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Anticancer activity of Heat Shock Protein 70 (HSP70) Inhibitor, JG-98, against human cervical cancer HeLa and ovarian cancer SKOV-3 cells

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Abstract

Cervical and ovarian cancer are two aggressive neoplasms for women, still with high mortality and morbidity. Among the molecules and compounds that have anticancer activity, it was studied the JG-98, a heat shock protein 70 (HSP70) inhibitor. It demonstrated inhibitory effects on the growth of neoplastic cells, mediated by the induction of apoptosis, with anti-proliferation activity on neoplastic cells via the apoptotic pathway. The authors investigated the antiproliferative effects of JG-98 on human cervical cancer HeLa and ovarian cancer SKOV-3, examined by a standard XTT assay. Apoptotic effects and oxidative status were also evaluated by flow cytometry, ELISA, and total oxidant status assays in HeLa cells, respectively. The IC50 values of JG-98 in HeLa and SKOV-3 cells were recorded as 1.79 and 2.96 µM, respectively. Flow cytometry results showed that JG-98 treatment remarkably increased the proportion of apoptotic cells at IC50 concentration. The JG-98 treatment significantly increased the proteins Bax, cleaved caspase 3, cleaved PARP, and 8-oxo-dG levels, all indicators of cellular apoptosis. These findings show that JG-98 significantly decreased cell proliferation and increased apoptosis in HeLa cells, suggesting that JG-98 has a promising anti-tumor effect in cervical and ovarian cancers.

Keywords: Cervical cancer, ovarian cancer, HSP70, JG-98, HeLa cells, SKOV-3 cells, apoptosis

Introduction

Among the gynecological cancers, cervical and ovarian ones are frequent neoplasms with challenging management options, including chemotherapy modalities [1,2]. Nowadays, several types of molecules and compounds and related molecular mechanisms are leading research topics to discover novel and effective drug candidates and targets for these gynecological cancers as well as for other types of cancers [3].

Heat-shock proteins (HSP) have recently attached the scientific interest of researchers, as novel chemotherapeutic targets, since current molecular data support that HSP-70s have important roles in cancer biology with their unique functions and their interaction with other molecular pathways in cancer biology [4]. In fact, the HSPs constitute one of the main families of ATP-dependent molecular chaperones that positively affect malignant growth in many cancers through proliferation, metastasis, anti-apoptosis, and angiogenesis [1,2]. Among these HSPs, several critical roles for the HSP-70 protein in cancer initiation and progression have been demonstrated by mechanistic studies

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from cell culture and animal models [5]. This attracts attention to this protein as a promising molecule and an important target for investigating drug candidates for a variety of cancers [6].

Cancer cells need several stress-reducing survival factors, including the HSP-70 protein. The expression of HSP-70 enhances sharply during a stress response; although, at a low level, it also takes place under normal conditions [3]. Unlike normal cells, many cancer cells largely express the HSP-70 protein to resist many biological modifications, either during tumorigenesis or during cancer treatment. Therefore, the addiction of cancer cells to HSP-70 is the main reason for its suitability as a target for the development of novel chemo-therapeutic alternatives [7].

Blocking the HSP-70 expressions by the siRNA approach promotes cancer cell death, especially after dual silencing of HSC70 and HSP-70 translations. In addition, these interventions also selectively lead the malignant cells to be more susceptible to other chemotherapeutics [8]. The effects of the HSP-70 protein on malignant cells consist of (a) suppressing several steps of apoptotic pathways; (b) regulating necrosis; (c) preventing entry to the cellular senescence program; (d) negatively interacting with tumor immunity; and (e) enhancing angiogenesis and helping metastasis. Thus, the direct involvement of HSP-70 protein in various cancer types explains the phenomenon of the tight link between cancer cell survival and modulation of HSP-70 expression [7,9]. Moreover, cancer cells also intensively release HSP-70 into the extracellular microenvironment, resulting in adverse outcomes in cancer patients [4,8].

