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*Research Article*

# Antagonistic interaction of HSP90 inhibitor XL-888 and 5-FU combination treatment in breast cancer cells

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ABSTRACT

Breast cancer is a serious global health problem, and investigation of innovative therapeutic approaches in its treatment is important to increase survival. Combination therapy targets more than one mechanism simultaneously and has recently emerged as an effective treatment strategy by using different therapeutic agents together. The purpose of this study was to determine the combined effects of the conventional chemotherapeutic agent 5-Fluorouracil (5-FU) and the HSP90 inhibitor XL-888 on breast cancer cell lines. MDA-MB-231 and MCF-7 cells were subjected to varying concentrations of XL-888 and 5-FU as individual treatments and in combination. The MTT test was employed to determine cell viability, and the Chou-Talalay technique was utilized to compute combination indices. Contrary to expectations, the HSP90 inhibitor XL-888 and 5-FU coadministration showed antagonistic effects in MDA-MB-231 and MCF-7 breast cancer cells. The results highlight the importance of careful consideration when combining these agents in breast cancer treatment regimens because their co-administration may not produce the expected synergistic results. The implications of the present research are anticipated to contribute to the developing of enhanced and focused treatment modalities for various cancers, with a particular emphasis on breast cancer.

## 1. Introduction

Breast cancer is an important and universal health problem for which new treatment strategies must be continually sought to improve survival rates[1,2]. The commonly used drug in the treatment of breast cancer is 5-FU. However, its clinical usage is limited due to side effects and dosage limits. A popular strategy for cancer treatment, called combination therapy, is being explored to overcome these limitations. Combination therapy involves using drug molecules to increase the effectiveness of existing treatments while relieving unwanted side effects. HSP90 has recently come into play as a key target in combination treatments. HSP90 is associated with many proteins within the cell and plays an important role in facilitating the maturation of client proteins (CPs), which are crucial for cellular survival, differentiation, and growth [3]. HSP90 appears to be an interesting therapeutic target due to its association with critical client proteins such as estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2), and progesterone receptor (PR), which are prominent in breast cancer [4]. Many HSP90 inhibitors that exhibit high selectivity and significant anti-cancer potency despite different chemical structures have been discovered. Among these inhibitors, XL-888, an advanced oral drug designed to target HSP90, has demonstrated the capacity to suppress HSP90 activity significantly without affecting other kinases [5]. XL-888 inhibits HSP90 activity, which may impair the survival of cancer cells under stress conditions and may, therefore, increase the effectiveness of chemotherapy drugs when used combined with chemotherapy drugs [6-7]. Ongoing preclinical trials have investigated the efficacy of XL-888 in melanoma and advanced pancreatic/colorectal cancer, lung cancer, and breast cancer [8–10].

Breast cancer is classified into two molecular subtypes: hormone receptor-positive and triple-negative. The majority, about 80%, fall into the hormone receptor-positive category, while the remaining 20% are classified as triple negative. Combining different drugs in the treatment of breast cancer, for example, using two different chemotherapy drugs or a targeted drug and a chemotherapy drug together to target different biological properties of cancer cells, is one of the effective treatment strategies [11-13].

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This research aimed to investigate how 5-FU and XL-888 combination therapy affected MCF-7 and MDA-MB-231 cell lines. When administered alone or in combination, the cytotoxic potential of these agents was evaluated at 24-hour and 48-hour periods. Evaluation of drug interactions was done using the Chou and Talalay methodology.

## 2. Materials and Methods

### *2.1 Materials*

MTT (3- (4,5- dimethylthiazol -2 - yl)-2,5-diphenyltetrazolium bromide) was provided by Serva. XL-888 was purchased from AdooQ® Bioscience. 5-FU was obtained by Gold Biotechnology**.** Dulbecco's Modified Eagle's medium High Glucose (DMEM), fetal bovine serum heat-inactivated (FBS), penicillin-streptomycin solution and other necessary chemicals (L-glutamine, phosphate buffer saline (PBS), and trypsin–EDTA) for use in cell culture were purchased from Biological Industries. The breast cancer cell lines, MDA-MB-231 and MCF-7 were purveyed from American Type Culture Collection.

### *2.2 Cell Culture*

To culture the MDA-MB-231 and MCF-7 cancer cell lines, we employed DMEM High Glucose medium supplemented with 10% FBS. The cancer cell lines were maintained in an incubator at a temperature of 37°C within a humidified environment containing 5% $CO_2$.

