RESEARCH ARTICLE

Hsp90 Inhibitor; NVP-AUY922 in Combination with Doxorubicin Induces Apoptosis and Downregulates VEGF in MCF-7 Breast Cancer Cell Line

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Abstract

Objective: Breast cancer is one of the most prevalent malignancies and leading causes of females' mortality worldwide. Because of resistance to various treatment options, new treatments based on molecular targeting has introduced as noticeable strategies in cancer treatment. In this regard, heat shock protein 90 (Hsp90) inhibitors are proposed as effective anticancer drugs. The goal of the study was to utilize a combination of the doxorubicin (DOX) and NVP-AUY 922 on the MCF-7 breast cancer model to investigate the possible cytotoxic mechanisms. **Methods**: MCF-7 breast cancer cell line was prepared and treated with various concentrations of DOX and NVP-AUY922 in single-drug treatments. We investigated the growth-inhibitory pattern by MTT assay after continuous exposure to NVP-AUY922 and DOX in order to determine dose-response. Then the combinatorial effects were evaluated in concentrations of $0.5 \times IC_{50}$, $0.2 \times IC_{50}$, $1 \times IC_{50}$ and, $2 \times IC_{50}$ of each drugs. Based on MTT results of double combinations, low effective doses were selected for Real-time PCR [caspase3 and vascular endothelial growth factor (VEGF)] and caspase 3 enzyme activity. **Results:** A dose-dependent inhibitory effects were presented with increasing the doses of both drugs in single treatments. The upregulation of caspase 3 and downregulation of VEGF mRNA were observed in double combinations of NVP-AUY922 and DOX versus single treatments. Also, in these combinations in low doses of examined drugs ($0.5 \times IC_{50}$, $0.2 \times IC_{50}$), higher caspase 3 activity were presented in comparison to single treatments (p<0.05). **Conclusions:** Our findings indicate an effective action of NVP-AUY922 in combined with DOX in this cell line. These results can predict the treatment outcome in this model.

Keywords: Breast cancer- doxorubicin- NVP-AUY922- Caspase 3- vascular endothelial growth factor- apoptosis

Introduction

Breast cancer is considered as a one of the most prevalent malignancies and leading causes of females' mortality worldwide (Maroufi et al., 2020). The resistance to different treatments, including chemotherapy, is not commonly exclusive. It appears that the tumor could be resistant to multiple treatment options. Nevertheless, the underlying mechanisms are complicated and need more studies. Moreover, drug resistance is a common problem in treatments of all breast cancer types, although the various treatment strategies could be applied (Chun et al., 2017). In this regard, targeted therapy in various types of malignancies including solid tumors considered as promising treatment options (Jensen et al., 2008). Indeed, cancer cells often use the specified molecular

mechanism to increase their capacity for growth (Arshad et al., 2018). One main target in effective cancer therapies is to induction of effective cytotoxic response after chemotherapy using drug response assays (Mohamadi et al., 2017; Mohammadian et al., 2019; Mohammadian et al., 2017; Moradi et al., 2019; Zeynali et al., 2018). Doxorubicin (DOX) is one of the furthermost effective chemotherapeutic agents in the treatment of breast cancer. Unfortunately, resistance to DOX is mutual and considered as a significant problem to successful treatment. In this regard, evaluating the new approaches which are related to treatment response may permit effective therapy to be proposed (Smith et al., 2006).

The heat shock protein 90 (Hsp90) as a main molecular chaperone has an essential role in functionality of its client proteins in cells. The Hsp90 client proteins comprise the

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numerous signal transducing molecules including protein kinases (Miyata et al., 2013). The Hsp90 machinery plays an important roles in the folding, maturation, and assembly of several proteins that involved in cell cycle control. Indeed, HSP90 as multichaperone complex has a significant function in cancer growth and could be considered as a promising target for effective cancer treatment. (Mayor-López et al., 2014; Miyata et al., 2013). So, Hsp90 inhibition with specific inhibitors including geldanamycin and its derivatives decreases the tumor growth. Hsp90 inhibitors can be potential cancer treatment options (Miyata et al., 2013).

the novel small molecule NVP-AUY922 as highly potent HSP90 inhibitor, has acceptable pharmacological and pharmaceutical properties, and show potent antitumor activity in human cancer cell models with inhibition of cell proliferation in low dose potency (Jensen et al., 2008).