In accordance with the confirmed roles of the HSP-70 protein as a survival supporter for cancer cells, several studies revealed the major contribution of HSP-70 to the proliferation of cancer cells by enhancing their resistance to chemotherapy [10]. After the development of resistance to cisplatin, there is a significant increase in the expression of the HSP-70 protein in human ovarian cancer cells [10]. Reversing drug resistance of cancer cells leads to HSP-70 down-regulation. This biological effect may be a crucial factor in the inhibition of apoptosis by returning HSP-70 up-regulation, leading both upstream and downstream of the mitochondrial signaling molecules [11]. Hence, to increase the clinical efficacy of HSP-70 blockage, it would be beneficial to determine promising molecules, reducing the interaction of HSP-70 with other co-chaperone molecules [11]. Considering candidate molecule groups targeting the HSP-70 protein, laboratory data supported the potency of protein JG-98, an allosteric HSP-70 inhibitor, in decreasing the proliferation of cancer cells. The expression of HSP-70 protein increases in several cancers with regard to the increase in tumor grade, metastasis, and poor prognosis; in concordance with these data, the decrease in HSP-70 expression reduces cancer progress in mouse models [5].

To examine the merit of novel molecules to be a chemotherapeutic agent and the mechanisms of their effects, cell lines are vital as a first step to understanding molecular and behavioral changes, altering the proliferation and survival of cancer cells [12]. The JG-98 has an anticancer HSP-70 inhibitor effect in the HeLa human cervical cancer cell line and in the ovarian cancer SKOV-3 cell line. This significant cytotoxic effect on cancer cell lines has been mainly related to the increase in oxidative stress and apoptosis [13]. Further investigation is needed to better understand the mechanisms of action of the JG-98 protein in HeLa and SKOV-3 cells. Since HeLa cells have been detected to be more susceptible to the JG-98 protein compared to the SKOV-3 cells in the current study, mechanistic studies were performed within this cell line. In this study, the effects of the JG-98 protein, as an HSP-70 inhibitor, on the proliferation of human cervical cancer HeLa and ovarian cancer SKOV-3 cells, were evaluated.

Material and Methods

Laboratory experiments of this research were carried out between January 2021 and April 2021 at the Sivas Cumhuriyet University Faculty of Medicine Research Center. This study was approved by the Human Research Ethics Committee of the Sivas Cumhuriyet University, Sivas, Turkey (Approval number: 2021-11/56).

Cell lines and cell culture

The American Type Culture Collection (ATCC, USA) was used to obtain HeLa (CCL-2) and SKOV-3 (HTB-77) cell lines. Cells were found as standard in the modified Dulbecco Eagle environment (Sigma-Aldrich, USA). During this procedure, cells were grown in a humidified atmosphere with 5% CO2 at 37 ˚C. When examined, the contents of the media mixture contained 10% bovine fetal serum (FBS) (Sigma-Aldrich, USA), 1% L-glutamine (Sigma-Aldrich, USA), and 1% mixed penicillin/streptomycin antibiotics (Sigma-Aldrich, USA). The JG-98 protein (Medchem, USA) was then dissolved in DMSO in DMSO. By dilution, less than 0.1% of the final DMSO content was obtained prior to the treatment of cancer cells.

Cell viability assay

A standard XTT test (Roche Diagnostic, Germany) has been used to detect the cytotoxic activity of JG-98 against HeLa and SKOV-3 cell lines. The cells were used as triplicate in 96-well cell culture plates with an adjusted density of 1x104 cells per well. Cells were incubated at concentrations of 0.1, 1, 2.5, 5, 10, 20 and 40 μM of JG-98 for 24 hours. In the study, viable cells were measured by adding 50 ml of XTT labeling mixture. Cells were incubated for 4 hours. Repeated absorbance measurements were obtained using an ELISA (Epoch, Biotech, United States) microplate reader at 450 nm. Cell viability was recorded in % with controls (100% viability). The IC50 values of JG-98 in HeLa and SKOV-3 cell lines were demonstrated using Graph Prism 8 software (GraphPad, United States). According to the favorable CI50 of JG-98 in HeLa cells, they have been used in ELISA and flow cytometry experiments.