### *2.3 Cell Viability Assay*

The cell proliferation assay, generally preferred and known as MTT, was chosen to evaluate the in vitro cytotoxic impact of XL-888 and 5-FU. Culturing the cancer cell lines was performed in 96-well plates with a density of $5x10^4$ cells per well. Subsequently, the cells were exposed to varying concentrations of XL-888 (100 nM -1.5625 nM) and 5-FU (10 µM-0.156 µM) for 24 h and 48 h. Following the incubation period, a solution of MTT (5 mg/mL) was introduced to each well and then allowed to incubate at 37°C for 3 hours. After the formed formazan product was dissolved in dimethyl sulfoxide, the absorbance of each well was measured at 570 nm, and the viability of the cancer cells was calculated as a percentage relative to the control group. Moreover, the initial concentrations for cytotoxicity analyses of the drugs, 100 nM for XL-888 and 10µM for 5-FU, were determined by preliminary testing following literature research. The combination ratio was based on these initial concentrations, and serial dilution was studied by keeping the ratio of 100 nM: 10 µM, i.e. 1:100, constant.

### *2.5 Combination Index*

In drug combination studies, the Chou and Talalay method, a type of software, generally stands out in determining combination effectiveness. This approach relies on the utilizing of the median effect equation as its fundamental principle. The derivation of this method from the law of mass action has made it more preferred in practical applications [14-15]. The widespread adoption of the Chou and Talalay method in practical applications began in 2005 with the introduction of CompuSyn software [16]. This program calculates effective dose-dependent combination indexes by comprehensively analyzing drug interactions using cell viabilities and combination ratios depending on drug concentrations [17]. To assess the effectiveness of the XL-888 and 5-FU combination, CompuSyn software version 1.0 was used to calculate the combination index (CI). The Chou-Talalay method was employed for CI determination.

### *2.4 Statistical Analysis*

GraphPad Prism 8.0 program was utilized with significance regarded as $p<0.05$.

## 3. Results and Discussion

The MTT assay, a method for assessing cell viability, was used to evaluate the impact of XL-888 and 5-FU, individually and in combination, on MDA-MB-231 and MCF-7 cell lines. As drawn in Figures 1A, 1B, 1C and 1D, both XL-888 and 5-FU exhibited time- and dose-dependent inhibition of cell viability in MDA-MB-231 and MCF-7 cells.

According to the IC50 values calculated in Table 1, the HSP90 inhibitor XL-888 had a cytotoxic effect on the cells at low concentrations in both cell lines. Compared to the MDA-MB-231 cell line, MCF-7 cells were more sensitive to HSP90 inhibition. Since the expression of HSP90 protein is significantly higher in tumor cells, inhibition of HSP90 in tumor cells is an important strategy for cancer therapy [18]. Previous research has demonstrated that decreasing HSP90 alone or in combination inhibits the development of breast cancer cells. Ganetespib, an HSP90 inhibitor, acted as an inhibitor of oncogenic signaling in MDA-MB-231 cells at low doses and suppressing tumor growth by increasing the apoptotic markers Parp and Bim [18]. Phase II/III clinical studies of the HSP90 inhibitor 17-AAG in breast cancer and other tumors showed up-and-coming [20]. Moreover, tanespimycin, a different version of 17-AAG, showed promising antitumor activity in phase II trials when combined with trastuzumab in the scope of HER2-positive metastatic breast cancer [21]. HSP90 inhibitors have significant potential to induce apoptotic cell death pathways in breast cancer [22]. In a previous study, our research focused on the HSP90 inhibitor Debio-0932, this compound was found to effectively inhibit human Hsp90 ATPase activity and reduce cell proliferation by inducing apoptotic pathways in MDA-MB-231 and MCF-7 breast cancer cell lines [23]. Proia et al. evaluated the apoptotic potential of the combination of ganetespib with doxetaxel, DOX and paclitaxel. The combination of DOX and ganetespib caused an increase in Parp expression in MDA-MB-231 cells.