Apoptosis is programmed cell death that has a main role in the assessment of response to the cytotoxic and tumor cell damage. Caspases are main effectors of apoptosis proces that among these mediators, caspase-3 is the central protease activator with critical role in apoptosis induction (Moradi et al., 2019). On the other hands, angiogenesis is essential to tumor growth and invasion. The vascular endothelial growth factor (VEGF) signaling pathway has essential role in controlling the tumor angiogenesis. VEGF as a therapeutic target has been confirmed in different types of human malignancies (Niu et al., 2010).

So, in the present study, we examined the antitumor effects of NVP-AUY922 and DOX in combination cases with targeting angiogenesis and apoptosis main mediators in MCF-7 breast cancer cells.

Materials and Methods

Cell culture, drug treatments and MTT assay

The MCF-7 cell line was obtained from the Pasteur Institute (Tehran, Iran).

Cells were seeded in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and 1% of penicillin and streptomycin, with 95% humidity and 5% CO2 at 37°C.

DOX and NVP-AUY922 were provided by Sigma and LC companies respectively. DOX and NVP-AUY922 stock solution was prepared and kept at 4 and -20 ° C, respectively.

DOX were applied into the culture medium at the concentrations of 0.1, 0.2, 0.25, 0.5, 1, 2, 3 and 5 μM. Also, MCF-7 cells treated with NVP-AUY922 in the doses of 1, 2, 4, 5, 8, 10, 20, 40 and 50 nM,.

Cytotoxic effects of DOX and NVP-AUY922 on MCF-7 cells were assessed using the MTT test as describe in manufacture protocol (Kia zist, Iran). Briefly, 1×10^4 viable cells were cultured in 96-well culture plates with and without examined drugs in a final volume of 100 μl, as triplicates. After 24 h, drugs were added. Cells were allowed to grow in the following 24 h after treatments. The cell viability test performed, and the dose-response curve were drown. The IC_{50} concentrations were assessed by Compusyn software. In following, the various doses

(2× IC_{50}, 1× IC_{50}, IC_{50}, 0.5× IC_{50}, and 0. 2× IC_{50}) of each drugs were examined in double combinations, and effective dosages for Real-Time polymerase chain reaction (PCR) and caspase3 activity were selected(0.5 × IC_{50} and 0.2 × IC_{50} of each drugs in combinatorial cases). Definitely, to evaluate the combined effects of these drugs, we utilized lower effective treatments of them that approved with MTT assay (0.5× IC_{50} and 0. 2× IC_{50}) for further studies.

RNA extraction and Real-Time PCR

In this study, 24 h After various treatments, total RNA was extracted by using the RNA extraction kit (Gene all,South Korea) according to the manufacturer's protocol. Reverse transcription was carried out to synthesize the cDNA based on the manufacturer's instructions. Then cDNA was subjected to Real-Time PCR assay. Indeed, the expression of VEGF and caspase 3 at the mRNA levels was studied. The specific primers sequences of caspase-3 (Atari-Hajipirloo et al., 2017), VEGF (Zeynali et al., 2018) and β-actin (Atari-Hajipirloo et al., 2017) as a housekeeping gene presented in Table 1.

Real-time PCR was carried out by Real Q Plus 2x Master Mix Green (Amplicon, Denmark). The PCR conditions were as follow: 15 min at 95°C, then 40 cycles of 95°C for 20 sec; 58°C for 60 sec and 72°C for 5 minutes for VEGF (7)(Zeynali et al., 2018) and at 30 cycles of denaturation for 30 s at 95°C, annealing for 30 seconds at 59°C, and extension for 30 seconds at 72°C for caspase3. Levels of caspase 3 and VEGF were normalized to β-actin (housekeeping gene). The Ct (threshold cycle) value of each sample was measured. Results were calculated using the $2^{-\Delta\Delta Ct}$ method.