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Annexin V binding assay

Approximately 5×105 HeLa cells were seeded into 6-well plates and allowed to adhere overnight. The following day, they were grown in the presence of the JG-98 at 1 and 2.5 µM concentration and incubated for another 24 h. After trypsinization, the cells were collected and suspended in PBS containing at least 1% FBS. During these procedures, it was blended with an equal volume of Annexin V and Dead Cell reagent (Merck Milli-pore, United States) following the manufacturer's instructions. Live, dead, early apoptotic, and late cells were assessed by quantification with the Muse Cell Analyzer (Merck Millipore, United States).

Bax protein, cleaved caspase 3, BCL-2, cleaved PARP, and 8-hydroxy-deoxyguanosine expression analyses

The human Bax ELISA kit (BT Lab, catalog #E1825HU), the human cleaved caspase 3 ELISA kit (BT Lab, catalog #E6970HU), the human BCL-2 ELISA kit (BT Lab, catalog #E1832HU), and the human cleaved PARP ELISA kit (BT Lab, catalog #E6971HU), and the human 8-Hydroxy-Desoxyguanosine ELISA kit (BT Lab, catalog #E1436HU) were used to determine the levels of Bcl-2-associated X protein (Bax), cleaved caspase 3 (Asp175), BCL-2 family proteins, cleaved PARP (Asp214), and 8-hydroxy-deoxyguanosine (8-oxo-dG) levels in the JG-98-treated and untreated HeLa cells. HeLa cells were seeded in a 6-well plate and treated to JG-98 concentrations of 1 and 2.5 µM for 24 hours. JG-98 cells were collected, diluted with PBS, and destroyed by multiple freeze-thaw cycles, both treated and untreated. The levels of Bax, cleaved caspase 3, BCL-2, cleaved PARP, and 8-oxo-dG in the cell lysates were measured in the order recommended by the manufacturer. The BCA assay was used to compare the total protein contents in treated and untreated HeLa cells (Pierce Biotechnology, United States).

Measurement of Total Oxidant Status (TOS) in JG-98-treated and untreated HeLa cells

The TOS assay kit was used to determine the value of total oxidative status (TOS) in treated and untreated HeLa JG-98 cells (Rel Assay Diagnostics, Turkey). JG-98 was given to HeLa cells at doses of 1 and 2.5 µM for 24 hours, according to the manufacturer's instructions. In the case of TOS, the results were expressed in mol H_2O_2 equivalent/L [14].

Statistical analysis

Statistical analysis was performed using IBM SPSS v23 (IBM SPSS, United States). The Kruskal-Wallis ANOVA test with post hoc Dunn's test was used to examine the laboratory data, which were provided as mean standard deviation. Statistical significance was defined as a p-value lower than 0.05.

Results

First, the cytotoxic effect of the JG-98 was determined in the HeLa and the SKOV-3 cells. As seen in Figure 1, the JG-98 inhibited the growth of cancer cell lines in a concentration-dependent manner (P<0.01).

The IC50 values of JG-98 in the HeLa and the SKOV-3 cells were recorded as 1.79 and 2.96 µM, respectively, for 24 h. The JG-98 treatment also considerably impacted HeLa cell density and cell morphology (Figure 2 a, b, and c).

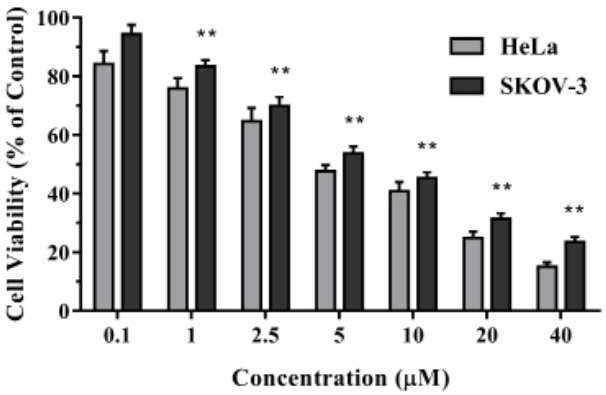

Figure 1. The effects of JG-98 on the viability of HeLa and SKOV-3 cells. JG-98 was applied to the cells for 24 h concentrations ranging from 0.1 to 40 µM. The XTT assay was used to determine the number of viable cells. When compared to untreated control, the results are expressed as a percentage of viable cells. The data represent the mean SD of two separate experiments. The differences are statistically significant when **p<0.01 compared to the control. All cell viability results for HeLa cells are significantly different from the control for 0.1, 1, 2.5, 5, 10, 20, and 40-µM concentrations (p<0.01)