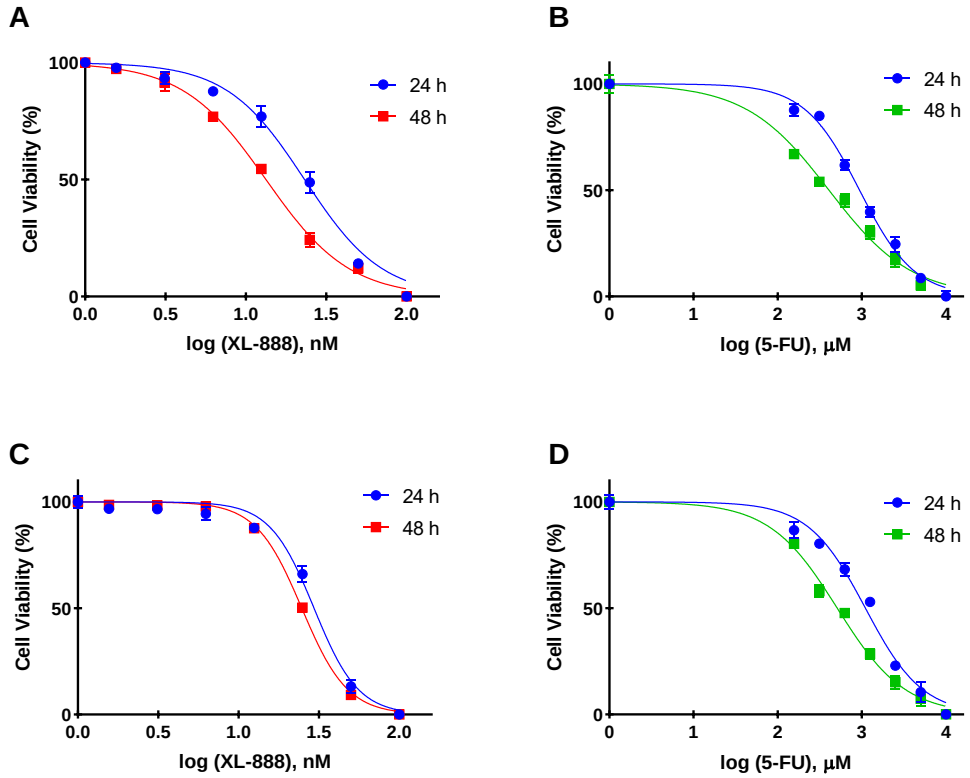


Figure 1. The antiproliferative effects of XL-888 (A) and 5-FU (B) were examined in the MCF-7 cell line, while XL-888 (C) and 5-FU (D) were assessed in the MDA-MB-231 cell line at both 24 h and 48 h.

Further, ganetespib treatment increased the expression of Parp and Caspase-7 in BT-20 cells [24]. Another combination therapy study by Muhammedyan et al. showed that the effect of NVP-AUY922, an HSP90 inhibitor, combined with DOX on the MCF-7 cell line was stronger than the effects of the individual drugs [25].These findings emphasize the prospective therapeutic effects of HSP90 inhibitors in the treatment of breast cancer by inducing apoptosis and inhibiting metastasis.

In this research, 5-FU had a strong cytotoxic impact on MDA-MB-231 and MCF-7 cells after 24 and 48 hours. 5-FU is a pyrimidine analog antimetabolite that inhibits thymidylate synthase activity. This molecule has the ability to arrest the cell cycle at the S phase and induce apoptosis [26]. In first- and second-line breast cancer treatment, 5-FU is used in combination with other drugs such as doxorubicin, methotrexate, and cyclophosphamide [27]. However, 5-FU treatment is restricted by drug resistance caused by breast cancer resistance proteins and dangerous side effects. As a result, finding novel selective breast cancer drugs that may be employed as a single dosage or in combination with other cytotoxic treatments is critical [28].

The clinical efficacy of 5-Fluorouracil (5-FU)-based chemotherapy is limited by issues of multidrug resistance and dose-dependent cytotoxicity. To address these issues, studies are being conducted using 5-FU and additional anticancer drugs as a new combination that interacts with cells.

In this study, the combined use of XL-888 and 5-FU revealed an antagonistic effect on MDA-MB-231 and MCF-7 cell lines. These results show that contrary to the synergistic effect usually sought, the combined use of these two drugs completely reverses the expected effect (Figures 2A and 2B). Analysis results show that the antiproliferative effects of the two agents on breast cancer cells are higher when used alone compared to their combined use. To confirm this antagonism, the combination of two drugs prepared at a fixed ratio was serially diluted and treated to the cells, and then CompuSyn software, designed with the methodology introduced by Chou and Talalay, was used to calculate the combination index (CI) [29]. This analysis showed the following combination effects: CI > 1 demonstrates antagonism, CI <1 indicates synergism, and CI = 1 suggests additivity [30]. The combined treatment of XL-888 and 5-FU exhibited an antagonistic effect in both MCF-7 (Figure 2C) and MDA-MB-231 (Figure 2D) cell lines.