Caspase3 enzyme activity assay

The caspase-3 colorimetric assay kit (Abnova) was utilized to detect the caspase-3 enzyme activity. Briefly, after 24 h of different treatments, the supernatant was removed, and cells were trypsinized. Then cells were collected and centrifuged at 14,000 rpm for 5 min. in following, cell lysis buffer was added, and cells were retained on ice and centrifuged. Protein concentration was measured with the Bradford method (16)(Bradford, 1976). Proteins (50 μg) diluted by addition of cell lysis buffer. Then 50 μL 2× reaction buffer and 5 μL DEVD-pNA 4 mM substrate was added. The subjected plates were maintained at 37 °C for 2 h and read at 405 nm. The change in caspase 3 activities was assessed by comparing these data with the level of the untreated control.

Statistical analysis

Data were assessed with, one-way ANOVA followed by post hoc Tukey test for the study of multiple group comparisons by SPSS statistical software. Results are shown as the mean±Standard deviation. P<0.05 were considered as statistically significant.

Results

The effects of the DOX and NVP-AUY922 on cellular viability after 24 h of exposure in the MCF-7 cancer cell line were drawn as the percentage of the viable cells to the
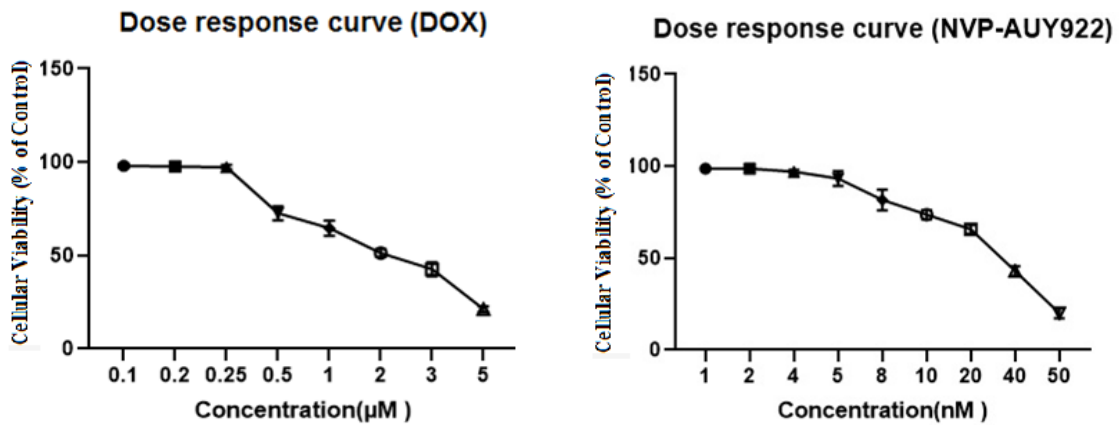
Figure 1. Cellular Viability Results from MTT Analysis (Cytotoxic Effects) of DOX and NVP -AUY922 in Single-Drug Treatments with Different Concentrations after 24 h. Data presented as mean±SD. DOX; Doxorubicin.

Table 1. Primers Sequences to Study the β-actin, VEGF and Caspase-3 Gene Expression Levels in MCF-7 Cell Line

Gene	Primer	Product size (bp)
VEGF (Forward)	5' AGGAGGAGGGCAGAATCATC 3'	141
VEGF (Reverse)	5' GGCACACAGGATGGCTTGAA 3'	
Caspase 3 (Forward)	5'AGAACTGGACTGTGGCATTGAC3'	191
Caspase 3 (Reverse)	5' GCTTGTCGGCATACTGTTTCAG 3'	
β-actin (Forward)	5' CTGGAACGGTGAAGGTGACA 3'	161
β-actin (Reverse)	5' TGGGGTGGCTTTTAGGATGG3'	

untreated control cells. (Figure 1). Our findings show that the exposure of MCF-7 cells to various concentrations of each single drugs decreased cellular viability in the dose dependent manner. Higher IC_{50} values were obtained in DOX compared to NVP-AUY922.

Various concentrations of DOX and NVP-AUY922 were selected to examine the cytotoxic effects of both drugs in order to assay the effects of drug combinations in MCF-7 cell line. Cellular inhibitory effects of double combinatorial cases were shown in Figure 2.