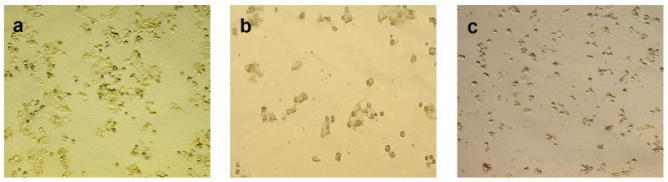

Figure 2. The effect of JG-98-treatment on HeLa cell morphology. Control cells are shown on the left (a), cells treated with 1 µM JG-98 in the middle (b), and cells treated with 2.5 µM JG-98 on the right (c)

Flow cytometry investigation of the apoptotic effects of JG-98 on HeLa cells revealed that JG-98 treatment significantly increased the proportion of apoptotic cells at IC50 concentration for 24 h compared to untreated cells (Figure 3).

The percentage of early and late apoptotic cells in the control group (3.78±0.79% and 0.91±0.11%, respectively) increased considerably to 10.61±1.35%, 13.69±0.97% and 58.33±2.45%, and 67.79%±3.11 respectively, in 1 and 2.5 µM JG-98-treated HeLa cells (P<0.01).

The effects of the JG-98 therapy on pro and anti-apoptotic protein expression in HeLa cells were also assessed using ELISA assays. The compound treatment significantly increased Bax (P<0.01), cleaved caspase 3 (P<0.05 for 1 µM and P<0.01 for 2.5 µM), cleaved PARP (P<0.01), and 8-oxo-dG (P<0.05) levels, while anti-apoptotic BCL-2 levels dropped.

The TOS assay kit analysis showed the TOS values of 2.81±0.27 in control cells and 5.62±0.51 and 7.79±0.63 in 1 and 2.5 µM JG-98-treated cells, respectively (P<0.01), as shown in Figure 5.

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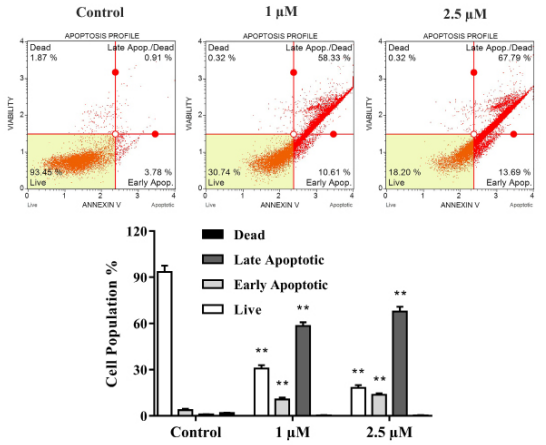

Figure 3. Annexin V staining revealed that JG-98 caused apoptosis in HeLa cells for 24 hours in a concentration-dependent manner. As detailed in the Material and Methods section, the cells were exposed to 1 and 2.5 µM JG-98, and the number of apoptotic cells was determined using the Muse cell analyzer (Merck Millipore, Billerica, USA). The percentages of early and late apoptotic cells increased significantly in cells treated with 1 and 2.5 µM JG-98. Three times each experiment was carried out. **P value <0.01 as compared to the untreated HeLa cells and the 1 or 2.5 µM JG-98-treated groups