In previous studies, combining Hsp90 inhibitors with chemotherapy agents has typically shown synergistic effects. In combination with doxorubicin, the HSP90 inhibitor AUY-922 increased caspase-3 expression, a marker of mitochondrial apoptosis, and decreased VEGF mRNA levels [25].

In a preclinical study, 17-AAG demonstrated a positive effect on breast cancer cells treated with bevacizumab

(VEGF inhibitor) [31]. In another study, colorectal cancer cells treated with the HSP90 inhibitor AUY-922 were shown to be more sensitive to 5-FU-based chemotherapy in vitro and animal models. This suggests that the coadministration of 5-FU with AUY-922 may be a valid treatment strategy [32]. In the other study, Liu and colleagues investigated the effect of the combined use of 5-FU and the HSP90 inhibitor SNX-2112 in esophageal cancer. Unexpectedly, the combined use of these two agents resulted in antagonistic results in the cells. Further investigation into the molecular mechanisms behind this response revealed several plausible factors [33]. Based on these possible factors, the combination of SNX-2112 and 5-FU could potentially lead to opposite results by countering G2/M cell cycle arrest, reducing Hsp90 client proteins and suppressing caspase-dependent apoptosis, inhibiting the initial reduction of MMP (Mitochondrial Membrane Potential).

In a study investigating the combined effect of the chemotherapeutic drug DOX and the HSP90 inhibitor XL-888 on liver cancer cell lines, it was shown that the simultaneous use of both drugs strongly triggered apoptosis [34]. In addition, the combination of DOX and the HSP90 inhibitor MPC-3100 was found to be more effective in triggering apoptosis in breast cancer cells than either drug alone and showed a synergistic effect [35]. In a study examining the combined effect of 5-FU and the HSP90 inhibitor MPC-3100 on liver cancer cell lines HepG2 and HUH-7 [36]. It was observed that both drugs individually showed dose- and time-dependent cytotoxic effects. However, particularly the combined use of the two drugs resulted in an antagonistic effect rather than the expected additive or synergistic effects on HepG2 and HUH-7 cell lines.

Table 1. $IC_{50}$ values of XL-888, 5-FU, and combination form in MDA-MB-231 and MCF-7 cell lines at 24 h and 48 h.

| **$IC_{50}$ (nM)** | | | | | | |
|---|---|---|---|---|---|---|
| | **XL-888** | | **5-FU** | | **XL-888 + 5-FU** | |
| **Cell Lines** | **24 h** | **48 h** | **24 h** | **48 h** | **24 h** | **48 h** |
| **MCF-7** | 23.20 ± 0.004 | 13.34 ± 0.003 | 939.1±0.003 | 406.6 ± 0.004 | 1544 ± 0.005 | 572.9 ± 0.003 |
| **MDA-MB-231** | 29.48 ± 0.004 | 24.74 ± 0.001 | 1103 ± 0.004 | 509.9 ± 0.004 | 951.9 ± 0.003 | 912.2 ± 0.005 |

