after transfection with specific anti-Hsp27 (si27), anti-Hsp72 (si72), or anti-Hsp90 (si90) siRNA and subsequent osthole (0) and tamoxifen (T) treatment; C – control; TR – transfection reagent; TRsi – transfection reagent with specific siRNA; OT- pre-incubation with osthole followed by tamoxifen incubation; TO- pre-incubation with tamoxifen followed by osthole treatment; O+T – simultaneous drug treatment; *$p < 0.05$;

a



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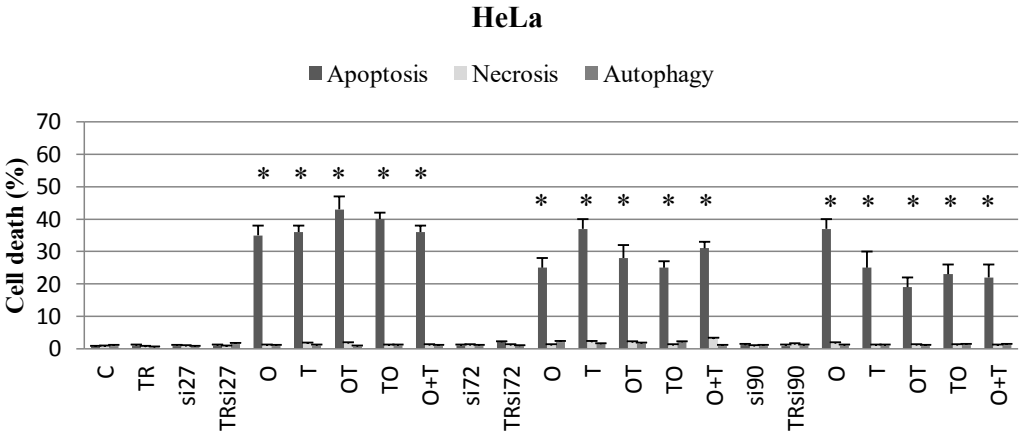

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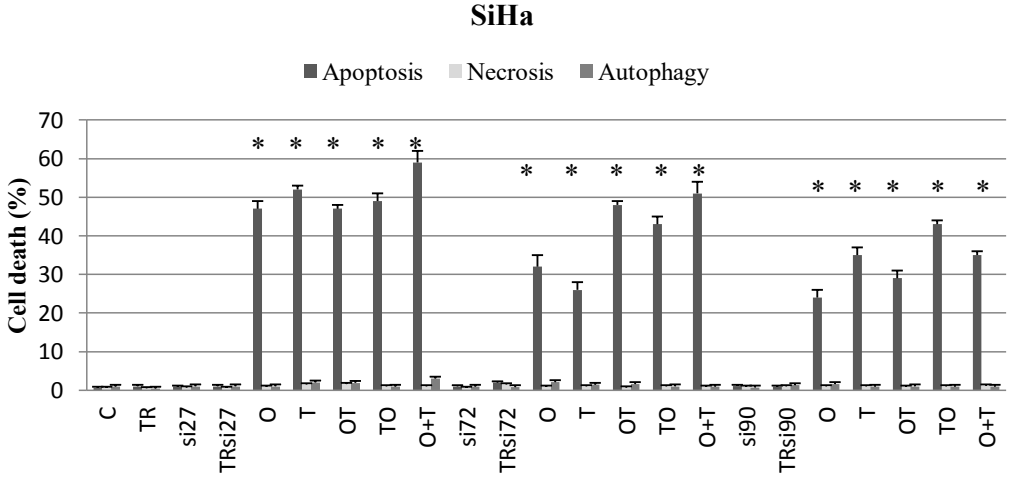

Fig. 4. Effect of osthole (O) and tamoxifen (T) on apoptosis, necrosis, and autophagy induction in CaSki (a), HeLa (b), and SiHa (c) cells transfected with specific siRNA anti-Hsp27 (si27), anti-Hsp72 (si72), and anti-Hsp90 (si90); C- control; TR transfection reagent; OT- pre-incubation with osthole followed by tamoxifen incubation; TO- pre-incubation with tamoxifen followed by osthole treatment; O+T – simultaneous drug treatment; *$p < 0.05$

in terms of survival and response to therapy. An elevated Hsp level in malignant cells plays a key role in protection from spontaneous apoptosis associated with malignancy. The role of Hsps in cervical cancer has not been sufficiently studied and some aspects are still under consideration. It was observed that Hsp27 in this type of cancer was a marker of squamous metaplasia. Hsp70 was responsible for increased cell proliferation and increased tumor size. In the case of Hsp90, which plays a regulatory role by forming complexes with other functional proteins, its level had no effect on the prognosis [5].