As presented in Figure 2, in double combination treatments, drug concentrations were $2 \times IC_{50}$, $1 \times IC_{50}$, $0.5 \times IC_{50}$, $0.2 \times IIC_{50}$. In all double combinations of DOX and NVP-AUY922, decreased cellular viability were presented compared to single drugs treated cases at IC_{50} doses(P<0.05).

Also, cellular viability decreased significantly in all double combinations compared to untreated controls (P< 0.05). Also, double combinations in $0.5 \times IC_{50}$ concentrations had no significant difference with $0.2 \times IC_{50}$ concentration of each drug(P> 0.05). So, in this study, we utilized lower effective doses of both
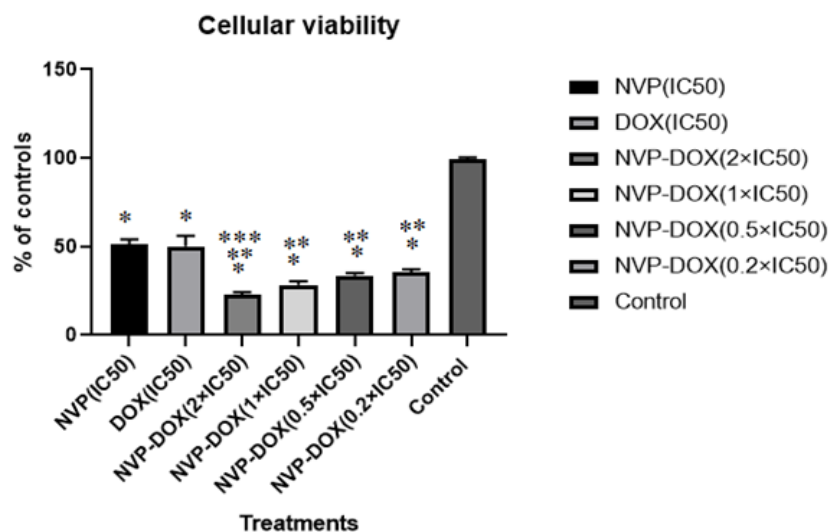

Figure 2. Cellular Viability Assay Results of Various Treatments in Single (IC_{50} Doses) and Double Combinations($2 \times IC_{50}$, $1 \times IC_{50}$, $0.5 \times IC_{50}$ and $0.2 \times IC_{50}$) after 24 h. *; significant differences in comparison to untreated control<0.05; **; significant differences in comparison to single treatments (IC50 doses). P<0.05; ***; significant differences in comparison to double treatments($0.5 \times IC_{50}$ and $0.2 \times IC_{50}$). P<0.05
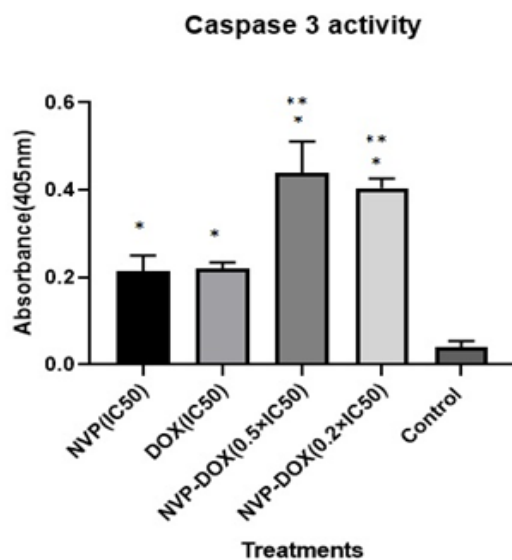
Caspase 3 activity

Figure 3. Activities of Caspase 3 in the MCF-7 Breast Cancer Cells after Treatment with the Various Concentrations of NVP-AUY and DOX for 24 h. Each value shows the mean ± SD. *, Indicates a significant difference versus untreated control p<0.05; **, Indicates a significant difference versus single-drugs treatments p<0.05.

drugs for real-time PCR and caspase 3 enzyme activity ($0.5 \times IC_{50}$ and $0.2 \times IC_{50}$).