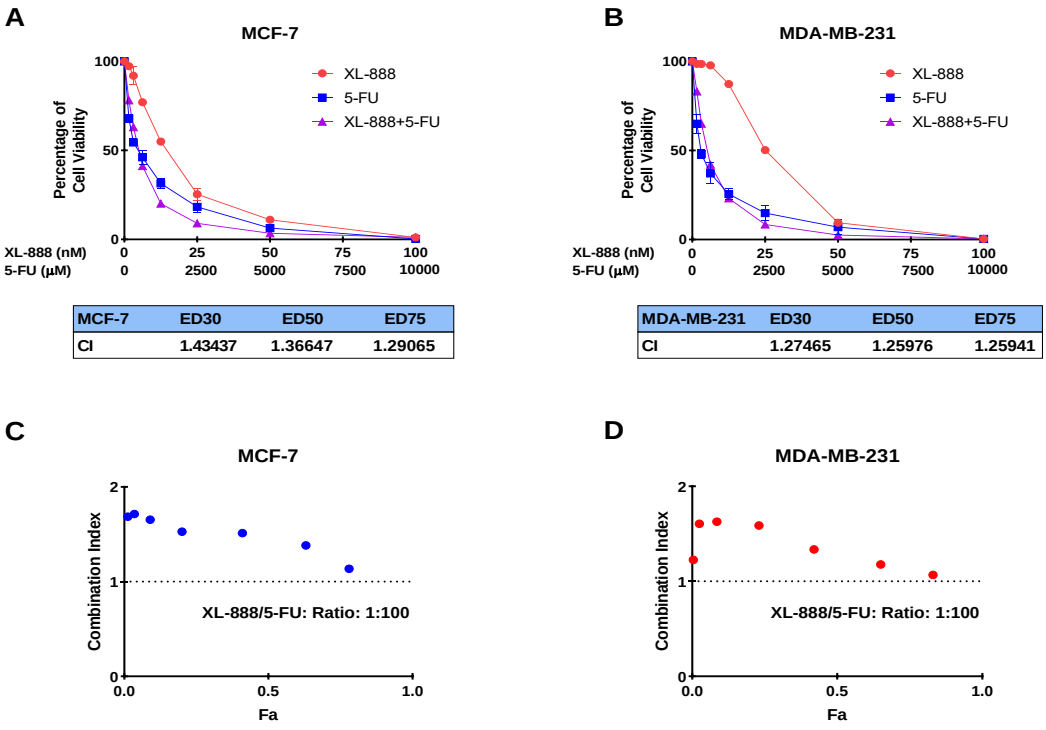


Figure 2. Survival graphs of MCF-7 (A) and MDA-MB-231 (B) XL-888 and 5-FU combination-treated cancer cell lines; graphs were generated based on combination index data plotted against fraction affected (Fa) values obtained from Chou-Talalay median effects analysis for MCF-7 (C) and MDA-MB-231 (D) cell lines.

* CI: combination index, Fa: affected fraction; ED50, ED75, and ED90 refer to the amount of dose required to inhibit 50%, 75%, and 90% of cells, respectively, and reveal the potential of a drug or treatment.

Furthermore, in a study investigating the effectiveness of combined therapy of Hsp90 inhibitors and anticancer agents in pancreatic cancer cell cultures, it was found that although both inhibitors have the same mechanism of action, the use of different Hsp90 inhibitors with the same anticancer agent may lead to different effects. It has been suggested that these differences may be due to the different structures and activities of both compounds [37].In addition, another study, pathway analyses of the anticancer activities of Hsp90 inhibitors XL-888 and Debio0932 on the neuroblastoma cancer cell line SH-SY5Y showed that these inhibitors are important in regulating many cancer-related pathways (such as invasion, metastasis, angiogenesis, and apoptosis) [38]. In a study conducted to overcome the resistance to trastuzumab treatment in HER2-positive breast cancer, the Hsp90 inhibitor HVH-2930 was reported to prevent angiogenesis and tumor growth in trastuzumab-resistant xenograft mice in vivo. In addition, in this study, it was reported that the combination of the Hsp90 inhibitor HVH-2930 with paclitaxel exhibited a synergistic effect in JIMT-1 xenografts [39].

In our research, possible explanations for the antagonistic effects of combining 5-FU with an HSP90 inhibitor in breast cancer cells may overlap with these findings. In a broader context, the data obtained in our study should further investigate the antagonistic interaction between HSP90 inhibitor and 5-FU across various types of cancer. It is recommended that the combination of 5-FU and HSP90 inhibitors be considered when planning clinical applications.

## 4. Conclusions

In this study, the potential of combined use of the conventional chemotherapy agent 5-FU and HSP90 inhibitor XL-888 in the treatment of breast cancer was investigated. The findings separately revealed the dose- and time-related cytotoxic impacts of each agent on MDA-MB-231 and MCF-7 breast cancer cell lines. However, data from the combined use of the two drugs showed surprising effects. Co-administration of 5-FU and XL-888 had an antagonistic rather than predicted additive or synergistic effect on MDA-MB-231 and MCF-7 cell lines. This unexpected result underscores the complex interaction between different drugs and demonstrates the importance of comprehensive evaluation of all variables during combination therapy development. Although combining XL-888 and 5-FU was not effective in increasing cytotoxicity in breast cancer cells, information gained from studies such as ours will contribute to the creation of more targeted and effective treatments against breast cancer and other malignancies.

## Declaration

The author affirms that the article is original and was prepared in compliance with international publication and research ethics standards. No potential conflicts of interest are declared by the author regarding the research, authorship, and publication of this article. Additionally, the author states that no special permission or ethical committee approval was necessary for this study.

## Author Contributions

Nazan GÖKŞEN TOSUN: Investigation, Methodology, Formal analysis, Writing-review & editing, Visualization, Methodology, Resources.

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