In our experiments, three cervical cancer cell lines differing in the type of HPV infection were used. As cell death inducers, a new combination of naturally occurring coumarin osthole and tamoxifen, classified as a Selective Estrogen Receptor Modulator (SERM), was investigated. SiHa cells infected with HPV 16 appeared to be the most sensitive to initiation of apoptosis, especially when both drugs were used in combination. The other cell lines, HeLa and CaSki, were not sensitive to programmed cell death induction upon osthole and tamoxifen incubation, irrespective of the separate or combined drug treatment. Thus, our experiments revealed that the separate action of the furanocoumarin or TAM was not satisfactorily effective in programmed cell death initiation in the cervical cancer cells. The results for osthole were in contrast to observations made in medulloblastoma and ovarian or laryngeal cancer cells, where pro-apoptotic properties of the furanocoumarin were noted [9, 10]. At the molecular level, death was mediated via a mitochondrial (internal) pathway with the activation of caspase 3 and caspase 9. The drug also increased the expression of pro-apoptotic Bax and diminished that of anti-apoptotic Bcl-2 [9, 10]. Tamoxifen, reported to exert anti-proliferative effects toward breast cancer cells [14, 16], failed to eliminate the cervical cancer cells via programmed death. Our observations are in agreement with the study performed by Khar et al. [11], who found no inhibitory action of TAM on HeLa cell proliferation in the presence of fetal calf serum in culture medium. Other authors observed an inhibitory effect of the drug at the concentration 5 μM on HeLa cell growth, but after a longer (even 6 days) incubation time. Interestingly, this concentration applied for 6 days was cytotoxic to CaSki cells [7]. Concentration dependence in TAM action on HeLa cells was noticed by Majmudar, who observed multiplication of the inhibitory effect on HeLa growth after incubation with a lower drug concentration (up to 5 μM) and toxicity after treatment with higher concentrations [13].

Such differences in the cell sensitivity to the osthole and TAM treatment may be explained by the level of expression of heat shock proteins. After separate osthole and tamoxifen incubation, the level of Hsp90 and Hsp72 in the SiHa cells was comparable to the control untreated cells. Only the expression of Hsp27 was an exception; a significantly lower level of the protein was noticed, but it seemed to be insufficient to direct the cells into the death pathway. The action of both drugs was correlated with a diminished Hsp27, Hsp72, and Hsp90 level. The HeLa and CaSki cell lines were characterized by an unchanged or elevated level of expression of heat shock proteins in comparison to the control. In the CaSki cells, inhibition of Hsp72 was only noticed after the simultaneous drug treatment, but it did not elevate the level of apoptosis. Since the results suggested a correlation between the increased sensitivity of cancer cells to cell death, the expression of Hsps was blocked with specific siRNA in the next step of the experiments. The level of apoptosis after the additional osthole and/or tamoxifen treatment increased significantly in comparison to that of the non-transfected cells. The SiHa cells were the most sensitive to apoptosis induction. Similar observations were made in the non-transfected cell line, which may suggest that the inhibition of Hsps is most effective in cell death induction in cervical cancer cells infected with HPV 16. In the other cell lines with the integrated HPV 18 genome, the increase in the number of apoptotic cells was also significant, but to a smaller extent than that in SiHa. Therefore, there may be a correlation between the level of Hsp expression and the type of HPV infection. Correlation studies between the grade of neoplasia and the Hsp level showed that the level of Hsp70 but not Hsp90 was increased after HPV infection [4]. In HPV18 positive oral sarcoma cancer cells, there was 17.3-fold higher expression of Hsp27 in comparison to their paired normal tissues [19].

In all the experimental cases, with or without blocking the Hsp expression, autophagy was observed at a very low, insignificant level. In light of recent investigations reporting that autophagy may represent a survival mechanism, such results are very encouraging. Silencing of Hsp expression did not induce necrosis, which is also an important and promising observation.

Our results indicate that osthole and tamoxifen applied separately or in combination are not very effective in apoptosis initiation in cervical cancer cells, which was correlated with the protective action of heat shock proteins against cell death. Blocking of Hsp expression facilitated effective elimination of HeLa, SiHa, and CaSki cells via programmed death upon application of the studied drugs.

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The authors have no potential conflicts of interest to declare.