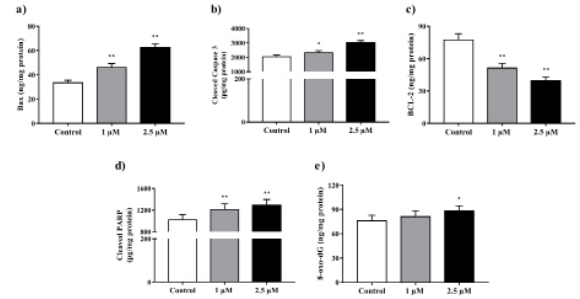

Figure 4. JG-98 treatment increased apoptosis and DNA damage in HeLa cells for 24 hours, as measured by ELISA. The levels of Bax, cleaved caspase 3, BCL-2, cleaved PARP, and 8-oxo-dG were measured using an ELISA kit after the cells were treated with JG-98 at 1 and 2.5 µM for 24 hours. The values are the mean SD of three samples of media taken from HeLa cell wells. *P value <0.05 vs. untreated HeLa cells and those treated with 1 or 2.5 µM JG-98

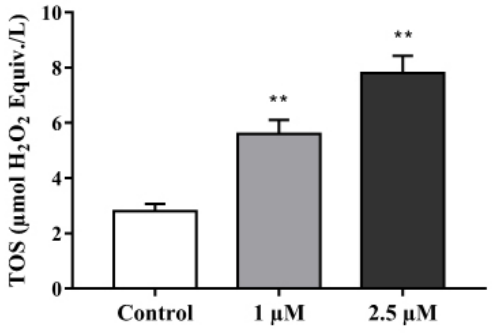

Figure 5. JG-98 treatment greatly increased the TOS of HeLa cells. The cells were treated for 24 h with 1 and 2. 5µM JG-98, and TOS was determined using a TOS assay kit. The values represent the mean SD of three samples of medium taken from wells containing HeLa cells. **P value <0.01 vs. untreated HeLa cells and 1 or 2.5 µM JG-98-treated groups

Discussion

This study screened the cytotoxic activity of the JG-98 protein in HeLa and SKOV-3 cells for the first time in the literature, showing a decreased cell viability for HeLa and SKOV-3 cells in a dose-dependent manner when treated with JG-98 for 24 hours. The authors also performed in vitro biological assays to determine the anticancer effect of the JG-98 on HeLa cells, which is the most sensitive to JG-98. Overall, the JG-98 provided an anticancer effect due to its apoptotic effects, under the influence of oxidative stress.

In cancer treatments, the development of new treatment modalities by the HSP-70s is a reliable option. The HSP-70s proteins have been reported to localize to the plasma membrane under pathophysiological states, including cancer, but apart from the corresponding normal cells [15,16]. HSP-70, which is a fundamental component of the anti-stress defense system, supports the survival of cancer cells with its interaction at key points in cellular apoptotic pathways. However, high expression of extracellular HSP-70 is indicative of a worse prognosis during the cancer process. The HSP-70 inhibition leads to anti-tumor system activation and apoptotic process in some tumors [17]. Research on HSP-70 inhibitors could provide novel drugs for physicians, to use alone and in combination, as a promising tool to develop more successful anti-cancer strategies, improve the patient's outcomes, and for long-term survival [18].

A review of related studies reveals the relationship between HSP-70 and its support for cell survival during different types of ongoing cell death, such as caspase-dependent/independent apoptosis, necrosis, autophagy, or programmed cell death [19]. Besides, cancer cells are exposed to many environmental, physiological, and pathophysiological stress conditions within the tumor microenvironment. These include high oxidative stress, limited nutrients, hypoxia, and increased expression of mutant proteins which, in combination, affect cancer cell survival [19].

Liu et al. investigated the effect of 2-Phenylethinsulfonamide (PES), a small molecular inhibitor of HSP-70, used with cisplatin to inhibit the proliferation of cervical cancer cells in vitro and tumor growth after in vivo animal models [20]. The authors showed that PES could enhance the killing effect of cisplatin on them [20]. Moreover, PES combined with cisplatin could inhibit the proliferation and development of cervical cancer cells in vitro and the transplanted tumor growth of cervical cancer cells, through the mitochondrial apoptosis pathway [20].

Garg et al. evaluated the contribution of the expression of HSP-70-2, a member of the HSP-70 chaperones protein family, to the malignant properties of cervical cancer [21]. The authors detected that HSP-70-2 is highly expressed in many cervical cancer cell lines and neoplastic tissues of cervical cancer patients, suggesting that HSP-70-2 has several significant roles in cell migration, invasion, and tumorigenesis in cervical cancer [21].