According to our results (Figure 3), DOX and NVP-AUY922 in single treatments had higher caspase-3 activity in IC_{50} doses versus untreated control cells (p<0.05). In double treatments of both drugs, data showed significant increased caspase 3 enzyme activity compared to control treatment (p<0.05). Likewise, treatment at $0.2 \times IC_{50}$ and $0.5 \times IC_{50}$ concentrations of each examined drug increased caspase-3 activity significantly (P < 0.05) in compared to single treatments (Figure 3). In comparing these two combinatorial cases ($0.2 \times IC_{50}$ and $0.5 \times IC_{50}$), there were no significant differences (p>0.05).

Based on MTT results, combinations of DOX and NVP-AUY922 at concentrations of $0.2 \times IC_{50}$ and $0.5 \times IC_{50}$ were used for complementary tests. According to real time PCR results (Figure 4A) there were significant fold increase in caspase-3 mRNA as the result of $0.2 \times IC_{50}$ and $0.5 \times IC_{50}$ (P< 0.05) treatments of both drugs compared to the control group. Also, the single treatments in IC_{50} doses of single drugs increased the level of caspase-3 mRNA versus the untreated control group (P< 0.05) (Figure 4A). These results showed that NVP-AUY922 elevated caspase-3 gene expression levels more efficiently when combined with DOX. The higher caspase 3-gene expression were observed in double combinations compared to single treatments of each drug (p<0.05).

Real-time PCR results (Figure 4 B). showed the (insignificant) decreased VEGF gene expression levels in NVP-AUY922 treated cells versus controls (p>0.05). Also, the decreased VEGF levels in a single treatment of DOX at IC_{50} level in compared to untreated cells did not reach a significant level (p>0.05). Double treatments at both examined concentrations showed significantly decreased VEGF gene expression levels compared to controls (p<0.05). In addition, decreased VEGF mRNA were presented in all double combinations versus single treatments (p<0.05) (Figure 4B).

Discussion

Breast cancer is a heterogeneous disease and prevalent malignancy in women worldwide (Amiri et al., 2016; Kanaani, 2017; Maroufi et al., 2020; , Sajjadiyan et al., 2016). Chemotherapy is one of the major treatment strategies for various types of cancers (Kanaani et al., 2017; Poy et al., 2018). But resistance against anticancer drugs considered as a main problems in unsuccessful cancer treatment (Ebrahimifar et al., 2017).

In this regards, the effectiveness of chemotherapy as an imperative breast cancer treatment strategy has been rigorously restricted because of cancer cells
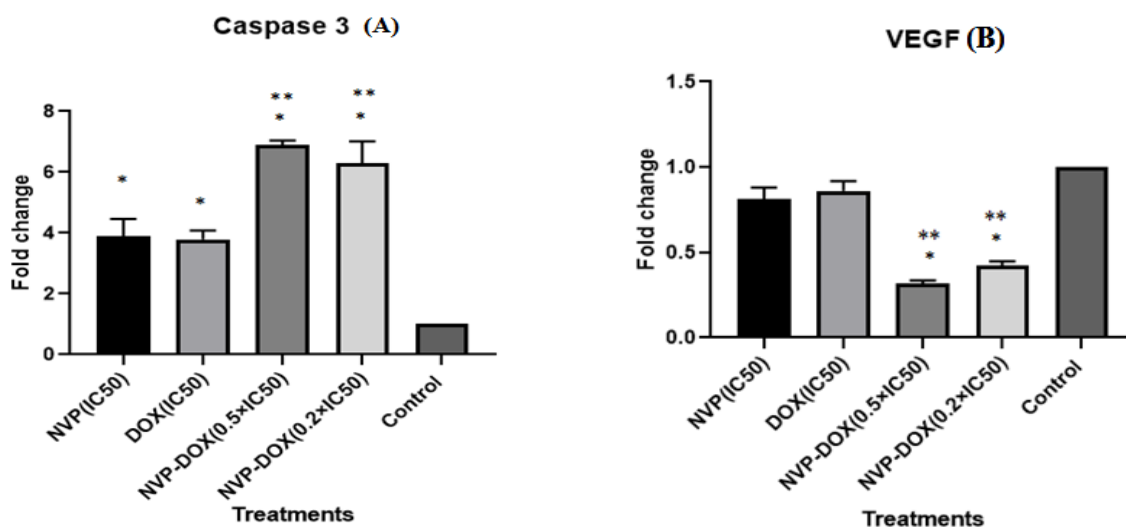