Court et al. investigated gene expression profiles during magnetic

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fluid hyperthermia in ovarian cancer cells to determine cellular response and molecular targets to promote its effect in vitro and in vivo experiments [22]. The authors evaluated whether there was an up-regulation of several HSPs and the HSP-70 was found to be the most up-regulated gene. They showed that the inhibition of HSP-70 by RNA interference, as well as by PES, a novel HSP-70 inhibitor, enhanced the effect of magnetic fluid hyperthermia, both in vitro and in vivo experiments in ovarian cancer cell lines and animal models [22].

In a recent investigation on the mRNA profile in 196 patients with ovarian cancer by microarray technology, the HSP-70 (also known as hsp72 or HSPA1A protein), the single-chain type-1 glycoprotein (MIC2 or CD99), the member of the RAS oncogene family RAB3A and the POM121L9P (POM121 transmembrane nucleoporin like 9, pseudogene) were overexpressed and the combination of, at least, two overexpressed genes were found as further associated with advanced grade, chemotherapy resistance, and progressive disease [23].

The HSP-70s modulates protein homeostasis and cell survival, as a promising anti-cancer target based on expression data, knockdown studies, and the promising cytotoxic activity of first-generation inhibitors [24].

The research on a wide range of effective HSP-70 inhibitors highlighted proteins of Bcl-2 associated with the athanogene family (BAG) [25]. Of these co-chaperones, the BAG3 protein gained interest as a new anti-cancer target because this protein was selectively up-regulated in response to a stressful event in cancer cells. Moreover, its expression is accompanied by HSP-70 expressions in many cancer types. The BAG3 has also been shown to interact with HSP-70 to aid cancer development, through several molecular pathways, such as the cell cycle and suppression of oncogene-induced senescence [25].

The inhibition of HSP-70s can provide an interesting advantage when added to chemotherapy regimens, because the levels of HSP-72 are dramatically increased after exposure to other therapies, such as HSP-90 inhibitors, proteasome inhibitors, and radiation [26]. As an important side effect, HSP-90 inhibition causes a compensatory induction of HSP-70 expressions, a potent negative regulator of cell death. The HSP-70 overexpression can reduce the death of cancer cells induced by HSP-90 inhibitors and therefore reduces the effectiveness of these compounds [27]. The HSP-70 is a crucial factor for tumor cell survival and tumor growth in life. An association has been shown between high HSP-70 levels in tumors and poor prognosis for cancer patients [28]. This molecular pathway may be another opportunity for HSP-70 inhibitors to compensate for this disadvantage [28].

Our study had some limitations regarding its study design including in vitro assays without the inclusion of normal cells, although it has important findings in terms of supporting the literature, mainly associated with increased oxidative stress and apoptosis.

Since ovarian and cervical cancers also include several subtypes with very different genetic alterations and pathological features, and the Hela and the SKOV3 cannot represent all cervical or ovarian cancers, the translation of research into clinical practice could be undermined by these premises. This research could be a forerunner for other studies on other cervical or ovarian cancers.

Conclusion

The HSP-70 can promote cancer progression and facilitate tumorigenesis in all stages and the cytotoxicity as-says elucidate the cytotoxic effect of the JG-98 against the HeLa and the SKOV-3 cancer cell lines [29,30]. To this, we can add our data, in which additional in vitro assays to measure the anticancer effect of the JG-98 on HeLa cells provided the highest cytotoxic activity in all doses. The efficacy of the JG-98, as a selective agent inhibiting the in vitro proliferation of human cervical cancer HeLa cells, was remarkable. The in vitro anti-cancer activity of the JG-98 against HeLa cells was developed, at least in part, by the promotion of apoptosis and this may be a consequence of increased oxidative stress. The findings of the current study justify the performance of further in vitro cell-based and in vivo studies in cervical and ovarian cancer, to explore in more depth the potential beneficial uses of HSP-70 inhibitors.

Conflict of interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical approval
This study was approved by the Human Research Ethics Committee of the Sivas Cumhuriyet University, Sivas, Turkey (Approval number: 2021-11 / 56).

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