Figure 4. Real Time-PCR Analysis to Determine the Effects of NVP-AUY922 and DOX in Single and Double Drug Treatments on Caspase-3 (A) and VEGF (B) mRNA levels with β-actin as the internal control. Vertical bars show the mean fold changes ± Standard deviation *: significant difference to control.**: significant differences to single-drugs treatment.

resistance. Since the molecular chaperone HSP90 dysregulated in response to cellular stress (Jokar et al., 2019), the aim of this study was to investigate the anti-cancer effects of HSP90 inhibitor; NVP-AUY922 in combination with conventional standard drug DOX in breast cancer cell line and test their possible mechanism. Human breast cancer cell line was tested for sensitivity against NVP-AUY922 and DOX in vitro. We investigated whether NVP- AUY922, in combination with DOX, could be effectively used to treat breast cancer (in vitro), and also detect the related mechanisms. Based on our results, it seems that NVP-AUY922 might potentiate the effects of DOX. Indeed, combination of NVP-AUY922 and DOX in low doses could inhibit breast cancer cell growth, and upregulate caspase3 as major apoptosis component. Also, these combinations of NVP-AUY922 and DOX in very low concentrations of each drugs down-regulate VEGF as angiogenesis main effector. Indeed these combinations could acts in two way including apoptosis activation and inhibiting angiogenesis which both of these effects confirmed their anti-cancer activities.

VEGF is one of the most potent angiogenic factors that have an essential role in tumor angiogenesis. Increased expression of VEGF was presented in numerous human cancers cells (Adams et al., 2000). Indeed, our double treatments downregulate the VEGF that shows the anti-angiogenic effects of NVP-AUY922 in combined with DOX. Decreases in VEGF may be considered as one of the possible mechanisms of NVP-AUY922- DOX combination in low doses.

Based on the previous report, NVP-AUY922, as a potent small molecule HSP90 inhibitor, display imperative activity against breast cancer. NVP-AUY922 potently binds to HSP90 in a competitive biochemical assay. These effects associated with the NVP-AUY922 ability to interfere with the HSP90-p23 complex in breast cancer cells in culture and xenografts (Jensen et al., 2008).

In other study, HER2-positive breast cancer cell lines showed significant sensitivity to NVP-AUY922 in vitro, with IC_{50} values. NVP-AUY922, in combination with trastuzumab, significantly increased growth inhibition in cell lines, which in accordance with our results (Canonici et al., 2018). Also, in a similar study, NVP-AUY922 treatment decreased VEGF-A excretion in the breast cancer cell line (van Scheltinga et al., 2014).

In a comparable study, Gamitrinib, as HSP90 inhibitor in combination with-doxorubicin, showed increased cytotoxicity in various cancer cell lines, including breast cancer cell lines, which is parallel to our results. Also, drug combination increased caspase activity and cell death in breast cancer cells (Park et al., 2014). Similarly, Ganetespib, as HSP90 inhibitor, potentiated the DOX cytotoxic effects by increased mitotic arrest and DNA damage (Proia et al., 2014). Based on author knowledge, we didn't find any similar study to compare with our results.

Our data showed the anti-cancer effects of NVP-AUY922 in combinatorial cases with DOX. The effectiveness of NVP-AUY922 in combination with DOX should be evaluated in clinical practice. To our knowledge these combinations has not yet been investigated in humans and assessment of the other related mechanism should be conducted by preclinical studies.

In conclusion, our results indicate an effective action of NVP-AUY922 in combination with DOX in MCF-7 cell line. The possible mechanism of these cytotoxic effects might be related to apoptosis induction, cell growth inhibition and likewise downregulation of VEGF as the main angiogenic factor. It could be concluded that these altered levels of caspase 3 and VEGF can predict the treatment outcome in this model.

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